

# ABSTRACTS FOR POSTER PRESENTATION

## Student Competition Finalists

### EFFECT OF MACROMOLECULES FOR BOVINE OOCYTE VITRIFICATION

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Vitrification could become the procedure of choice to preserve oocytes of valuable cows. Most successful vitrification protocols include fetal calf serum (FCS) as a component, which varies from batch to batch and may contain viruses. The aim of this study was to determine the effectiveness of different macromolecules to replace FCS in vitrification solutions for oocyte cryopreservation. Oocytes were matured as described below for 21 h, at 38.5 °C in 5% CO<sub>2</sub> in air, and then partially denuded with 100 IU/ml of hyaluronidase in holding medium (Hepes-TCM 199 with 20% FCS to prevent zona hardening; HM). Oocytes in groups of 24 were then held in HM before vitrification. All vitrification and warming procedures were carried out at 37 ± 2 °C. Subgroups of 4 oocytes were pre-equilibrated for 30 s in 100 µl of vitrification solution 1 (Hepes-TCM 199 with 10% DMSO, 10% ethylene glycol (EG) and the corresponding macromolecule). Then, oocytes were rinsed in two 100-µl drops of vitrification solution 2 (Hepes-TCM 199 with 20% DMSO, 20% EG, 0.5 M of galactose and the corresponding macromolecule), loaded onto cryoloops [Lane et al., Nat Biotechnol 1999;17:1234], and immersed in liquid nitrogen after 25 s. Oocytes were warmed by plunging the cryoloop into 0.5 M galactose in HM and then placed in 0.5, 0.25, 0.125, and 0 M galactose in HM for 3 min each. Oocytes were then placed back in maturation medium for 0.5 to 2 h to complete a total of 23 h incubation. Frozen-thawed semen from one of three bulls was used for fertilization in a chemically defined medium [Olson and Seidel, J Anim Sci 2000;78:152; F-CDM] at 1 million sperm/ml. Zygotes were vortexed after fertilization, cultured in CDM-1 for 48 h, and then in CDM-2 for 144 h at 38.5 °C in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub>. Experiment 1: Two maturation media were used: TCM 199 versus CDM, both with the addition of 10% FCS, 1 µg/ml E<sub>2</sub>, 1 µg/ml LH, and 15 ng/ml FSH. Macromolecule treatments were: A: no macromolecules; B: 20% FCS; C: 6% BSA; D: 2% BSA; E: 18% Ficoll 70,000; F: 6% Ficoll 70,000; G: non-vitrified control; H: non-vitrified but cumulus partly removed control. Experiment 2: Maturation medium was CDM with the addition of 0.5% Fatty Acid Free-BSA, 1 µg/ml E<sub>2</sub>, 1 µg/ml LH, 15 ng/ml FSH and 50 µg/ml of EGF. Macromolecule treatments were: A; B; G; H (see above); I: 20% PVP; J: 6% PVP; K: 1% PVA, and L: 0.3% PVA. Cleavage was evaluated at 72 h post-fertilization, and blastocyst rate was evaluated on Days 7, 8 and 9. Each experiment was replicated six times. Data were arcsin transformed and analyzed by ANOVA. In Experiment 1, there were no differences between maturation media, so data were pooled. Cleavage rates were not different among treatments ( $P > 0.05$ ) ranging from 63 to 80%. LS means for blastocyst formation rate (per oocyte) were different in H versus A and C, and G versus A ( $P < 0.05$ ) A: 9; B: 18; C: 12; D: 21; E: 15; F: 15; G: 28 and H: 34%. In Experiment 2, there were no significant differences in cleavage rates (70–87%;  $P > 0.05$ ), but blastocyst rates were different between G versus L and A, and in H versus L ( $P < 0.05$ ) A: 17; B: 23; I: 21; J: 22; K: 20; L: 10; G: 43; H: 34%. In conclusion, vitrification solutions for bovine oocytes containing no macromolecule, 0.3% PVA, and 6% BSA (likely due to high viscosity) were inferior to non-vitrified controls. A wide range of other macromolecules and concentrations were, however, suitable for vitrification of bovine oocytes.

## OXYGEN-REGULATED GENE EXPRESSION IN BOVINE BLASTOCYSTS

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Hypoxia-inducible factors (HIFs) are transcription factors mediating O<sub>2</sub>-dependent regulation of genes such as glucose transporters, glycolytic enzymes, and angiogenic growth factors. Activation of HIF DNA binding is regulated by protein stabilization and nuclear translocation in response to hypoxia. Our hypothesis is that post-compaction bovine embryos require hypoxic O<sub>2</sub> levels, as postulated to occur in the uterus [Fischer and Bavister, *J Reprod Fert* 1993;99:673], to regulate the expression of genes facilitating further development. We have previously demonstrated expression of HIF1 $\alpha$  and HIF1 $\beta$  throughout bovine pre-elongation embryo development. Here we determine the effect of O<sub>2</sub> concentration on the expression of HIF1 $\alpha$ , HIF2 $\alpha$ , glucose transporter 1 (GLUT1) and vascular endothelial growth factor (EGF), cell allocation and the localization of HIF1 $\alpha$  protein within bovine blastocysts.

In vitro-produced bovine embryos were generated using standard protocols. Culture to Day 5 was performed using Cook Bovine Cleave medium (Cook Veterinary Products, Bloomington, IN) under 7% O<sub>2</sub>. Post-compaction (Day 5) embryos were cultured under 2% (hypoxia), 7 and 20% O<sub>2</sub> for 72 h in Cook Bovine Blast medium. Resulting blastocysts were pooled and total RNA isolated. In vivo-derived blastocysts were collected from superovulated cows. RT-PCR was performed using random primers and Superscript II and real-time reactions carried out using SYBR green master mix (Applied Biosystems). Reactions were normalized by measuring 18S rRNA for each sample. Differential staining, for cell number determination, and immunofluorescent localization of HIF1 $\alpha$  protein were undertaken using separate cohorts of embryos. Immunofluorescence utilized a monoclonal antibody against HIF1 $\alpha$  (Affinity Bioreagents) and a Texas-Red conjugated secondary antibody. Embryos were counterstained with H33342 to localize nuclei and examined using confocal microscopy. Statistical analyses were performed by ANOVA.

Although no difference was found in total cells counts ( $178 \pm 7$  cells), hypoxic culture significantly increased ( $P < 0.05$ ) allocation to the ICM cells ( $32.3 \pm 1.6\%$  versus  $26.7 \pm 1.6\%$  and  $26.1 \pm 1.5\%$  for 2, 7 and 20% O<sub>2</sub>, respectively). Real-time PCR analysis demonstrated that under the culture conditions used, O<sub>2</sub> concentration did not affect the expression of HIF1 $\alpha$  or HIF2 $\alpha$  (data not shown). GLUT1 expression was significantly altered by O<sub>2</sub> concentration ( $P < 0.01$ ), with higher levels in embryos incubated in 2% O<sub>2</sub>. VEGF tended to be upregulated under hypoxic culture, although not significantly ( $P = 0.081$ ). Substantial variability existed in the expression of genes from the two replicates of in vivo-derived embryos. Immunolocalization of HIF1 $\alpha$  protein in embryos cultured under hypoxia revealed primarily a nuclear localization.

These results reveal that O<sub>2</sub> concentration regulates specific genes in bovine blastocysts thought to be important for further development in a hypoxic environment. Perturbed expression of genes at the blastocyst stage during in vitro embryo production can partly be attributed to non-hypoxic conditions. These results further support our hypothesis that O<sub>2</sub> concentration is a physiological regulator of gene expression in vivo, mediated by the HIF family of transcription factors (Table 1).

Table 1  
Levels of GLUT1 and VEGF gene expression in response to oxygen concentration

Gene (n)	2% O <sub>2</sub>	7% O <sub>2</sub>	20% O <sub>2</sub>	In vivo (n = 2)
GLUT1 (4)	188.9 $\pm$ 14.4 <sup>a</sup>	112.7 $\pm$ 15.2 <sup>b</sup>	133.6 $\pm$ 2.8 <sup>b</sup>	193.7, 654.9
VEGF (4)	2.91 $\pm$ 0.28	1.92 $\pm$ 0.59	1.54 $\pm$ 0.15	0.46, 1.19

Values for mRNA abundance are arbitrary units normalized to 18S rRNA. Superscripts represent significant differences ( $P < 0.01$ ). All replicates (n) are pools of 23–33 blastocysts from independent IVP experiments.

## SEX-SORTING AND RE-CRYOPRESERVATION OF FROZEN-THAWED RAM SPERM FOR IN VITRO EMBRYO PRODUCTION

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Application of sperm sorting to breeding of livestock and wildlife is limited when the sorter is a long distance from the male(s) but would be facilitated by the sorting of frozen-thawed sperm [Lu et al., *Theriogenology* 1999;52:1393–1405] and re-freezing it. High purity sorting with maintained quality of frozen-thawed ram sperm has been achieved after processing to remove the cryodiluent [Hollinshead FK et al., *Proc Soc Reprod Biol* 2002, submitted]. The aim of this study was to evaluate the functional capacity of frozen-thawed sperm after sorting and a second cryopreservation/thawing step. Frozen semen from two rams ( $n = 2$  ejaculates per ram) was used throughout. Post-thaw sperm treatments comprised (i) unsorted (Control); (ii) sorted (Froz-Sort); and (iii) sorted then re-frozen (Froz-Sort-Froz). X and Y sperm were separated using a high-speed sorter (SX MoFlo<sup>®</sup>, Cytomation, CO, USA) after incubation with Hoechst 33342 and food dye to eliminate non-viable sperm. Reanalysis revealed high levels of purity for X- and Y-enriched samples for all treatments ( $87.0 \pm 4.5\%$ ). For IVF, 472 IVM oocytes were inseminated with  $1 \times 10^6$  motile sperm/ml. After 3 h in SOF medium, oocytes were transferred to Sydney IVF cleavage medium (Cook<sup>®</sup>, QLD, Australia) for 4 days followed by Sydney IVF blastocyst medium (Cook<sup>®</sup>) for an additional 3-day culture in 5% O<sub>2</sub>:5% CO<sub>2</sub>:90% N<sub>2</sub>. Oocytes were assessed for cleavage at 24 and 48 h post-insemination (p.i.). At 52 h p.i., uncleaved oocytes were stained with orcein for assessment of maturation and fertilization. Data from three replicates were analyzed by ANOVA, chi-square, and Fisher Exact Test. At insemination, percentage of motile sperm ( $\pm$ S.E.M.) was higher ( $P < 0.001$ ) for Froz-Sort ( $85.8 \pm 2.4\%$ ) and Froz-Sort-Froz ( $66.7 \pm 7.7\%$ ) than Control ( $36.7 \pm 2.1\%$ ). Maturation rate was 95.6% (451/472). Cleavage of oocytes in a parthenogenetic control group (no sperm) was low (2/56; 3.6%). Polyspermic fertilization was low (9/451; 2.0%) and did not differ among treatments. Fertilization and cleavage rates were consistently high across treatments. Blastocyst development rate was higher for oocytes fertilized with Froz-Sort-Froz than with Control sperm. These results demonstrate that frozen-thawed ram sperm can be sex-sorted for either immediate or future use in an IVF system after re-cryopreservation (Table 1).

Table 1

Fertilization and early embryo development of oocytes after incubation with frozen-thawed unsorted (Control), frozen-thawed and sorted (Froz-Sort) and frozen-thawed, sorted then frozen-thawed (Froz-Sort-Froz) ram sperm

Treatment	No. of mature oocytes fertilized <sup>a</sup>	No. of mature oocytes undergoing cleavage after insemination		No. of cleaved oocytes forming blastocysts		
		24 h	48 h	Day 5	Day 6	Day 7
Control	40 (67.8)	26 (44.1)	36 (61.0)	4 (11.1)	13 (36.1)	16 (44.4) <sup>a</sup>
Froz-Sort	110 (63.6)	67 (38.7)	109 (63.0)	24 (22.0)	34 (31.2)	57 (52.3) <sup>ab</sup>
Froz-Sort-Froz	94 (57.7)	71 (43.6)	91 (55.8)	23 (25.3)	37 (40.7)	59 (64.8) <sup>bc</sup>

<sup>a</sup> Monospermic fertilization. Within column, values with different superscripts differ ( $P < 0.05$ ). Values in parentheses are percentages.

REDUCTION OF POLYSPERMIC PENETRATION AFTER TEMPORARY ARREST  
OF IN VITRO-MATURED METAPHASE I PIG OOCYTES

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In vitro production of pig embryos remains challenged by poor cytoplasmic maturation of oocytes, polyspermy and high incidences of apoptosis in developing embryos. Insufficient oocyte IVM systems contribute to non-synchronous nuclear and cytoplasmic maturation of the oocyte, and can result in the formation of embryos with compromised developmental competence. The objective of this study was to decrease polyspermy and increase normal blastocyst development by using taxol, a microtubule stabilizing agent, to temporarily arrest nuclear maturation at metaphase I (MI), while facilitating more time for cytoplasmic maturation. Pre-pubertal gilt COCs, aspirated from 2- to 6-mm follicles of slaughterhouse ovaries, were matured in NCSU23 with 10% sow follicular fluid, 0.1% BSA, 0.1 mg/ml cysteine, 25 mM  $\beta$ -mercaptoethanol, 0.1  $\mu$ g/ml db-cAMP, 10 IU/ml eCG and hCG, and antibiotics for 22 h. From 22 to 42 or 48 h, the COCs were placed in NCSU23, as above, but without db-cAMP, eCG and hCG. Between 30 and 36 h of IVM, COCs were cultured with (Tx) or without (C) 1  $\mu$ M taxol, followed by taxol-free culture. At either 42 h (normal) or 48 h (extended) IVM, oocytes were denuded and fertilized in modified Tris-buffered medium (mTBM) with 0.1% BSA and 1 mM caffeine. IVF wells contained 500  $\mu$ l mTBM and 40 to 50 denuded oocytes, which were incubated with 10  $\mu$ l washed, fresh sperm (final concentration of 50 sperm/oocyte) for 6 h. Presumptive zygotes were then cultured in NCSU23 with 0.4% BSA up to 150 h to determine the blastocyst rate. All blastocysts were fixed and stained with TUNEL and Hoechst 33342 to evaluate apoptosis and cell number (mean  $\pm$  S.E.M.). Incidence of apoptosis: % blastocysts with  $\geq 1$  apoptotic nuclei; Apoptotic Index (ApI): (no. TUNEL-labeled nuclei/total no. nuclei)  $\times 100$ . Fertilization and embryo development data (three replicates) were analyzed by Fisher's Exact Test, and blastocyst cell number and apoptosis by ANOVA. At 42 h IVM, 57% of MI Tx oocytes had remained in MI (6% MI in C), and had not changed by 48 h IVM. Results showed no difference between C and Tx in penetration at 42 or 48 h IVF. IVF at 48 h led to a reduction in penetration, while normal fertilization rate was not affected. Polyspermy was reduced in the Tx 48 h group. Percentage blastocyst development was poorer after 48 h IVF, but no differences were seen in the number of nuclei/blastocyst. When fertilized at 42 h though, Tx blastocysts contained fewer nuclei. IVF at 48 h IVM tended to decrease the incidence of apoptosis and the number of apoptotic nuclei/blastocyst denoted by the ApI ( $P > 0.05$ ). In conclusion, taxol treatment significantly reduced polyspermy and while it did not increase blastocyst formation, it showed the propensity to reduce the occurrence and degree of apoptosis in resultant embryos. The tendency of both taxol and 48-h IVF to decrease blastocyst apoptosis and increase the development of non-apoptotic blastocysts is noteworthy, and further studies should be conducted to refine and further elucidate the efficacy of taxol treatment of IVM pig oocytes.

Treatments	C 42 h	Tx 42 h	C 48 h	Tx 48 h
<i>n</i>	55	132	37	126
Penetration (%)	50 <sup>a</sup>	66 <sup>a</sup>	19 <sup>b</sup>	29 <sup>b</sup>
Normal fertilized (%)	31 <sup>ab</sup>	42 <sup>a</sup>	16 <sup>b</sup>	25 <sup>b</sup>
Polyspermic/penetrated (%)	39 <sup>a</sup>	37 <sup>a</sup>	14 <sup>ac</sup>	14 <sup>bc</sup>
<i>n</i>	94	134	96	136
Evenly cleaved 48 h post-IVF (%)	47 <sup>a</sup>	33 <sup>b</sup>	24 <sup>b</sup>	27 <sup>b</sup>
D6 blasts (%)	33 <sup>a</sup>	19 <sup>b</sup>	16 <sup>b</sup>	11 <sup>b</sup>
<i>n</i>	29	24	13	12
Mean cell no. D6 blasts	39 $\pm$ 4 <sup>a</sup>	26 $\pm$ 2 <sup>b</sup>	34 $\pm$ 4 <sup>ab</sup>	39 $\pm$ 4 <sup>ab</sup>
Incidence of apoptosis	24 <sup>a</sup>	26 <sup>a</sup>	14 <sup>a</sup>	7 <sup>a</sup>
ApI	0.80 <sup>a</sup>	0.85 <sup>a</sup>	0.45 <sup>a</sup>	0.21 <sup>a</sup>

<sup>a,b,c</sup> Within rows values with different superscripts are significantly different:  $P < 0.05$ .

## EFFECT OF THE 1:29 ROBERTSONIAN TRANSLOCATION ON THE SEGREGATION AND IN VITRO DEVELOPMENT OF BOVINE BLASTOCYSTS

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The effect of the bovine 1:29 Robertsonian translocation on the development of oocytes matured, fertilized and cultured in vitro was assessed by means of microsatellite analysis. This translocation has no phenotypic effect on the carrier. Fertilization differences and embryonic mortality in all stages of development may contribute to an alteration of the offspring's ratio from heterozygous carriers of the 1:29 Robertsonian translocation. The aim of this study was to examine the in vitro development of the bovine embryos and to determine the rate of Day 8 embryos carrying the translocation. Six microsatellites (AGLA17, BM6438 and TGLA49 on chromosome 1 and BM4602, BMC2228 and BMS1857 on chromosome 29; [Joerg et al., J Anim Breed Genet 2001;118:371–377]) were selected to type both the animals and the embryos, which were produced in vitro. Typing of these six microsatellite markers in Brown Swiss cattle resulted in a haplotype combination (222, 262, 116, 113, 173 and 159, respectively) which segregates with the 1:29 Robertsonian translocation. Bovine ovaries were obtained from 73 slaughtered Brown Swiss and Simmental cows. Cumulus enclosed oocytes were matured, fertilized and the denuded potential embryos were cultured in vitro under standard conditions (TCM 199 for IVM, Talp-HEPES for IVF and SOF for IVC, respectively). Fertilization was performed using sperm from a Brown Swiss bull heterozygote for the translocation with proved natural fertility records. Blastocysts were washed three times in phosphate buffered saline solution supplemented with 6 mg/ml polyvinylpyrrolidone, pipeted in 1  $\mu$ l into PCR tube containing 9  $\mu$ l of lysis buffer supplemented with 0.20  $\mu$ g/ $\mu$ l of proteinase K and overlaid with 30  $\mu$ l of mineral oil. After an incubation at 56 °C for 60 min and proteinase K heat-inactivation, a preamplification polymerase chain reaction (PEP-PCR) was performed. A standard 50-cycle PCR was completed using primers for the six microsatellites and 4  $\mu$ l of the PEP-PCR reaction. To exclude a potential carrier of the translocation within the oocyte donors, the 73 presumptive mothers of the embryos were characterized for the six microsatellites. The DNA of the parent animals was extracted from muscle or sperms, and a subsequent 35-cycle PCR was performed under the same conditions. An ABI PRISM<sup>®</sup> 377 DNA Sequencer in combination with GeneScan<sup>®</sup> Analysis Software (Applied Biosystems, Foster City, CA, USA) was used to determine the size of the amplified products. The blastocyst rate obtained at Day 8 was 39%, a result similar or even slightly higher than with the in-house control bulls. None of the cows had the six alleles linked to this centromere fusion. From the 93 embryos obtained in this study, 48 (51%) showed the defined haplotype combination segregated with the 1:29 Robertsonian translocation. The results showed that a 1:1 ratio ( $\chi^2 = 0.097$ ,  $P > 0.05$ ) exists for fertilization and, up to Day 8, this translocation did not impair the in vitro development of the blastocysts.

## DURATION OF CRYOPRESERVATION HAS NO EFFECT ON FERTILIZING ABILITY OF BOAR SPERMATOZOA

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Preserving genetic diversity in swine is important for the maintenance of healthy production populations, as well as the conservation of rare breeds that have genetic traits of merit. One method by which to accomplish this goal is the cryopreservation and banking of semen. However, semen cryopreservation in swine is not as efficient as that in other domestic species. The effect of long-term storage of cryopreserved boar semen on the fertilizing ability of the sperm after thawing is unknown. The objective of this research was to evaluate boar semen after varying periods of cryopreservation for its ability to successfully penetrate oocytes and support early embryonic development *in vitro*. Oocytes derived from abattoir ovaries were matured in TCM199-based defined maturation medium for 40 to 42 h. Before fertilization, oocytes were denuded by vortexing with hyaluronidase and placing into modified Tris buffer (mTBM) fertilization medium. Straws were thawed at 50 °C for 45 s and 2.5 ml of semen was layered onto a 45/90% Percoll (Sigma, St. Louis, MO) gradient and centrifuged for 20 min at 1000 × *g*. The remaining semen was used for post-thaw analyses. After Percoll separation, the sperm pellet was washed with 4 ml D-PBS (Gibco, Grand Island, NY) with 1 mg/ml BSA (Sigma). For each boar (*n* = 47), the percentage of motile sperm (0–100%), the rate of forward progression (0, no motility to 5, rapid forward movement) and initial concentration were evaluated post-thaw. Oocytes were co-incubated with sperm ( $5.0 \times 10^5$ /ml) for 5 h. Oocytes were randomly selected 12 h post-insemination to assess pronuclear formation. The remaining oocytes were cultured in NCSU23 for 144 h, at which time blastocyst development was recorded. Each boar was examined in two replicates. The following additional parameters were analyzed; % cleavage (CLG), % morula and blastocyst (MOR/BLST), % blastocyst (BLST), % normal penetration (2PN), % polyspermic penetration (PPN) and % total penetration (PN). Data collected for each boar were averaged. Results were analyzed using simple linear regression to determine correlation of measured parameters with duration of cryopreservation. The duration of storage time of cryopreserved samples varied from 10 to 231 months. Of the 47 boars, sperm of 83% exhibited >20% motility post-thaw. Sperm from only one boar did not penetrate oocytes after IVF. Sperm from 87% of the boars penetrated more than 15% of inseminated oocytes. *In vitro* fertilization with sperm from 91% of the boars resulted in embryonic cleavage >50%, and sperm from 77% of the boars resulted in blastocyst development >10%. None of the parameters examined were significantly correlated with the duration of cryopreservation (Table 1). This study demonstrates that boar semen can be successfully cryopreserved and stored more than 19 years without reducing the ability of the sperm to be used successfully in an *in vitro* embryo production system. Thus, successful germplasm preservation has been achieved by cryopreservation of boar semen.

Table 1

Lack of correlation between length of cryopreservation of boar semen and fertilization and development parameters *in vitro*

	% Motile	Progression Score	CLVG	% MOR/BLST	% BLST	% 2PN	% PPN	% PN
Mean ± S.E.M.	30.55 ± 1.48	2.65 ± 0.08	64.62 ± 1.64	22.40 ± 1.61	16.30 ± 1.16	31.00 ± 1.80	16.68 ± 1.74	46.00 ± 3.13
Correlation coefficient <sup>a</sup>	0.03	-0.19	0.26	0.15	0.10	-0.15	-0.38	-0.27
(R <sup>2</sup> ) <sup>b</sup>	0.001	0.04	0.07	0.02	0.01	0.02	0.14	0.07

<sup>a</sup> Pearson's correlation coefficient values are between -1 and 1; values near zero indicate no linear relationship.

<sup>b</sup> Coefficient of determination values are between 0 and 1; values near zero indicate no linear relationship.

# Artificial Insemination

## DEEP INTRAUTERINE INSEMINATION IN SOWS: FIRST FIELD TRIAL IN A USA COMMERCIAL FARM WITH A NEWLY DEVELOPED DEVICE

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A procedure for deep intrauterine insemination (DUI) in non-sedated sows has been recently described [Martinez et al., *Reproduction* 2002;123:163–170]. However, this technology has not been applied in the USA under field conditions. The objective of this study was to assess the reproductive performance of weaned sows when DUI with a reduced number of sperm was used in a commercial swine unit in the USA. A total of 105 weaned sows (average 21 days post-farrowing, parity 2 to 6) were divided into two treatment groups. In both groups, heat detection was performed once a day and all sows were inseminated at 0 and 24 h after the onset of estrus. Sows were also inseminated at 48 h after first heat detection if they were in estrus at this time. One group ( $n = 54$ ) was inseminated with  $150 \times 10^6$  total sperm in 5 ml semen extender using a new catheter (1.8 m length, 4 mm outer diameter) that allows the placement of the sperm deep inside one uterine horn. This dose of semen was taken directly from the same batch of semen used to inseminate the control sows. For deep intrauterine insemination procedures, a standard AI spirette was introduced into the vagina and a cervical lock was obtained. The DUI catheter was introduced through the AI spirette, the cervix was cleared, and the DUI catheter was advanced deep inside one uterine horn. Semen ( $150 \times 10^6$  total sperm) was deposited and the catheter was flushed with 4 ml of semen extender to clear the semen from the catheter. As a control, 51 weaned sows were inseminated following standard AI procedure for the farm. A total dose of  $3 \times 10^9$  total sperm in 100 ml semen extender was inseminated intracervically with the aid of an AI spirette. Pregnancy diagnosis was performed 30 to 35 days after onset of estrus by ultrasound. Pregnancy and farrowing rate were analyzed using CATMOD procedure of SAS. Litter size was analyzed using SAS-GLM procedure. Results of the field trial are presented in [Table 1](#). Pregnancy and farrowing rate did not differ ( $P > 0.25$ ) between DUI and control groups. However, differences ( $P < 0.01$ ) were found in the number of total piglets born and born alive between the groups. The results obtained in this first field trial are very encouraging. Although differences between treatments were found in the number of total piglets born and born alive, the overall performance of the DUI group seems promising considering a 20-fold reduction in the number of sperm inseminated under field conditions. Further experiments are underway to establish the minimal number of sperm necessary to obtain fertility results that are equivalent to the standard AI technique.

Table 1  
Results of DUI field trial with a reduced number of sperm

Treatment	No sows	Pregnant (%)	Farrowed (%)	Total born $x \pm$ S.E.M.	Born alive $x \pm$ S.E.M.	Stillborns $x \pm$ S.E.M.	Mummies $x \pm$ S.E.M.
Control ( $3 \times 10^9$ sperm)	51	47 (92%)	46 (90%)	$12.9 \pm 0.49^a$	$11.2 \pm 0.50^a$	$1.3 \pm 0.33$	$0.3 \pm 0.08$
DUI ( $150 \times 10^6$ sperm)	54	46 (85%)	45 (83%)	$10.5 \pm 0.49^b$	$9.0 \pm 0.51^b$	$1.27 \pm 0.34$	$0.2 \pm 0.08$

<sup>a,b</sup>Values within a column having superscripts are different ( $P < .01$ ).

EFFECT OF ECG ON PREGNANCY RATES OF LACTATING ZEBU BEEF COWS TREATED WITH CIDR-B DEVICES FOR TIMED ARTIFICIAL INSEMINATION

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The aim of this trial was to verify the efficacy of eCG treatment at the moment of CIDR-B device withdrawal on the pregnancy rates (PR) in lactating Zebu beef cows submitted for fixed-timed artificial insemination (FTAI). Two hundred and fifteen lactating Nelore cows (*Bos indicus*) with a post-partum period of  $75.0 \pm 19.4$  days were randomly assigned to two different homogenous groups. On Day 0, cows in Group eCG ( $n = 108$ ) and Control ( $n = 107$ ) received an i.m. injection of 2 mg of estradiol benzoate (EB, Benzoato de Estradiol, Syntex, Argentina) and a CIDR-B device (1.9 g of P4, Pharmacia, Brazil). On Day 8, all cows were injected i.m. with 150 µg de D(+) cloprostenol (Prolise, Tecnopec, Brazil) and the CIDR-B was removed. Cows in eCG Group received 400 IU of eCG i.m. (Novormon, Tecnopec, Brazil) at the time of CIDR-B removal. Cows in both groups received 1 mg of EB i.m. 24 h after CIDR-B withdrawal and were FTAI 30 to 34 h after EB administration. Ovarian ultrasonography (Aloka SSD 500, Aloka Corp., Japan) was performed on Day 0, to determine ovarian status and 30 days after FTAI to determine pregnancy status. Cows were classified, based on the ultrasound examination on Day 0, in: A = cows with a CL; B = cows with follicle(s) >8 mm; C = cows without a CL or follicles >8 mm. Pregnancy rates were compared by chi-square test. Pregnancy rate of the eCG Group (55.1%; 59/107) was higher ( $P < 0.05$ ) than that of the Control Group (38.9%; 42/108). Results summarized in the table, show the pregnancy rate in each group based on the ovarian ultrasound examination on Day 0. We concluded that eCG treatment at the time of CIDR-B withdrawal increased pregnancy rates after FTAI in lactating *Bos indicus* beef cows, and the effect was more evident as the anestrus condition was more pronounced.

Acknowledgments: Tecnopec and Pharmacia.

	Control % PR (no.)	eCG % PR (no.)	% difference on PR eCG vs. Control	P
A	55.5 (15/27)	64.0 (16/25)	+8.5	0.37
B	34.4 (22/64)	50.0 (29/58)	+15.6	0.06
C	29.4 (05/17)	56.5 (13/23)	+27.1	0.08

## THE EFFECTS OF ESTRADIOL CYPIONATE AT CIDR REMOVAL AND INTERVAL TO FIXED-TIME AI ON PREGNANCY RATES IN BEEF CATTLE

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The objectives of this research were to compare pregnancy rates following treatment with estradiol cypionate (ECP) at two intervals or estradiol benzoate (EB) 24 h after CIDR removal and to determine the effect of insemination time on pregnancy rate and fetal sex ratio. In Experiment 1, Angus crossbred beef heifers ( $n = 300$ ) were scanned ultrasonically for cyclicity and reproductive tract abnormalities, and received a CIDR device (Vetrepharm Can Inc., Belleville, Ont., Canada) plus 5 mg of estradiol-17 $\beta$  and 100 mg of progesterone i.m. (E-17 $\beta$  + P4; Sigma Chemical Co., St. Louis, MO, USA). Seven days later, CIDR were removed and 25 mg of dinoprost (Lutalyse, Pharmacia Animal Health, Orangeville, Ont., Canada) were given. Heifers were randomly assigned to three groups to receive 0.5 mg of ECP (Pharmacia Animal Health) at CIDR removal (ECP0;  $n = 98$ ), or 24 h later (ECP24;  $n = 99$ ) or 1.0 mg of EB (Sigma Chemical Co.) 24 h after CIDR removal (EB24;  $n = 103$ ). The interval from CIDR removal to fixed-time AI (FTAI) was 54 to 56 h (ECP0 group) or 56 to 58 h (ECP24 and EB24 groups). Pregnancy diagnosis was conducted by ultrasound 28 days after FTAI. Forty-nine heifers (16.3%) were prepuberal at the beginning of the experiment, but pregnancy rate was not different from those that were cycling (61.2 and 64.1%, respectively;  $P < 0.7$ ). Moreover, pregnancy rates did not differ among groups (62/98, 63.3%, 64/99, 64.6%, and 65/103, 63.1% for ECP0, ECP24 and EB24 groups;  $P = 0.96$ ). In Experiment 2, 78 lactating beef cows and 34 beef heifers received a CIDR and 2 mg of EB on Day 0. On Day 7, CIDR were removed and 500 mg of cloprostenol (Estrumate, Schering Plough Can Inc., Pointe-Claire, Que., Canada) were injected, and 1 mg of EB was injected 24 h later. Cattle were divided into two groups for FTAI (one bull for cows and another for heifers) at 24 or 36 h after EB treatment. Heifers were examined ultrasonically every 12 h for 72 h after EB treatment to determine time of ovulation. Ultrasonographic examinations for pregnancy diagnosis (all cattle) and fetal gender determination (heifers only) were done 28 and 61 days after FTAI, respectively. Two heifers (5.8%) did not ovulate and were excluded from data analysis. Overall, pregnancy rates were 49.1 and 69.8% ( $P < 0.03$ ), for inseminations done at 24 and 36 h, respectively (61.1 and 85.7% for heifers, and 43.5 and 64.1% for cows). Two heifers (5.8%) ovulated between 24 and 36 h after EB treatment and 30 heifers (88.2%) ovulated between 36 and 60 h after EB treatment. Two of 18 heifers (11.1%) inseminated 24 h after EB treatment ovulated within 12 h, whereas, 11 of 14 heifers (78.5%) inseminated 36 h after EB treatment ovulated within 12 h. Numerically, more heifers inseminated at 24 h after EB treatment had male fetuses (7/11, 63.6%) than those inseminated at 36 h (6/12, 50.0%;  $P = 0.4$ ). In summary, ECP treatment at CIDR removal resulted in acceptable fertility to FTAI in heifers when E-17 $\beta$  was used to synchronize follicular wave emergence. Pregnancy rate was higher following FTAI at 36 h than at 24 h after treatment with EB to synchronize ovulation (60 h versus 48 h after CIDR removal).

EFFECT OF ECG TREATMENT IN POSTPARTUM BEEF COWS SYNCHRONIZED WITH PROGESTERONE VAGINAL DEVICES AND ESTRADIOL BENZOATE AND INSEMINATED AT A FIXED-TIME

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Although treatments with progesterone-(P4) releasing devices and estradiol benzoate (EB) have resulted in synchronous ovulation and acceptable pregnancy rates at a fixed-time AI (FTAI) in cows and heifers, results in postpartum beef cows have often been lower than expected. Two experiments were designed to evaluate if treatment with eCG at the time of P4 device removal, would increase pregnancy rates in a FTAI program in postpartum beef cows. Experiment 1 was performed in 481 lactating beef cows (Angus and Polled Hereford, 60 to 80 days postpartum) with a body condition score of 2.5 to 3.5 (1–5 scale) in 4 replicates (Replicate 1,  $n = 150$ ; Replicate 2,  $n = 69$ , Replicate 3,  $n = 143$  and Replicate 4,  $n = 119$ ). At the beginning of each replicate (Day 0), all cows received a P4 device (1 g of P4, Triu-B, Elast écnica, Argentina) and 2 mg EB (Syntex, Argentina). On Day 8, Triu-B devices were removed and all cows received 150 µg D(+) cloprostenol (Preloban, Intervet, Argentina). Cows were randomly divided to receive 400 IU eCG (Novormón 5000, Syntex, Argentina) on Day 8 (eCG group), 1 mg EB i.m. on Day 9 (EB group) or eCG on Day 8 and EB on Day 9 (eCG + EB group). Cows were not observed for signs of estrus and were FTAI 52 to 56 h after Triu-B removal. Ovarian activity was estimated by ultrasonography on Day 0, and there were 336/481 (69.9%) cows with a CL and 145/481 (30.1%) cows with follicles  $\geq 8$  mm in diameter. Experiment 2 was designed to evaluate the same treatments in 181 lactating postpartum Braford (1/3 Brahman, 5/8 Hereford) cows (60–90 days postpartum) with a body condition score of 1.5 to 2.5, except that PRID devices (1.55 g of P4; Sanofi, France) were used. Ovarian activity was estimated by rectal palpation on Day 0, and there were 66/181 (36.5%) cows with a CL, 67/181 (37.0%) with palpable, medium-size follicles and 48/181 (26.5%) with ovaries containing no detectable structures. In both experiments, pregnancy rates were determined by ultrasonography 30 days after FTAI and data were analyzed by logistic regression (Infostat<sup>®</sup>). In Experiment 1, there was a replicate effect ( $P < 0.05$ ), due to a lower pregnancy rate across treatments in replicate 3 than in the other three replicates. There was no effect of ovarian status at the time of treatment ( $P = 0.3$ ); however, there was a treatment effect, attributed to a lower pregnancy rate ( $P < 0.05$ ) in the eCG Group than in the EB or eCG + EB groups (Table 1). In Experiment 2, there was no effect of ovarian status at the time of treatment on pregnancy rates to FTAI ( $P > 0.7$ ), but there was a treatment effect ( $P < 0.05$ ), attributed to a lower pregnancy rate in the eCG group than in the eCG + EB group (Table 1). The EB group was intermediate between the eCG + EB and eCG groups, and not different from either. Results suggest that the use of eCG at the time of removal of a P4 releasing device in a FTAI program in postpartum beef cattle is less satisfactory than EB 24 h after P4 device removal. This is probably a reflection of a more variable interval from treatment to ovulation in cows treated with eCG than in those treated with EB. Although the combination of eCG + EB did not significantly improve pregnancy rates, the numerical increase in cows in Experiment 2 warrants further investigation.

Table 1

Pregnancy rates in postpartum beef cows treated with P4 releasing devices and eCG at device removal (Day 8), EB on Day 9 or eCG on Day 8 plus EB on Day 9

Experiment	eCG (%)	EB (%)	eCG + EB (%)
1	76/158 (48.1) <sup>b</sup>	101/163 (62.0) <sup>a</sup>	89/160 (55.6) <sup>a</sup>
2	11/63 (17.4) <sup>b</sup>	15/56 (26.7) <sup>ab</sup>	22/62 (35.5) <sup>a</sup>

<sup>a,b</sup>Proportions within the same row with different superscripts differ ( $P < 0.05$ ).

## ASSESSMENT OF MITOCHONDRIAL ACTIVITY IN RAM SEMEN THROUGH STAINING WITH THE FLUORESCENT PROBE JC-1

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Mitochondria, which are localized on the midpiece, provide the necessary energy for sperm motility. Hence, any changes in mitochondrial metabolism have a reflect on cell movement ability. Therefore, development of assays to detect alterations in the mitochondrial membrane potential is of importance in sperm assessment. The aim of the present work is to evaluate the use of the fluorescent probe JC-1 (molecular probes) as an indicator of mitochondrial function in pre-frozen and post-thawed ram semen, and its assessment through microscopy or flow cytometry. This cationic compound has been reported to differentially stain mitochondria with high (orange) and low (green) membrane potential. The orange fluorescence is due to formation of multimers known as J-aggregated. Also, JC-1 can stay as monomers and emit green fluorescence. Ejaculates of five Churra breed rams (five replicates of each animal) were evaluated before freezing and after thawing. Semen (5 µl) was diluted in 1.5 ml of Hepes-BSA medium which contained 2 µl of 0.1 mM JC-1. Samples were incubated at 37 °C for 30 min. The fluorescence was quantified manually by epifluorescent microscope (M) and automatically by flow cytometer (FC). We observed two sperm subpopulations: orange and green stained spermatozoa. Also, individual (IM) and progressive motility (PM), were determined by a computerized analysis system (Motility Analyzer 7.4G, Hamilton Thorn Research<sup>TM</sup> [IGER-OUADA M, Verstegen, Theriogenology 2001;55:733–749]). The statistical analysis was carried out using the SAS<sup>TM</sup> program. Normality of the variables was verified (UNI ARIATE), and the Student's *t*-test was used to compare data from these techniques. The correlation coefficients were also determined (coefficient of Pearson) among orange population and motility (IM, PM). While the results show no significant differences ( $P < 0.05$ ) in pre-frozen semen among M and FC (67.66% versus 62.43%, respectively), in post-thawed semen there are significant differences between these techniques (24.54% versus 38.12%, respectively). Hence, in post-thawed semen, the method is a factor of variation. We have also found some correlations. In pre-frozen semen, IM is significantly correlated with JC-1 staining (0.62 and 0.68 by M and FC, respectively), while PM presents a significant correlation only for FC ( $r = 0.54$ ). In post-thawed semen, IM and PM are significantly correlated with JC-1 staining both for M and FC (0.62 and 0.58, respectively, in IM; 0.58 and 0.66, respectively, in PM). The correlations obtained among JC-1 staining and motility show that motility is a parameter which depends on the mitochondrial status of the spermatozoa. However, these correlations are not very high, due to the fact that motility is a variable which depends on a lot of factors. Our results show the ability of the fluorescent probe JC-1 to distinguish functional mitochondria in ram sperm both by microscopy or flow cytometry. We suggest the use of cytometry because it is a less time-consuming and more objective method for semen assessment. This study was supported in part by Diputacion Provincial de alladolid and Junta de Castilla y León.

	Individual motility	Progressive motility	Orange population	
			Flow cytometer	Microscope
Pre-frozen	87.89 ± 1.88 <sup>a</sup>	55.20 ± 2.60 <sup>a</sup>	62.43 ± 5.51 <sup>a,α</sup>	67.66 ± 3.83 <sup>a,α</sup>
Post-thawed	66.11 ± 3.38 <sup>b</sup>	49.924 ± 2.74 <sup>a</sup>	38.12 ± 5.27 <sup>b,α</sup>	24.54 ± 3.38 <sup>b,β</sup>

All of the data are expressed in mean ± S.E.M. Different superscripts (a, b) in each column indicate significant differences ( $P < 0.05$ ); different superscripts (α, β) in each row indicate significant differences ( $P < 0.05$ ) between M and FC.

PREGNANCY RATES AFTER FIXED-TIME ARTIFICIAL INSEMINATION IN BEEF CATTLE, USING ESTRADIOL BENZOATE ASSOCIATED WITH PGF<sub>2α</sub>

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The present work evaluated pregnancy rates after fixed-time AI, using low-cost treatments with estradiol benzoate, that have been shown to synchronize ovulation [Fernandes et al., *Theriogenology* 2001;55:1521–1532]. In Experiment 1, Nelore (*Bos indicus*) cows were assigned into three groups: GP, GPE and EPE. In the GP group ( $n = 99$ ), the animals were treated with a GnRH agonist (8 mg of buserilin acetate, Conceptal (R), i.m., D 0) followed by PGF<sub>2α</sub> 7 days later (150 mg of D-cloprostenol, Preloban<sup>®</sup>, i.m., D 7). After PGF<sub>2α</sub> injection, cows from the GP (control group) were observed twice a day to detect estrus, and AI was performed 12 h afterwards. Twenty-four hours after PGF<sub>2α</sub> administration, cows from GPE ( $n = 97$ ) group received an injection of estradiol benzoate (EB, 1 mg, Estrogen (R), i.m., D 8) and 30–36 h afterwards were inseminated without estrous detection. Group EPE ( $n = 99$ ), animals had a CL detected by rectal palpation, received EB (3 mg, i.m., D 0), followed 9 days later by PGF<sub>2α</sub> (150 mg of D-cloprostenol, i.m., D 9), and then EB again (1 mg, i.m.) 24 h after PGF<sub>2α</sub> (D 10). The cows were inseminated 30–36 h after the last EB injection. The rationale for EPE protocol is that, in the presence of a functional CL (progesterone), EB would induce follicular atresia and a new follicular wave in approximately 5 days. On Day 9, injection of PGF<sub>2α</sub> would cause luteolysis and a second administration of EB would induce LH surge and synchronization of ovulation, allowing timed AI. Experiment 2 was similar to the Experiment 1, except that the presence of CL was detected by ultrasonography in all animals and the Nelore cows were allocated into only two groups: GPE ( $n = 67$ , 66% with CL) and EPE ( $n = 67$ , 100% with CL). Pregnancy was diagnosed by ultrasonography 30–35 days after AI. The pregnancy rates after fixed time AI protocols are shown in the table. In the control group (GP, Experiment 1), although the conception rate was 61% (20/33), only 33% (33/99) of the cows were detected in estrus after PGF<sub>2α</sub> administration, consequently, the pregnancy rate was low (20%). Even though the cows were in good body conditions and cycling when the treatments started, the animals had gone through a period of inadequate pasture supply (a particularly long dry season). Therefore, the surprisingly low pregnancy rates (we have reported 43% using GPE protocol) observed after GPE (20–22%) and the other treatments perhaps could be explained by the pasture conditions before starting the protocols. The insufficient food intake, due to poor pasture condition, could have interfered with the development of follicles that would become the ovulatory follicles approximately 2 months later on, and consequently decreased the pregnancy rates after timed AI protocols. Even though the pregnancy rates were not as good as expected, it is concluded that EPE treatment was at least partially effective in synchronizing ovulation to allow timed AI. Additionally, due to EPE low cost and better results obtained, it is a less expensive alternative to the GPE protocol. However, it does require more intensive labor (ultrasonography or rectal palpation) than GPE treatment.

Experiment	Groups (%)		
	GP (control)	GPE	EPE
1	20/99 (20) <sup>a</sup>	19/97 (20) <sup>a</sup>	35/99 (35) <sup>b</sup>
2	–	15/67 (22) <sup>a</sup>	21/67 (31) <sup>a</sup>
1 + 2	20/99 (20) <sup>a</sup>	34/164 (21) <sup>a</sup>	56/166 (34) <sup>b</sup>

Values with different superscripts (a, b) within rows differ ( $P < 0.05$ , chi-square or Fisher's exact test).

THE EFFECTS OF GONADOTROPIN-RELEASING HORMONE (GnRH)  
OR ESTRADIOL CYPIONATE (ECP) TREATMENT AT CIDR INSERTION  
ON REPRODUCTIVE PERFORMANCE OF VIRGIN BEEF HEIFERS

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This study compared the effects of ECP (Pharmacia Upjohn Animal Health) or a GnRH analog (Factrel; Fort Dodge Animal Health) administered at initiation of a 7-day progestin treatment (CIDR; Pharmacia Upjohn Animal Health) on synchrony of estrus and pregnancy rates to AI in virgin beef heifers. Angus and Angus cross-beef heifers ( $n = 137$ ) 14–16 months of age received a CIDR intravaginal insert on Day 0 and 25 mg PGF<sub>2α</sub> (Lutalyse; Pharmacia Animal Health) at CIDR removal on Day 7. Heifers were blocked and randomly assigned by reproductive tract score (Odde et al., 1994) and weight within blocks to receive at CIDR insertion on Day 0 either: (1) no further treatment (control;  $n = 46$ ); (2) 0.5 mg of estradiol cypionate i.m. (ECP;  $n = 45$ ); or (3) 100 µg i.m. of GnRH (GnRH;  $n = 46$ ). Heifers were observed for signs of estrus on Days 7–11 (0–96 h after device removal) and were artificially inseminated 8–12 h after the onset of estrus. Pregnancy diagnosis was performed using ultrasonography 42 days after insemination. Data were analyzed using ANOVA and chi-square procedures.

Although treatment had no effect on the mean interval to estrus ( $P = 0.28$ ), a tendency ( $P = 0.07$ ) was observed for fewer ECP treated heifers (58%, 26/45) to be observed in estrus between 36 and 60 h post-CIDR removal compared to the control (78%, 36/46) and GnRH (76%, 35/46) treatments. A treatment difference in conception and pregnancy rates was not detected ( $P = 0.6$ ). Pregnancy rates were higher ( $P = 0.04$ ) for heifers with tract scores of 2–5 (47%, 53/113) than for heifers with a tract score of 1 (17%, 2/12). In conclusion, ECP at CIDR insertion tended to reduce synchrony of estrus compared to GnRH-treated or control heifers. Although the numeric trend in reproductive performance favored GnRH-treated heifers, the data set is of insufficient sample size to confirm this difference statistically (Table 1).

Table 1  
Reproductive performance after treatment with ECP or GnRH at CIDR insertion

	Control ( $n = 46$ )	ECP ( $n = 45$ )	GnRH ( $n = 46$ )
Estrus response (%)	95.6	93.3	97.8
Interval from CIDR removal to estrus (h)	48.8 ± 16.2	51.4 ± 22.4	45.0 ± 17.5
Conception rate (%)	45.2	40.5	51.1
Pregnancy rate (%)	43.2	37.5	50.0

## ANNEXIN V-FITC/PI ASSAY IN BULL AND RAM SEMEN

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We have carried out an assay using the fluorescentprobe Annexin V-FITC in ram semen in order to obtain some preliminary results for its application in this species. Annexin is a protein which binds specifically to phosphatidylserine, which is situated in the inner leaflet of the plasmatic membrane and only appears in the outer leaflet when it is disrupted. Annexin V-FITC, that fluoresce green, is used jointly with propidium iodide (PI), which fluoresce red and stains cells with a damaged plasmatic membrane. Thereby cells with a slightly damaged plasma membrane (that are not stained by PI, but with Annexin V-FITC) can be detected. This technique has been used to assess human and bull sperm. We have used bull samples because there are previous reports of this species, but not of ram sperm. We used ram (18 males, Churra breed) and bull (13 males, Morucha breed) frozen semen doses, one straw per male. Straws were thawed and motility was visually assessed on a phase contrast microscope (400×). Each sample was defined as of good or bad quality, according to its progressive motility (PM, see Table 1). This was considered high if  $PM \geq 40\%$  or low if  $PM < 40\%$ . Annexin V-FITC Apoptosis Detection Kit II (Pharmingen) was used for Annexin assay, and we followed the protocol included with the kit. A Becton-Dickinson FACScalibur™ flow cytometer was used to detect red (PI) and green (Annexin V-FITC) fluorescence. We obtained four cell subpopulations for each sample: red stained (PI+, Annexin V-FITC-), green stained (PI-, Annexin V-FITC+), red and green stained (PI+, Annexin V-FITC+) and unstained cells (PI-, Annexin V-FITC-). Statistical analysis of the data was carried out with the SAS™ package. A non-parametric test (NPAR1WAY procedure, Wilcoxon test) was used to compare groups of data, considering motility quality as a factor of variation.

In bull sperm, we observed significant differences between high and low motility groups within the unstained population. No differences were found for ram sperm. These differences were expected, as bull samples were more heterogeneous than ram samples (S.D. of progressive motility were 21.92 for bull and 12.23 for ram). Furthermore, the number of samples we used in this experiment was rather low, so better results would be expected when more samples are analyzed. Cytometer calibration and settings were the same for bull and ram semen, and the behavior of the samples when analyzing was quite similar. However, further research is necessary to determine the usability of this technique for ram semen.

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Table 1

Results of the Annexin V-FITC/PI assay (mean  $\pm$  S.E.M.), by straw quality

Species	PM*	n	PI+ Annexin+	PI+ Annexin-	PI- Annexin+	PI- Annexin-
Bull	High	4	5.69 $\pm$ 3.54	30.41 $\pm$ 7.86	1.15 $\pm$ 0.96	62.76 $\pm$ 3.41 <sup>a</sup>
	Low	9	21.68 $\pm$ 9.36	47.67 $\pm$ 7.43	0.72 $\pm$ 0.42	29.93 $\pm$ 5.43 <sup>b</sup>
Ram	High	9	45.34 $\pm$ 3.17	25.68 $\pm$ 4.67	1.53 $\pm$ 0.43	27.45 $\pm$ 2.54
	Low	9	44.58 $\pm$ 7.66	30.26 $\pm$ 6.18	1.62 $\pm$ 0.68	23.53 $\pm$ 4.29

Different superscripts (a, b) indicate significant differences within species. \*Bull: high 52.50  $\pm$  4.79%; low 10.67  $\pm$  2.99% (mean  $\pm$  S.E.M.) \*Ram: high 44.44  $\pm$  1.76%; low 24.44  $\pm$  2.69% (mean  $\pm$  S.E.M.).

## USED CIDR AND INJECTABLE PROGESTERONE FOR RESYNCHRONIZATION OF ESTRUS IN FIXED-TIME INSEMINATED BEEF HEIFERS

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The objective was to investigate the efficacy of a previously used CIDR device, with or without a commercial progesterone preparation, for resynchronization of estrus in beef heifers not pregnant to fixed-time AI (FTAI) in CIDR-based protocols. In Experiment 1, a once-used CIDR (Vetrepharm Can, Inc., Belleville, Ont., Canada) was placed in 79 heifers from 13 to 20 days after FTAI, and an additional 80 heifers served as untreated controls. Mounting activity was monitored electronically (HeatWatch; DDx Inc., Denver, CO, USA) from 10 days after FTAI to 6 days after CIDR removal; re-inseminations were done 6–12 h after the onset of estrus. Ultrasonographic pregnancy diagnosis was conducted 28 days after FTAI and was repeated 52 days after FTAI in heifers that had been rebred. For the two resynchronization groups: 32 and 34 heifers, respectively, were not pregnant to FTAI; the interval from FTAI to the onset of estrus was  $22.1 \pm 1.2$  days (range, 21.3–25.2) versus  $19.0 \pm 2.1$  days (range, 15.3–22.4; means,  $P < 0.001$ ; variance,  $P < 0.07$ ); estrus rates were 71.9% versus 85.3% ( $P < 0.07$ ); conception rates were 60.9% versus 72.4% ( $P < 0.4$ ); and pregnancy rates (in heifers not pregnant to FTAI) were 43.3% versus 61.8% ( $P < 0.14$ ). In addition, three heifers given a used CIDR and one control heifer that were detected in estrus 18–23 days after FTAI (and inseminated) were subsequently diagnosed pregnant to FTAI. In Experiment 2, a once-used CIDR was placed in 979 heifers  $13 \pm 1$  days after FTAI and was removed 7 days later. Estrus was detected (by visual observation) between 21.5 and 25.5 days after FTAI in 336 heifers (mean and mode, 22.5). Rebreeding and pregnancy diagnosis were conducted as described in Experiment 1. Pregnancy rate to FTAI was 56.4% and the conception rate to rebreeding was 70.8% (overall pregnancy rate, 80.7%). Ninety (21.1%) heifers diagnosed non-pregnant to FTAI were not detected in estrus. In Experiment 3, 616 heifers were given a once- or twice-used CIDR, with or without a concurrent injection of 150 mg progesterone (progesterone 5%; Vétoquinol N.-A Inc., Lavaltrie, Que., Canada) in a  $2 \times 2$  factorial design,  $13 \pm 1$  days after FTAI. The CIDR devices were removed after 7 days. Estrus detection, rebreeding and pregnancy diagnosis were conducted as described in Experiment 2. Pregnancy rate to FTAI was 47.2%; estrus was detected in 71.7 and 66.0% of non-pregnant heifers that received a once- or twice-used CIDR, respectively (NS). Estrus rate (63.8% versus 73.8%,  $P < 0.051$ ) and conception rate (60.5% versus 70.6%,  $P < 0.1$ ) tended to be reduced in heifers injected with progesterone at the time of used CIDR reinsertion. In summary, reinsertion of a previously used CIDR 13 days after FTAI with removal on Day 20 synchronized returns to estrus over a 4-day interval, with acceptable conception rates. However, injecting progesterone at CIDR insertion tended to reduce both estrus and conception rates. Although resynchronization of estrus increased the proportion of heifers rebred over a short interval, additional studies are needed to improve estrus detection efficiency.

EFFECT OF COLLECTION METHOD, TIME AND TRANSPORT MEDIUM ON A PCR TEST FOR *TRITRICHOMONAS FOETUS* IN BULLS

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*Tritrichomonas foetus* is a flagellated protozoon causing significant reproductive losses in susceptible females. Infection in bulls is inapparent. Semen donors must be confirmed free of the disease before semen is utilized. Samples collected for culture are susceptible to the effects of time delay, sample handling and contamination by other organisms [Skirrow et al., Vet Bull 1988;58:591–603]. The objectives of this study were to investigate the effect of sampling method, the addition of guanidinium thiocyanate (GuSCN), and sample storage on the sensitivity and specificity of a PCR diagnostic test for *T. foetus*.

Five infected and eight control bulls were used. The positive bulls were sampled six times over a period of 18 days. Control bulls were sampled three times ( $n = 6$ ), four times ( $n = 1$ ) or six times ( $n = 1$ ). Sheath washing and sheath scraping were performed in that order. A GuSCN stock solution was added to half of each sample. A portion of the GuSCN-free sample was cultured, while both the GuSCN-treated and GuSCN-free samples were subjected to DNA extraction within 6 h, after 30 h and after 5 days of storage at 4 °C. DNA isolation was done using a GuSCN and silica method [Boom et al., J Clin Microbiol 1990;28:495–503]. PCR was performed using the method of Felleisen et al. with minor modifications [Felleisen et al., J Clin Microbiol 1998;36:513–519]. Primer TFR3 and TFR4 were used with an annealing temperature of 60 °C. PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. A two-tailed chi-square test was used to test for differences between treatments. Results for infected bulls are given in the Table 1. The sensitivity of culture was 83% for both sampling methods. The sensitivity of the PCR ranged from 0.9 in sheath washing samples extracted within 6 h to 0.41 in sheath scrape samples with GuSCN extracted after 5 days. Holding time reduced sensitivity for samples collected by both methods at 5 days, but there was no significant effect at 30 h. Sampling method had no effect with the exception of samples held for 5 days with GuSCN, where sheath washing gave higher sensitivity than sheath scraping. The addition of GuSCN had no effect on test sensitivity. No samples from the eight control animals subjected to any of the 12 treatments gave a positive PCR result. We conclude that the current PCR is as sensitive as culture when performed within 6 h and is highly specific. PCR tests done after a time delay of up to 30 h will render adequate sensitivity if serial sampling is done. The addition of the chaotropic agent guanidinium thiocyanate has no effect on test results. PCR is more rapid than culture.

Table 1

Sensitivity of testing of samples from infected bulls collected by different methods and held for differing times prior to PCR testing<sup>a</sup>

	Culture		PCR after 6 h		PCR after 30 h		PCR after 5 days	
	Wash	Scrape	Wash	Scrape	Wash	Scrape	Wash	Scrape
Positives	24	24	26	24	20	18	18	12
Number tested	29	29	29	29	29	29	29	29
Sensitivity <sup>#</sup>	0.83	0.83	0.9 <sup>a</sup>	0.83 <sup>b</sup>	0.69	0.62 <sup>a'</sup>	0.62 <sup>a'</sup>	0.41 <sup>a'b'</sup>

<sup>a</sup> Data for samples with GuSCN are not shown.

<sup>#</sup> Treatments marked a and a', and b and b' differ in sensitivity significance ( $P < 0.05$ ).

EFFECTS OF A CIDR OR MGA, REPRODUCTIVE STATUS, AND ESTRADIOL,  
GNRH OR PLH TREATMENT ON PREGNANCY RATE TO  
FIXED-TIME AI IN BEEF HEIFERS

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The objectives were to investigate the effects of progestins, reproductive status, and treatments to synchronize follicular wave emergence and ovulation, on pregnancy rates to fixed-time AI (FTAI) in 2379 beef heifers in several experiments. Heifers were either given a CIDR (Vetrepharm Canada Inc., Belleville, Ont.) or fed 0.5 mg per days melengestrol acetate (MGA; Pharmacia Animal Health, Orangeville, Ont.) for 7.0–8.5 days. At the start of progestin treatment, ultrasonographic imaging was done and heifers were classified as: proestrus (CL < 15 mm, largest follicle  $\geq$  10 mm); metestrus (CL < 15 mm, largest follicle < 10 mm); diestrus (CL  $\geq$  15 mm); or prepuberal (no CL). Pregnancy rates (26–30 days after FTAI) were not different ( $P < 0.85$ ) between heifers given a CIDR (53.6%, 995/1856) and those fed MGA (53.0%, 277/523). Pregnancy rates were 51.8% (99/191), 49.3% (141/286), 55.9% (623/1115), and 50.0% (132/264) for proestrus, metestrus, diestrus, and prepuberal CIDR-treated heifers, respectively ( $P = 0.11$ ); in MGA-treated heifers, pregnancy rates were 65.5% (19/29), 51.0% (25/49), 59.2% (122/206), and 46.4% (111/239;  $P < 0.03$ ). In cycling versus prepuberal heifers, pregnancy rates were 54.2% versus 50.0% ( $P < 0.21$ ) in CIDR-treated heifers and were 58.4% versus 46.4% ( $P < 0.01$ ) in MGA-treated heifers. In a subset of 257 CIDR-treated heifers, pregnancy rates for cycling versus prepuberal heifers were 54.8% (23/42) versus 90.0% (9/10;  $P < 0.07$ ) in heifers given estradiol benzoate (Sigma Chemical Co., St. Louis, MO; 2 mg at CIDR insertion and 1 mg 24 h after CIDR removal), and were 61.4% (105/171) versus 55.9% (19/34;  $P < 0.6$ ) in heifers given either 100  $\mu$ g GnRH (Cystorelin; Merial Canada Inc., Victoriaville, Que.) or 12.5 mg pLH (Lutropin; Vetrepharm Canada Inc.) in lieu of estradiol benzoate. In another subset of 983 CIDR-treated heifers, there was no significant difference in pregnancy rates between heifers given 1 mg estradiol cypionate (ECP; Pharmacia Animal Health, Orangeville, Ont.) plus 50 mg progesterone (progesterone 5%; Vétquinol N.-A Inc., Lavaltrie, Que.) versus 100  $\mu$ g GnRH (Fertagyl; Intervet Canada Inc., Whitby, Ont.) at CIDR insertion for heifers that were metestrus, diestrus or prepuberal (means: 51.9%, 81/156; 58.2%, 350/601; and 51.3%, 61/119). However, for proestrus heifers, pregnancy rate was lower in heifers given ECP versus GnRH (40.7%, 24/59 versus 62.5%, 30/48;  $P < 0.04$ ). These 983 heifers were given 0.5 mg ECP (at CIDR removal or 24 h later) or were given 100  $\mu$ g GnRH at FTAI (52 h after CIDR removal); pregnancy rates were 51.2, 64.2 and 51.0%, respectively ( $P < 0.01$ ). For these three treatments, pregnancy rates were: 50.0% (19/38), 75.9% (22/29) and 32.5% (13/40) for heifers in proestrus ( $P < 0.002$ ); 47.1% (24/51), 57.9% (33/57) and 50.0% (24/48) for heifers in metestrus ( $P < 0.51$ ); 51.6% (99/192), 63.0% (128/203) and 59.7% (123/206) for heifers in diestrus ( $P < 0.06$ ); and were 56.1% (23/41), 70.7% (29/41) and 24.3% (9/37) for prepuberal heifers ( $P < 0.001$ ). In heifers without a functional CL at CIDR insertion (proestrus and prepuberal), pregnancy rates were significantly reduced when GnRH was used to synchronize ovulation. In conclusion, pregnancy rates were similar in heifers receiving short-term treatment with either a CIDR or MGA. Although pregnancy rates in prepuberal heifers approached those of cycling heifers, stage of the estrous cycle and the hormones used to synchronize follicular wave emergence and ovulation seemed to have a greater influence on pregnancy rates.

EFFECTS OF ESTRADIOL AND PROGESTERONE ON PLASMA STEROID  
AND GONADOTROPIN CONCENTRATIONS IN CIDR-TREATED  
OVARIECTOMIZED COWS

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Four experiments were conducted to evaluate the effects of estradiol and progesterone on gonadotrophin release in CIDR-treated cows that had been ovariectomized (OVX) approximately 1 year prior to use. In Experiment 1, 16 cows received either a new CIDR or nothing on Day 0 (beginning of the experiment) and on Day 5, half of each group received an intramuscular injection of either 5 mg estradiol-17 $\beta$  (E-17 $\beta$ ) or 5 mg E-17 $\beta$  + 100 mg of progesterone (P4) in oil. Plasma P4 concentrations increased by 24 h after CIDR insertion to  $6.2 \pm 0.8$  ng/ml ( $P < 0.01$ ) and then declined to  $2.8 \pm 0.2$  ng/ml by 72 h. Plasma LH concentrations declined by 6 h ( $P < 0.05$ ) and surged 18–24 h after E-17 $\beta$  treatment, whether animals received injectable P4 or not. There was a decrease in plasma FSH concentrations by 6 h ( $P < 0.01$ ) and resurgence by 48 h after E-17 $\beta$  treatment. In Experiment 2, 16 cows receiving a used CIDR and 5 mg E-17 $\beta$  on Day 0 were assigned to four groups to receive 0, 25, 50, or 100 mg i.m. of P4. Plasma P4 concentrations were elevated by 24 h in all groups ( $P < 0.01$ ). Plasma LH concentrations decreased by 36 h ( $P < 0.01$ ), returning to pretreatment concentrations at 84 h after treatment. There was no significant effect of progesterone treatment on plasma FSH concentrations. In Experiment 3, 16 cows received a used CIDR on Day 0 and were assigned to three groups to receive 5 mg E-17 $\beta$  or 5 mg E-17 $\beta$  + 100 mg P4 on Day 0, or 5 mg E-17 $\beta$  on Day 1. CIDR were removed on Day 7 and 1 mg i.m. estradiol benzoate (EB) was administered to all cows 24 h later. There was an effect of time and a treatment-by-time interaction on plasma P4, estradiol, LH, and FSH concentrations ( $P < 0.01$ ). Administration of 100 mg of P4 at CIDR insertion resulted in an increase ( $P < 0.01$ ) of 2.0 ng/ml plasma P4 over the used CIDR alone ( $4.0 \pm 0.6$  ng/ml). The use of CIDR alone suppressed LH for 6 h. Estradiol-17 $\beta$ , with or without P4, induced an LH surge by 24 h and suppressed plasma FSH for approximately 48 h. Plasma P4 concentrations declined to baseline by 12 h after CIDR removal and 1 mg EB 24 h later suppressed LH and FSH for 6 h ( $P < 0.02$ ), followed by an LH surge at 24 h. In Experiment 4, 16 cows received a used CIDR and 5 mg i.m. of E-17 $\beta$ , EB or estradiol valerate (EV) plus 100 mg P4 on Day 0. There was an effect of time and a treatment-by-time interaction on plasma estradiol, LH, and FSH concentrations ( $P < 0.01$ ). Elevated plasma estradiol concentrations occurred sooner and reached a higher peak after E-17 $\beta$  treatment than after EB or EV ( $P < 0.01$ ). Plasma estradiol returned to baseline by 36 h in E-17 $\beta$ -treated and by 96 h in EB- and E-treated cows. Plasma FSH decreased by 12 h after all estradiol treatments ( $P < 0.01$ ), reached a nadir at 24 h and increased by 60 h in all groups. However, plasma FSH remained higher in E-17 $\beta$ -treated than in EB- or EV-treated cows ( $P < 0.02$ ). While both new and used CIDR devices increased plasma P4 in OVX cows, an injection of 100 mg P4 at CIDR insertion resulted in an additional 2.0 ng/ml. EB and EV suppressed FSH for longer intervals than E-17 $\beta$ . The administration of 1 mg of EB 24 h after CIDR removal resulted in an LH surge 24 h later.

## THE USE OF PLASMA PROGESTERONE CONCENTRATION TO PREDICT THE OPTIMAL BREEDING TIME IN BITCHES 6 DAYS IN ADVANCE

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Practicing veterinarians and owners need a method whereby they may find out in advance when a bitch should best be inseminated. Such predictability has various advantages. It will facilitate the use of chilled semen because it provides an early warning when the semen has to be collected, chilled and shipped; it also provides owners with an early indication when they should transport animals for mating. Such predictability may also reduce the number of visits to a veterinarian during the heat period of a bitch. The sooner one can predict when to inseminate a bitch, the greater these benefits. The identification of the LH peak may provide such an advanced warning, but LH assays are not generally available. In contrast, sensitive and accurate assays to determine the concentration of progesterone in serum or plasma (PPC) are more generally available. Concannon [Concannon et al., *Biol Reprod* 1977;17:604–613] has shown that PPC starts rising concomitantly with the onset of the LH surge in bitches. The aim of this study was to determine whether the first rise above 6 nmol/l of PPC can be used to predict when to best inseminate bitches. Fourteen bitches of nine breeds of a variety of sizes were each used during one estrous cycle. The heat periods were usually monitored daily throughout pro-estrus and estrus by means of vaginal cytology and vaginoscopy, using a simple Perspex tube and an otoscope as light source. PPC was usually measured daily but sometimes with 2-day intervals starting during late pro-estrus and ending in late estrus. The first decrease in edema of the vaginal mucosa, characterized by shrinking rounded folds, was evaluated as a predictor of the period during which PPC was likely to start rising. All bitches were inseminated twice with a 1-day interval between inseminations, except for one bitch that was inseminated once only, 6 days after the rise in PPC. The first insemination was done 4, 5, 6 or 7 days after the initial rise of PPC in 1, 3, 9 or 1 bitch, respectively. Frozen-thawed semen was deposited into the uterus in all cases. Semen quality and source varied. Eight bitches were inseminated 6 and 7 days after the onset of the rise and all conceived with a mean litter size of 5.6 (S.D. 3.11). Thirteen bitches were inseminated 5 days or more after the onset of the rise and all conceived with a mean litter size of 6.0 (S.D. 2.71). In the 10 bitches where all data are available, the edema in the vagina started to decrease 1.3 (S.D. 1.75) days before the rise in PPC, with a range of 3 days before to 2 days after the first decrease in edema. In these 10 bitches the edema in the vagina had sufficiently decreased to cause angularity of the mucosal folds 3.0 (S.D. 1.17) days after the rise in PPC. Bitches whelped 65.0 (S.D. 2.00) days after the rise in PPC, 59.3 (S.D. 2.07) days after the first insemination and 55.9 (S.D. 1.40) days after the onset of cytological dioestrus. The interval between the first decrease in edema and whelping was 63.6 (S.D. 5.10) days, whereas the interval between the onset of angularity of the vaginal folds and whelping was 60.8 (S.D. 3.96) days. Vaginoscopy alone is not sufficiently sensitive to determine the optimal time for insemination with frozen-thawed semen. This study shows that it is suitable to inseminate bitches 6 and 7 days after PPC first exceeds 6 nmol/l vaginal oedema starts to decrease at least 4 days before the first insemination is due, making vaginoscopy a suitable tool with which to determine when to start measuring PPC.

COMPARISON OF ANDROMED<sup>®</sup> AND TRIS-EGG YOLK EXTENDER BOVINE POST-THAW SPERM FUNCTION PARAMETERS AND IN VITRO FERTILITY

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AndroMed<sup>®</sup> (Minitüb, Germany) is a commercially available bull semen extender in which egg yolk is replaced by sterile, chemically defined ingredients. It was our interest to determine whether there are differences in post-thaw semen quality, classified by functional in vitro sperm tests and in vitro fertility, between the egg yolk-free AndroMed<sup>®</sup> extender and the widely used TRIS egg yolk-citrate extender (TRIS). Ejaculates (3) from three Simmental bulls (15–20 months, proven fertility) were collected, processed (0.25 ml straws,  $20 \times 10^6$  spermatozoa) as split-samples using AndroMed<sup>®</sup> or TRIS extender and cryopreserved under appropriate conditions. (1) After thawing (38 °C, 25 s), each straw was divided (v/v) and viable sperm were separated by different swim up (SU) procedures (non-capacitating/capacitating conditions, heparin 2 µg/ml), in TALP (2.6 mM CaCl<sub>2</sub>; 6 mg/ml BSA). Subsequently, both SU-supernatants were treated with Ca-Ionophore A23187. Acrosomal status was determined after permeabilization of sperm cells with 95% ethanol and staining with FITC-conjugated *Pisum sativum* agglutinin (FITC-PSA; 100 µg/ml, Sigma). Appearance of acrosome reaction (AR) was evaluated after SU, prior (spontaneous AR) to as well as after exposure to different in vitro capacitating conditions (induced AR). (2) Zona pellucida (ZP) binding ability (ZPBa) of SU-separated, in vitro capacitated (heparin, 2 µg/ml, 45 min) sperm onto bovine ZP (frozen stored, non-matured oocytes, –20 °C, >20 days) was examined (10 oocytes/400 µl, sperm concentration  $2 \times 10^6$  cells/ml). After 3 h coincubation, ZP/spermatozoa entities were washed by multiple pipetting in distilled water to remove loosely ZP-attached sperm. Remaining, tightly bound sperm were liberated by ZP-lysis (sodium citrate, pH 2, 2 min) and evaluated under phase contrast optics [Arch Anim Breeding 2001;44:118–120]. (3) In vitro embryo production (IVP), [Theriogenology 2000;53:411] with oocytes (TRIS,  $n = 2036$ ; AndroMed<sup>®</sup>,  $n = 1806$ ) collected from abattoir ovaries was performed. The cleavage rate and the rate of blastocyst formation was determined on the third and seventh day after fertilization, respectively. With the exception of bull individual differences, laboratory sperm function parameters resulted in no significant statistical differences between AndroMed<sup>®</sup> or TRIS-processed sperm. Experiment 1 revealed that the number of spontaneously acrosome-reacted sperm cells varied according to ejaculate, but spontaneous AR was not significantly different between the extenders. With respect to responsiveness of sperm cells to the different capacitating conditions, a ranking was observed (heparin < A23187 < heparin + A23187). Also in this regard, there was no extender-related effect. Results in ZPBa experiments (sperms/ZP:  $12.5 \pm 6.17$  versus  $12.4 \pm 5.62$ ) and IVP (cleavage rate 54% versus 57%, blastocysts rate 13% versus 13%, AndroMed<sup>®</sup>, TRIS, respectively) showed no differences between the ejaculate/extender used. According to our results, AndroMed<sup>®</sup> and TRIS extenders offer equal functional in vitro quality for bull semen cryoprocessing. However, considering various practical factors (e.g. extender preparation, quality standards, hygiene, cost of material, working temperature) AndroMed<sup>®</sup> seems to be superior to TRIS for routine use in bovine AI centers.

PREGNANCY RATES AFTER ESTROUS INDUCED BY PGF<sub>2α</sub>, CIDR+, AND LH TREATMENTS FOR LONG DAYS OPENED HOLSTEIN COWS

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The aim of this study was to compare different heat induction methods for long time opened Holstein cows. The averages of age, body weight and period of days open of the cows used in this study were  $8.2 \pm 2.0$  year,  $574.0 \pm 64.4$  kg and 284–328 days, respectively. After examination of the ovaries using an ultrasonographic probe (5.0 MHz, SA600, South Korea), they were grouped into three groups: G1; corpus luteum (CL) stage, G2; large follicle (follicle size: A 15 mm and not CL stage and small CL, and G3; middle follicles (size: 5–10 mm) and small ovary, not CL stage. G1 group was with PGF<sub>2α</sub> (Pharmacia & Upjohn, Belgium, 25 mg per head, IM), with G2 group was treated with CIDR+ (InterAg, NZ) was placed in the vagina for 7 days, with PGF<sub>2α</sub>, and GnRH hormone. G3 group was injected PGF<sub>2α</sub>, CIDR+ was placed in the vagina for 7 days. Fixed AI was conducted at 72 h after injection of PGF<sub>2α</sub> and 20 µg GnRH (buserelin acetate) was given at the time of AI. Pregnancy detected in all group was done at 60 days after AI by an ultrasonographic machine (Table 1).

This study suggest that for heat induction of long period open cows (G1 group cows were PGF<sub>2α</sub> injection, G2 group cows were CIDR+ + PGF<sub>2α</sub> and GnRH injection, G3 group cows were CIDR+ + PGF<sub>2α</sub> injection), all methods were effective methods.

Table 1  
Pregnancy rate after AI by ovaries and uterus stimulation for long days opened Holstein

Group	Methods	No. of heads	Open days (mean ± S.D.)	Pregnancy (%)
G1	PGF <sub>2α</sub>	23	284 ± 192.4	10 (43.5)
G2	CIDR + PGF <sub>2α</sub> + GnRH	22	232 ± 156.0	11 (50.0)
G3	CIDR + PGF <sub>2α</sub>	23	328 ± 181.1	12 (52.2)

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST RAM SPERMATOZOA AS CITOCEMISTRY PROBES TO EVALUATE SEMINAL QUALITY

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Conventional seminal parameters have little value as fertility predictors, so there is a tendency to consider physiological characteristics of sperm, such as capacitation, acrosome reaction or zona binding ability. Molecular Probes that bind cellular components can be used to analyze functional parameters of sperm. We have selected a group of monoclonal antibodies against ovine spermatozoa, which could be considered as tools for seminal evaluation. Inbred BALB/c mice were immunized intraperitoneally with 1 ml sperm suspension ( $25 \times 10^6$  cells/ml). Reinjection was done 4 weeks later and cell fusion 3–4 days later. Spleen cells from immunized mice were prepared in sterile RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, and 0.5% gentamicin. Cells were fused with myeloma SP2 cells in 50-ml centrifuge tubes, in a proportion of 5:1. After 15 min of centrifugation at 1200 rpm, a mixture of RPMI 1640 (55%) and PEG 1450 (45%) was added, resting for 5 min. Cells were then resuspended in RPMI, centrifuged for 10 min at 500 rpm, and resuspended in RPMI with 4% HAT (hypoxanthine 0.01 mM, aminopterin 0.1 mM, thymidine 0.016 mM) at  $1$  to  $2 \times 10^6$  cells/ml. Ninety-six-well plates (COSTAR) were seeded and kept in a humidified incubator (37 °C and 5% CO<sub>2</sub>). After a week, medium was substituted by RPMI with 4% HT (hypoxanthine 0.1 mM, thymidine 0.016 mM). Supernatants were screened on ovine spermatozoa smears, and positive hybridomas were cloned by limiting dilution ( $0.5 \times 10^6$  cells/ml). Positive clones were expanded into 24-well plates (COSTAR) and 100-mm Petridishes. Supernatants were stored at 4 °C. Hybridoma cells were frozen in cryovials with 75% RPMI, 20% fetal calf serum, and 10% DMSO. Screening of hybridoma supernatants was carried out by indirect immunofluorescence with ram spermatozoa (RSp). Cross-reactivity of positive supernatants was assessed with goat (GSp) and bull spermatozoa (BSp) and on frozen sections of ovine testis (OTe) and kidney (Oki). Sections were fixed with acetone for 5 min, washed with PBS and incubated with each supernatant for 1 h at room temperature in a humidified chamber. Next they were washed in PBS and incubated for 30 min with FITC-conjugated rabbit immunoglobulin (Dakopatts) at 1:40 in PBS. Sections were washed with PBS, contrasted with Carazzi hematoxylin, dehydrated, and mounted. We carried out five fusions, yielding 10 antibodies with a significant binding pattern: 6-5F11 (RSp+, GSp+, BSp+, OTe+, Oki+), 8-4B4 (RSp+, GSp+, BSp+, OTe+, Oki+), 10-3F2 (RSp+, GSp+, BSp+, OTe-, Oki-), 10-4E3 (RSp+, GSp+, BSp+, OTe+, Oki+), 10-5F1 (RSp+, GSp+, BSp-, OTe-, Oki-), 11-1F9 (RSp+, GSp+, BSp+, OTe+, Oki+), 11-1H2 (RSp+, GSp+, BSp+, OTe-, Oki-), 11-2A2 (RSp+, GSp-, BSp-, OTe-, Oki-), 11-2C3 (RSp+, GSp+, BSp+, OTe+, Oki+). The most specific of these antibodies were tested to define their binding pattern on spermatozoa (Table 1). Antibodies 10-3F2 and 11-1H2 have a high topographic specificity for the acrosomal region and the middle tract, respectively. However, the antibody 10-5F1 only labels the sperm head. These antibodies could be used as diagnostic probes of the sperm cell status. At this moment we are evaluating its application on ram semen in the many stages of the cryopreservation process.

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Table 1  
Binding pattern of the selected monoclonal antibodies

Antibody	Acrosomal region	Post-acrosomal region	Midpiece	Tail
10-3F2	+	–	–	–
10-5F1	+	+	–	–
11-1H2	–	–	+	–
11-2A2	+	+	–	+

+ : high fluorescence, – : no fluorescence.

## EFFECT OF SEMEN EXTENDER ON OOCYTE PENETRATION CAPACITY BY PORCINE SPERMATOZOA IN VITRO

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The objective of this experiment was to determine the effect of five different commercially available porcine semen extenders on spermatozoa penetration in oocytes. Four boars were collected and ejaculates were split among five different semen extenders (A, B, C, D, and G). Insemination doses were prepared with 3 billion total spermatozoa in 70 ml of extended semen. Two insemination doses for each extender were prepared at each time period for each boar. Only one dose was prepared if a boar had less than 42 billion spermatozoa in a single ejaculate. Two sub-samples were prepared from the extended dose for evaluating penetration and observations were made at the time of collection (Day 0) and on Days 3, 5, and 10 post-collection. If there were live spermatozoa on Day 15, data was recorded then as well. Each sample was stained on the day of observation and compared with a differentially stained sample from a fresh ejaculate from the same boar by co-incubation with an orange/red fluoro probe (DiQ) or a yellow/green fluoro probe (DiO, DiOC-16, or BODIPY-C16, Molecular Probes, Eugene, Oregon, USA) for 30–90 min. Fluoro probe co-incubation time depended upon the individual probe's binding affinity for each boar, and was predetermined for each boar separately, in order to optimize stain-binding and motility. Equal amounts of each aged and fresh sample were added to a droplet containing 20 cumulus-intact oocytes, at a ratio of 100 spermatozoa per oocyte. Penetration of extended samples was measured after 6 h by counting the number of each differently labeled-spermatozoa bound to the oocytes, and expressed as a percentage. The data were analyzed with a repeated measures design using ANOVA procedures for categorical data. The model included time (day), extender (treatment), and the time by extender interaction. There was a treatment by time interaction, so additional analyses were done to examine differences among extenders at each time point. The model was sorted by time and included extender and ejaculate as a block. Days 10 and 15 values for extender G were significantly higher than all other extenders. No other differences were observed. This data suggests that initial treatment of porcine spermatozoa with commercially available extenders can have a profound difference in penetration in oocytes following long-term storage (beyond 5 days)

Extender	Day 0 <sup>1,2</sup>	Day 3	Day 5	Day 10	Day 15
A	na (16)	40.1 ± 6.7 (16)	35.4 ± 6.9 (16)	15.4 ± 7.7 <sup>a</sup> (16)	6.3 ± 5.4 <sup>a</sup> (16)
B	na (16)	35.8 ± 8.2 (16)	31.3 ± 7.6 (16)	12.5 ± 5.4 <sup>a</sup> (16)	7.6 ± 4.1 <sup>a</sup> (16)
C	na (16)	41.8 ± 4.2 (16)	28.2 ± 4.4 (16)	20.1 ± 6.5 <sup>a</sup> (16)	3.3 ± 1.8 <sup>a</sup> (16)
D	na (16)	45.1 ± 6.1 (16)	30.1 ± 6.7 (16)	19.9 ± 7.3 <sup>a</sup> (16)	5.1 ± 3.4 <sup>a</sup> (16)
G	na (16)	45.4 ± 6.8 (16)	44.2 ± 5.7 (16)	41.9 ± 7.3 (16) <sup>b</sup>	25.9 ± 6.1 <sup>b</sup> (16)

Numbers within columns with different superscripts (a, b) are different ( $P \leq 0.05$ ).

<sup>1</sup> Numbers in parentheses under means are number of observations. Numbers in the table represent proportion of aged spermatozoa that penetrated cumulus-intact oocytes ± S.E.M., expressed as a percent.

<sup>2</sup> No observations were performed on Day 0, since there was no aged semen for comparison. Average number of spermatozoa penetrating cumulus-intact oocytes was, however, evaluated and found not different among extenders.

## RELATIONSHIP BETWEEN POSTPARTUM FOLLICULAR DYNAMICS AND FERTILITY IN DAIRY CATTLE

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Resumption of ovarian activity and involution of uterus play an important role in subsequent fertility of postpartum dairy cattle, and it has been known that most of postpartum follicular development shows wave-like manner as cyclic cattle. The postpartum first ovulation could be observed within 2 week after calving in dairy cows; however, it is still controversial whether the early occurrence of the ovarian cyclicity improves the reproductive performance of modern dairy cattle. The aim of this study was to investigate the postpartum follicular dynamics in relation to subsequent fertility in dairy cattle. Fifty lactating (26 primiparous and 24 multiparous) Holstein cows fed diet to meet maintenance, growth and lactation requirement were studied. The ovaries and uterine horns were examined by ultrasonography using a 5-MHz linear rectal transducer 3 × weeks beginning on Day 6–8 postpartum and continuing until at least confirmation of the second ovulation. A dominant follicle (DF) was defined as the largest follicle that grew to a diameter of  $\geq 10$  mm and at least 2 mm larger than other follicles. As an index for uterine involution, postpartum interval for the difference in diameter between the gravid and non-gravid horns becoming within 5 mm was recorded. The mean daily milk yield during 10 weeks postpartum was also recorded. Cows exhibited standing estrus or mounting activity were considered to be in estrus. After 45 days of voluntary waiting period, estrous cows with normal cyclicity were served AI. Conception was confirmed by ultrasonography at 35–40 days after AI. Data were analyzed by ANOVA or chi-square test, and  $P < 0.05$  was considered significant. The postpartum first DF ovulated in 23 (15 primiparous) cows (Group 1), the second to fourth DF did in 17 (8 primiparous) cows (Group 2), and in another 10 (three primiparous) cows ovulation occurred after more than four waves of DF emergence or after the first DF developing follicular cyst (Group 3). Postpartum intervals to the first ovulation were significantly different among the three groups (mean  $\pm$  S.D.:  $18 \pm 5$ ,  $32 \pm 8$  and  $58 \pm 14$  days, respectively). This difference in the timing of first ovulation positively affected the subsequent timing of first estrus ( $43 \pm 16$ ,  $57 \pm 18$  and  $80 \pm 16$  days) and first AI ( $63 \pm 11$ ,  $71 \pm 17$  and  $92 \pm 17$  days), and negatively affected the duration of first ovarian cycle ( $19 \pm 4$ ,  $14 \pm 6$  and  $12 \pm 5$  days). Uterine diameter involution was significantly more delayed in Group 1 ( $20 \pm 4$  days) than in Groups 2 and 3 ( $16 \pm 3$  and  $16 \pm 4$  days, respectively). There were no significant differences in the conception rate at 150 days postpartum (83, 82, and 90%), mean number of AI required ( $1.7 \pm 0.8$ ,  $1.6 \pm 0.8$  and  $1.2 \pm 0.7$ ), and mean days open ( $79 \pm 24$ ,  $86 \pm 27$  and  $92 \pm 14$ ). Mean milk yield in Group 3 ( $42 \pm 5$  kg per day) was significantly larger than that in Group 1 ( $33 \pm 8$  kg per day) but was not significantly larger than that in Group 2 ( $37 \pm 7$  kg per day). In conclusion, 80% of the postpartum first ovulation occurred within four follicular waves, and the differences in postpartum follicular dynamics affected some reproductive traits but might not affect days open in dairy cattle.

COMPARISON OF BOVINE SPERM QUALITY AFTER 6 DAYS OF STORAGE IN CAPROGEN<sup>®</sup> OR CEP-DILUTER

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A new diluter for fresh bovine semen, based on the composition of cauda epididymal plasma (CEP-diluter), has been developed in our laboratory. The efficacy of the CEP-diluter for prolonged sperm preservation was compared to that of the Caprogen<sup>®</sup> fresh semen of four bulls was stored at a concentration of  $10 \times 10^6$  spermatozoa/ml in either CEP- or Caprogen-diluter at 5 °C in airtight glass tubes. The sperm concentration was determined by means of a Bürker counting chamber. Sperm quality was determined before cooling and after 2, 4 and 6 days of storage. The percentage of membrane intact (MI) spermatozoa was determined by means of LIVE/DEAD<sup>®</sup> Sperm Viability Kit (Molecular Probes) and fluorescent microscopy. The following sperm motility parameters were determined with the Hamilton Thorne (HTM-Ceros 12.1) on at least 500 spermatozoa: velocity average path (VAP), velocity straight line (VSL), amplitude lateral head (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) and percentage of progressively motile spermatozoa (PMS). Before assessment, samples were incubated during 10 min in open air at 37 °C. The experiment was repeated three times, and the results were analyzed using the linear mixed effect model (SPSS, 2002). Time had a negative effect on all sperm quality parameters, except for the ALH and LIN (Table 1). No significant differences were found between the two sperm diluters for AP, PMS and percentage of MI spermatozoa. The VSL, BCF, STR and LIN were significantly higher for the spermatozoa stored in the CEP-diluter, while ALH was higher for the spermatozoa stored in Caprogen<sup>®</sup> (Table 1). The higher values for VSL, BCF, STR and LIN for spermatozoa stored in the CEP-diluter indicate that they are moving faster and more straight lined than the spermatozoa stored in the Caprogen<sup>®</sup>-diluter. The higher value for ALH for spermatozoa in the Caprogen<sup>®</sup>-diluter may indicate an increased proportion of hyperactivated spermatozoa. In conclusion, better sperm motility values are obtained during a 6-day storage period in the CEP-diluter than in the Caprogen<sup>®</sup>-diluter.

Table 1

Effect of time and medium, CEP- vs. Caprogen<sup>®</sup>-diluter, on the percentage of membrane intact spermatozoa (MI, %) and on motility parameters assessed by the Hamilton Thorne

Parameter	Time	CEP vs. Caprogen <sup>®</sup>
VAP	$P < 0.01$	$P = 0.28$
VSL	$P < 0.01$	$P < 0.01$
ALH	$P = 0.31$	$P < 0.01$
BCF	$P < 0.01$	$P = 0.10$
STR	$P = 0.01$	$P < 0.01$
LIN	$P = 0.16$	$P = 0.01$
PMS	$P < 0.01$	$P = 0.56$
% MI	$P < 0.01$	$P = 0.48$

# Cloning/Nuclear Transfer

## EFFECT OF TIMING OF OOCYTE-CELL FUSION ON DEVELOPMENTAL POTENTIAL OF BOVINE NUCLEAR TRANSFER EMBRYOS FROM CUMULUS CELLS

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We previously reported that in vitro developmental ability of nuclear transfer (NT) embryos from bovine adult skin fibroblasts was affected by timing of fusion and chemical activation [Akagi et al., *Theriogenology* 2001;55:252]. In this study, we compared in vitro and in vivo developmental potential of NT embryos produced by two different fusion and activation protocols. As donor cells for NT, bovine cumulus cells of passage 5 were used following culture in serum-deficient medium for 5–7 days. Oocytes were enucleated after in vitro maturation in TCM 199 supplemented with 10% fetal bovine serum. Enucleated MII oocytes were fused with cumulus cells by a DC pulse of 25 V/150  $\mu$ m for 10  $\mu$ s in Zimmerman mammalian cell fusion medium 21 or 24 h post-maturation (hpm). NT embryos fused at 21 hpm were activated chemically 3 h post-fusion (F21A24 group) and embryos fused at 24 hpm were activated chemically immediately post-fusion (F24A24 group). Chemical activation was accomplished by calcium ionophore (10  $\mu$ M) for 5 min and cytochalasin D (2.5  $\mu$ g/ml) + cycloheximide (10  $\mu$ g/ml) for 1 h then cycloheximide alone for 4 h. NT embryos were subsequently cultured in chemically defined medium (IVD-101, Research Institute of Functional Peptide Co., Ltd.) at 38.5 °C in 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>. On Day 7 after NT, embryo transfer was performed. Each synchronized recipient received one blastocyst. Fusion rates were 89% (157/176) and 91% (61/67) in F21A24 and F24A24 groups, respectively. Development rate to the blastocyst stage of NT embryos in F21A24 group was higher than in F24A24 group (Table 1). Pregnancy rates did not differ significantly between F21A24 and F24A24 groups, and 13 recipients are still pregnant after more than Day 210. These results suggest that timing of fusion and chemical activation affects development rate to the blastocyst stage of NT embryos from cumulus cells, but not pregnancy rate.

Table 1

In vitro and in vivo development of NT embryos derived from cumulus cells

Groups	No. of eggs	No. of cleaved (%)	No. of blastocysts (%)	No. of ET	No. of pregnancies (%)		
					Day 35	Day 120	Day 210
F21A24	147	106 (72.1) <sup>a</sup>	67 (45.6) <sup>c</sup>	13	11 (84.6)	10 (76.9)	9 (69.2)
F24A24	59	51 (86.4) <sup>b</sup>	12 (20.3) <sup>d</sup>	7	5 (71.4)	5 (71.4)	4 (57.1)

Different superscripts within columns differ significantly (a, b:  $P < 0.05$ , c, d:  $P < 0.01$ ; chi-square test).

PRODUCTION OF SHEEP NUCLEAR TRANSFER EMBRYOS FROM  
ROSCOVITINE-TREATED ADULT SOMATIC CELLS

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Cell cycle synchronization of the donor cell to the desired stage (G0/G1) of the cell cycle plays a major role in the success of nuclear transfer (NT) in mammals. This study was conducted to determine the developmental capacity of sheep MII oocytes reconstructed by NT using adult ear fibroblast donor cells synchronized with the CDK 2 inhibitor, roscovitine. A primary cell line of ear fibroblasts was established from a 4-year-old adult female sheep using typical cell culture techniques. Following first passage, the cells were frozen in DMEM-F12 supplemented with 20% FCS and 10% DMSO. After thawing, cells were cultured (DMEM supplemented with 10% FCS, penicillin/streptomycin) to approximately 80% confluence. Roscovitine (30  $\mu$ mol) was added to the culture medium 24 h prior to NT. The roscovitine-treated cells were exposed to the inhibitor until NT. As a control, serum-starved (72 h prior to NT, with 0.5% FCS) donor cells were prepared from the same established cell line. Differences between groups were analyzed using one-way ANOVA after arc-sine transformation of proportional data. The electro fusion rate was not significantly different ( $P \leq 0.05$ ) between the roscovitine-treated group (80.2%) and the serum starved group (82.1%) in six replications (240 couplets). The blastocyst development at Day 7, in the roscovitine treated group (19.2%) was significantly lower ( $P \leq 0.05$ ) than the serum-starved group (33.1%). The number of cells as determined on Giemsa stained embryo spreads in the blastocyst derived from roscovitine-treated group was significantly higher ( $P \leq 0.05$ ) than those derived from serum-starved group (roscovitine-treated group:  $134 \pm 8$  cells; serum-starved group:  $104 \pm 4$  cells). Although the blastocyst development rate is lower in the roscovitine treated group, this study highlights that the higher cell number of these blastocysts suggests an increased nuclear reprogramming capacity of the roscovitine-treated adult somatic cells. (Funded by NSERC, OMA-FRA, FSBC and Toticell, Inc.).

A COMPARATIVE INVESTIGATION OF THE CHARACTERISTICS OF HOLSTEIN COWS CLONED FROM COLOSTRUM-DERIVED MAMMARY GLAND EPITHELIAL CELLS IN AN AUTOMATIC MILKING SYSTEM

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Recently, many cloned cows have been produced using embryos or various somatic cells. However, little has been reported concerning the genetic traits and individual characteristics of cows cloned from somatic cells. This study examined the characteristics of the first cows in the world to be cloned from colostrums-derived mammary gland epithelial (MGE) cells [Kishi et al., *Theriogenology* 2000;54:675–684] in an automatic milking system (AMS). Two MGE cell-cloned cows derived from the colostrum of the same Holstein cow were compared to eight individual cows (control cows) produced by artificial insemination (AI). Cloned cows of the first lactation and control cows of the second lactation were housed in an AMS. We measured the number of times the cloned cows entered the AMS (times/week) and milk yield (kg/week) each day, and the body weight and body condition scoring (BCS) every 2 weeks, over a 20-week period after delivery. The cloned cows' milk compositions (milk fat percentage, milk protein percentage, milk lactose percentage, solids-non-fat percentage, and total solid percentage) were determined every 4 weeks over a 16-weeks period after delivery. In the cloned cows, the interval of the first to third postpartum ovulation and follicular development per any cycle were observed every other day using ultrasonography. The length of gestation, situation of delivery, and estrous cycle were compared between the cloned cows and the control cows. Dates were analyzed by ANOVA. Significant differences were seen between the two cloned cows regarding the number of times they entered the AMS (mean  $\pm$  S.D.,  $4.9 \pm 2.0$  versus  $5.7 \pm 2.4$  times) ( $P < 0.05$ ). In milk yield, significant differences were seen at Weeks 1, 9, 11, and 13 between the cloned cows, though in the other weeks they shared similar lactation curves. Concentrations of milk compositions between the cloned cows showed considerable resemblance. Similar changes were seen regarding body weight and BCS between the cloned cows. The first postpartum ovulation was retarded in both cloned cows, and an interval of the first to second postpartum ovulation was shortened. Cloned cows had two waves of follicular development per cycle. Cloned cows and control cows calved normally. No differences were found between cloned cows and control cows in the length of gestation and duration of estrous cycle (mean  $\pm$  S.E.M., 278 days versus 279 days versus  $279.5 \pm 3.1$  days,  $20.4 \pm 3.2$  days versus  $20.3 \pm 2.3$  days versus  $21.3 \pm 2.4$  days). These results demonstrate that cloned cows from colostrum-derived MGE cells are normal in regard to delivery, lactation, and growth, and were similar in regard to the functions of their reproductive physiology. However, differences were observed regarding their activities of entering into the AMS.

## EFFECT OF RECIPIENT CYTOPLAST ON THE KINETICS OF DNA REPLICATION DURING THE 1-CELL STAGE IN BOVINE NUCLEAR TRANSFER EMBRYOS

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In cow, the onset and the duration of the first DNA replication is correlated with the developmental potential of in vitro produced embryos. In the case of nuclear transfer embryos derived from somatic cells, it is known that the use of metaphase II oocytes as recipient cytoplasm generally results in a higher in vitro development of the reconstructed embryos than when using older stages of oocytes. In this study, we wanted to characterize the influence of recipient oocyte cell cycle stage on the kinetics of DNA synthesis in somatic nuclear transfer embryos. We have used bovine enucleated oocytes, either non-activated or activated, to investigate the onset and the length of DNA synthesis in somatic nuclei after they have been transferred into these recipient cytoplasts. Cumulus–oocyte complexes obtained from slaughterhouse ovaries were matured in vitro for 20 h, then enucleated at 22 h. In the non-activated group (NT-MII), the enucleated oocytes were fused with donor cells at 25–26 h post-IVM and activated immediately by incubation in 10 µg/ml cyclo-heximide (CH) and 5 µg/ml cytochalasin B for 5 h. For the activated group (NT-ACT), the enucleated oocytes were activated at 24 h post-IVM by incubation in 7% ethanol for 5 min and in CH for 2 h, fused with donor cells and incubated in CH for an additional 3 h. The donor cells were fibroblasts cultured in starvation condition (0.5% fetal calf serum) for 4–10 days. They were fused with cytoplasts by applying two DC pulses of 2.2 kV/cm for 30 µs. Nuclear transfer embryos were then cultured in B 2 medium supplemented with 2.5% fetal calf serum. At 1 or 2 h interval between 5 and 18 h post-fusion (hpf), embryos from each group were switched to a culture medium supplemented with 5'-bromo-2'-deoxy-uridine (BrdU) and fixed in 2.5% paraformaldehyde after 30 min of incubation. The continuous exposure to BrdU from 5 hpf and fixed at 18 hpf were also performed in both groups. The BrdU incorporation was then assessed by immunocytochemical procedure. At least two replicates at each time point were carried out for all experiments. Data were compared using chi-square test. At 5 hpf, DNA synthesis started in NT-ACT embryos (10 of 27 embryos), whereas it was not observed in NT-MII group (0 of 25 embryos,  $P < 0.001$ ). At 6 hpf, 10 of 53 and 11 of 14 embryos synthesized DNA in NT-MII and NT-ACT groups, respectively ( $P < 0.001$ ). The DNA replication ended at 18 hpf in NT-MII group; however, 9 of 25 embryos (36%) in NT-ACT group still synthesized DNA at this time ( $P < 0.001$ ). Almost all embryos in both groups synthesized DNA (30 of 31 and 26 of 29 embryos in NT-MII and NT-ACT groups, respectively,  $P > 0.10$ ). Development rates to the blastocyst stage were significantly higher in NT-MII group than in NT-ACT group (50.8% versus 25.6% of cultured embryos,  $P < 0.02$ ). These data demonstrate that the DNA synthesis is longer and starts earlier in somatic nuclei transferred into activated cytoplasts than in those transferred into non-activated cytoplasts. We are now trying to determine if this difference brings chromosomal abnormalities which could be associated with the lower number of developing embryos.

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EARLY PROPAGATION OF CLONED GOATS BY LAPAROSCOPIC OVUM  
PICK-UP AND IN VITRO EMBRYO PRODUCTION

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The use of laparoscopic ovum pick-up (LOPU) in young goats followed by IVP was evaluated in the early propagation of cloned goats. Ten kinder goats produced by somatic cell nuclear transfer technology were used as oocyte donors. All goats were hormonally induced into lactation at 2–4 months of age [Camuso et al., Anim Biotechnol 2000;11:1]. Half of the donor animals were subjected to LOPU at 2–3 months of age (prior to induction of lactation), whereas the other five goats were subjected to LOPU at 6–7 months of age (following induction to lactation). Donor goats were stimulated with 80 mg NIH-FSH-P1 (Folltropin<sup>®</sup>, Vetrepharm, Canada) together with 300 IU eCG (Novormon<sup>®</sup>, Vetrepharm, Canada) administered intramuscularly 36 h prior to LOPU. Follicle aspiration and IVM/IVF were performed as described previously [Baldassarre et al., Theriogenology 2002;57:275–284]. At 18–22 h after insemination, the resulting zygotes were transferred into the oviducts of 38 estrus synchronized recipient goats of standard breeds (Nubian, Saanen and Boer). Pregnancy was detected by ultrasound scan at 28 and 60 days of gestation. The number of follicles aspirated and oocytes recovered was higher in the young group of donors; however, oocytes from animals in the late prepubertal age group had higher developmental capacity resulting in higher pregnancy and development rate to term (see table). It is unknown at present if the quality of the oocytes recovered from the animals subjected to LOPU at the later age was affected by the estrogen/progesterone treatment used for induction of lactation prior to oocyte collection. In conclusion, laparoscopic ovum pick up in combination with in vitro embryo production techniques have proven to be a powerful tool for the early propagation of valuable female animals produced by somatic cell nuclear transfer.

Parameter/age at LOPU	2–3 months of age	6–7 months of age	<i>P</i> -value
Number of LOPU donors	5	5	na
Average follicles aspirated	57.0 ± 16	28.0 ± 5	<0.05
Average oocytes recovered	41.0 ± 9	25.8 ± 6	<0.05
Embryos transferred (ET) after IVM/IVF	139	105	na
ET/oocytes recovered (%)	67.8%	81.4%	<0.01
Recipients transferred	23	15	na
Pregnancy at 28 days (%)	9 (40%)	12 (80%)	<0.05
Pregnancy losses	1 (11%)	0	ns
Kids born (average/recipient)	15 (1.9)	27 (2.2)	ns

## APPLICATION OF EMBRYO CLONING TO THE GENERATION OF TRANSGENIC FOUNDER GOATS

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Early and late gestation losses in pregnancies from somatic cell nuclear transfer (NT) have been linked to the incomplete reprogramming of the donor karyoplast. A recent report [Heyman et al., *Biol Reprod* 2002;66:6–13] showed that the incidence of fetal losses in bovine embryo (blastomere) NT was significantly less than in somatic cell NT. We used a re-cloning protocol in an effort to improve the reprogramming of somatic cell donors. Blastomeres from NT embryos derived from transfected somatic cells were utilized as karyoplast donors in a transgenic goat founder program. Transgenes were introduced into female fetal fibroblast cells via lipofection. Cell lines were assayed by PCR, Southern blotting and FISH for the presence of the transgenes, and frozen for future use as nuclear transfer donors. Donor cells were thawed, washed, plated in TCM supplemented with 10% FBS and cultured for 48 h. Cells were then maintained in reduced-serum TCM (0.5%) for 4 days. Ten percent serum was added to cell culture before using as donor cells. Oocytes were collected from superovulated does and submitted to a NT protocol, as previously described [Behboodi et al., *Theriogenology* 2002;57:395]. NT embryos at 1- or 2- to 4-cell stages were embedded in 0.9 to 1.2% agarose chips and then transferred into the oviducts of intermediate recipients for 5 days. Embryos were flushed from the oviducts of intermediate recipients using PBS plus 10% serum. Developed NT embryos (16–32 cells) were selected for re-cloning. The zona pellucida was removed by incubating in 0.2% protease for 5 min. Blastomeres were then detached in PBS without Ca<sup>2+</sup>. After a 10-min incubation in cytochalasin-B, the blastomeres were introduced into the perivitelline space of enucleated oocytes. Couplets were fused by a single electrical pulse (80–100, 30  $\mu$ s) in 500 mm fusion chambers, followed by incubation in cycloheximide for 3 h. The fused couplets were cultured in M-199 for 33–48 h before transfer to oviducts of final recipient does. The pregnancy was confirmed by the detection of fetal heartbeats beginning at 30 days of gestation via ultrasonography. In conclusion, these results at [Table 1](#) indicate that somatic cell generated NT embryos at 16–32 cell stage could be used for embryo cloning in goat.

Table 1  
Recloning of goat NT embryos derived from somatic cell nuclear transfer using transfected fetal fibroblasts  
(*N* = 7)

Donor cells	No. of oocytes	No. of fused	No. of transferred	No. of embryo recovered	No. of pregnancies
Fetal. Fibroblasts	252	204	163 (7)	137 (84%)	N/A <sup>a</sup>
Blastomeres (16–32 cell)	175	147	106 (8)	N/A <sup>a</sup>	1 <sup>b</sup>

<sup>a</sup> Not applicable.

<sup>b</sup> Triplet pregnancy.

PRELIMINARY RESULTS OF BIOCHEMICAL COMPOSITION OF BOVINE  
ALLANTOIC FLUID ASPIRATED REPEATEDLY UNDER TRANSVAGINAL  
ULTRASOUND-GUIDANCE

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Regarding the relative high incidence of placenta abnormalities, conceptus death, dystocia and poor neonatal viability associated with the production of cloned offspring (so-called Large Calve Syndrome), factors contained in the fetal fluids might disclose the possible causes particularly pertaining to placental dysfunction. Little is known about the continuous compositional changes of amniotic and allantoic fluid in cattle, and initially, a technique allowing the systematic chronological analysis of the biochemical parameters of fetal fluids during a continuing bovine pregnancy established following AI is required. From the literature, it is known that up to five repeated transvaginal ultrasound-guided aspiration of fetal fluid may be conducted in the same animal between gestation Days 32 and 101; however, in that particular study in only one of seven animal's pregnancy continued to term [Vos et al., 1990]. The aim of the present study was to establish a method for regular aspirations and analysis of the composition of fetal fluid without interference with pregnancy and to collect preliminary data of the biochemical parameters. In a 15-month-old heifer, allantoic fluid was aspirated repeatedly in gestation week (GW) 10, 12, 16, 18, 20 and 22 using transvaginal ultrasound-guidance. Prior to aspiration, an epidural anaesthesia was applied, and only during the first two aspirations the heifer was sedated i.m. with 2 mg/100 kg detomidine hydrochloride to avoid unrestlessness. For aspiration, an ultrasound-scanner (Aloka SSD 500) equipped with a 5 MHz curved transducer (Aloka UST974-5), a needle guide (Corometrics) and a 17-g single lumen needle (Cook vet Products, V-BOAS-1760-S) were used. Before aspiration, the allantoic fluid was identified ultrasonographically by being less echogenic than the amniotic fluid, and the aspirated allantoic fluid was characterized by its clear, light yellow color and its watery consistency. The aspirated allantoic fluid samples were subjected to assays routinely employed at our laboratory, i.e. spectrophotometry (ADVIA 1650 Bayer) for glucose, creatinine, albumin, total protein and globulins, Ion-selective electrodes (ADVIA 1650 Bayer) for sodium, potassium, chloride and Atomic Absorption (AA800 PerkinElmer) for calcium and magnesium. A total of six repeated aspirations of allantoic fluid was conducted on the same animal between GW 10 and 22. The presence of a life fetus could be confirmed at the time of writing (GW 22). On each aspiration, 15 to 20 ml of fetal fluid were aspirated. The biochemical parameters in the allantoic fluid samples were found within the following ranges: glucose 0.14–0.18 mmol/l; creatinine 1.7–1.9  $\mu$ mol/l; albumin 0.26–0.53 g/l; total protein 0.57–1.77 g/l; globulins 0.22–1.48 g/l; sodium 4.3–125 mmol/l, potassium 0.97–73.5 mmol/l; chloride 27.5–92.8 mmol/l; calcium 0.54–3.0 mmol/l; magnesium 0.33–1.5 mmol/l; estrone sulphate 4.35–713 ng/ml. These preliminary results document the possibility of chronological collection and analysis of bovine allantoic fluid within the same animal throughout the first half of gestation. The study continues currently and results from four more animals will be presented. The described technique offers a valuable tool for future monitoring of the biochemical composition of bovine allantoic fluid during different stages of gestation and may be used to monitor deviations as may be anticipated in the course of pregnancies produced by nuclear transfer (cloned pregnancies).

## SEX-CHROMOSOMAL COMPLEMENT OF DONOR CELLS AND RECONSTRUCTED EMBRYOS DERIVED FROM THEM

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A recent preliminary study of the karyotype of bovine nuclear transfer (NT) embryos derived from somatic cells has revealed a high incidence of aneuploidy. Interestingly, the rate of these anomalies appeared to be similar to that of the donor cells. In order to confirm these results, we extended this study by comparing chromosomal complement in donor cells and NT embryos derived from two different cell lines for which different rates of successful pregnancy after transfer to recipients were observed. Two primary cultures of bovine granulosa and male fibroblast cells were established from follicle aspiration and tissue biopsy culture, respectively. A sub-population of these cells was isolated 48 h after confluency. These cells were separately used for nuclear transfer and for chromosomal analysis by fluorescent in situ hybridization (FISH) using painting probes specific for the bovine X and Y chromosomes (Cambio, UK). NT was carried out as previously described [Bordignon et al., *Biol Reprod* 1999;61:22–30]. The resulting reconstructed embryos were also subjected to fixation and FISH analysis as previously described [Slimane et al., *Biol Reprod* 2000;62:628–635]. A total of 97 cells and 83 embryos at 2 to 4 cell and morula stages were analyzed. Sex chromosome aneuploidy including monosomy and various type of polysomy such as (YY, XYY, XXX, XXXX, XXXXXX) were observed in donor cells (23%,  $n = 53$  and 7%,  $n = 44$  for granulosa and fibroblast cells, respectively) and NT embryos (30%,  $n = 26$  and 9%,  $n = 44$  derived from granulosa and fibroblast donor cells, respectively). The total rate of sex chromosome aneuploidy in donor cells did not differ significantly from that of reconstructed embryos (23% versus 30%,  $P > 0.5$ , for granulosa donor cells and NT embryos reconstructed from them and 7% versus 9%,  $P > 0.9$  for fibroblast donor cells and NT embryos reconstructed from them). However, the rate of abnormalities detected in the reconstructed embryos differed between types of donor cells (30% versus 9%,  $P < 0.001$  for NT embryos derived from granulosa and fibroblast cells, respectively). Overall, there were more anomalies in embryos reconstructed from cell cultures with a higher incidence of chromosomal aberrations. In addition, embryos produced using the cell-line leading to lower full-term pregnancy success rate showed a higher incidence of chromosomal anomalies. Based on these observations, we suggest that selecting NT donor cell lines with low rates of chromosome abnormalities may improve the success rate of the NT procedure. (Funded by NSERC and FSBC). The comparison between groups were made using Chi-square test, differences were considered significant for  $P < 0.05$ .

PRODUCTION OF TRANSGENIC GOATS BY SOMATIC CELL NUCLEAR  
TRANSFER USING TRANSFECTED GOAT ADULT SKIN CELLS

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The development of transgenic animals for the production of therapeutic proteins in milk holds remarkable potential. The use of transfected somatic skin cells as potential karyoplasts for nuclear transfer offers the unique ability to determine desirable specific genetic traits prior to transfection, coupled with ease of collection and longevity in culture. An expression vector was constructed using the goat  $\beta$ -casein promotor containing a transgene encoding a human monoclonal antibody. The expression vector was co-transfected with a neomycin vector into somatic skin cells from a 7-year-old female goat using LipofectAMINE (Gibco BRL, USA). Neomycin resistant colonies were isolated and characterized by PCR, Southern blot and FISH analysis. Selected recombinant cells were maintained in M199 (Gibco BRL, USA) with 0.5% FBS (JRH Biosciences, USA) plus G418 (Sigma, USA) for 3–4 days prior to nuclear transfer. Cells were re-fed with M199 + 10% FBS containing G418 several hours prior to nuclear transfer to initiate cells to re-enter the cell cycle. Ovulated in vivo MII oocytes from superovulated does were surgically collected then enucleated and reconstructed with the transfected donor karyoplast. Couplets were simultaneously fused and activated with a single electrical pulse between 2.6–3.2 kV/cm for 20  $\mu$ s. Embryos were then cultured in SOF/BSA at 38 °C in a humidified modular incubation chamber (Billurps-Rothenberg, USA) containing 6% O<sub>2</sub>, 5% CO<sub>2</sub> and 89% N<sub>2</sub> for 24–48 h. Nuclear transfer embryos with age appropriate development (up to two cells at 24 h or two to eight cell at 48 h post fusion and activation) were surgically transferred to the oviducts of surrogate recipient does. Six recipients were confirmed pregnant at Day 50. Five recipients produced term pregnancies and yielded seven offspring. One offspring died at 2 h post-natal. These results indicate that transfected adult somatic skin cells can be a viable source of donor karyoplasts for the production of transgenic goats by somatic cell nuclear transfer.

Cell	# Donors	# Oocytes	# Recon.	# Fused (%)	# Cleaved (% fused)	# Trans./ # Recip.	# Preg. Recips. (%)	# Offspring (% trans.)
A	16	93	88	64 (73)	32 (50)	51/10	2 (20)	1 (2)
B	24	214	188	156 (83)	74 (47)	108/19	4 (21)	6 (6)
Totals	40	307	276	220 (80)	106 (48)	159/29	6 (21)	7 (4)

EFFECT OF TREATMENT OF SOMATIC CELLS WITH APHIDICOLIN ON THE  
IN VITRO DEVELOPMENT OF CLONED SHEEP EMBRYOS

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Treatment of donor cells with aphidicolin halts the cell cycle in the late G1 or early S phase. In cattle, this treatment had a significant beneficial effect on both the in vitro [Kurosaka et al. *Theriogenology* 2002;57:424] and in vivo [Wells et al., *Theriogenology* 2002;57:456] development of nuclear transfer embryos. The aim of this study was to examine the effect of aphidicolin treatment on sheep donor cells. Frozen and thawed cumulus cells were cultured to passage 4 in Glasgow's medium containing 5% FCS and then serum starved in 0.2% FCS for 4 days. Cells were then passaged and cultured for 18–19 h in Glasgow's medium containing either 5% FCS with 0.2 µg/ml aphidicolin or 0.2% FCS until harvested for nuclear transfer. Oocytes were aspirated from Merino ovaries collected from the abattoir and matured in TCM199 based maturation medium for 18–23 h. Couplets of somatic cell and enucleated oocyte were electrically fused (one pulse, 1.25 kV/cm, 80 µs) at 23–24 h after maturation and activated in 10 µM Ca-Ionophore for 5 min and 2 mM 6-dimethylaminopurine for 2 h. The embryos were then transferred to a modified SOF-HCO<sub>3</sub> medium containing essential and non-essential amino acids and 4 mg/ml BSA and cultured at 38.9 °C in an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> for 7 days. Cleavage rates on Day 2 and blastocyst development were recorded and cell numbers of blastocysts on Day 7 counted under UV microscope after Hoechst 33342 staining. Data were analyzed by ANOVA software. Among the 303 pulsed couplets, the fusion rate, cleavage rate and blastocyst development rates were 76.9, 89.3, and 24.0%, respectively. As shown in the table, there were no significant differences between the two groups in fusion rate, cleavage rate, blastocyst development rate and mean cell numbers of blastocysts. This study indicates that treatment of cumulus donor cells with aphidicolin is not helpful in improving the in vitro development of nuclear transfer embryos in sheep as compared with serum starvation treatment.

Treatment	Couplets fused/pulsed (% ± S.E.)	Embryos cleaved (% ± S.E.)	Blastocysts at Day 7 (% ± S.E.)	Mean blastocyst cell numbers ± S.E.
Aphidicolin	123/156 (79.0 ± 7.9)	111 (90.3 ± 5.3)	27 (20.5 ± 5.1)	102.9 ± 35.1
Serum starved	110/147 (74.7 ± 10.4)	97 (89.1 ± 5.8)	29 (26.7 ± 4.5)	109.9 ± 18.0

Values within the same column are not different significantly ( $P > 0.05$ ) as tested by ANOVA.

PRODUCTION OF BOVINE TRANSGENIC CLONED EMBRYOS USING  
PROUROKINASE-TRANSFECTED SOMATIC CELLS: EFFECT OF TYPE  
AND SIZE OF DONOR CELLS

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In order to produce bioreactors that secrete human prourokinase (proUK) into milk using somatic cell nuclear transfer (SCNT), the present study was performed to determine the efficient type and size of donor cell for the production of proUK-transfected bovine clone embryos. Plasmid containing human proUK gene (target gene; 5 kb) driven by the bovine beta-casein promoter and green fluorescent protein (GFP) gene driven by the S 40 promoter was constructed (named as proUK-GFP). Three types of bovine somatic cells (fetal fibroblast, cumulus cell and adult ear fibroblast) as donor cells were isolated, cultured in vitro and transfected with proUK-GFP plasmid using lipid-mediated (FuGene6; Roche, Germany) method. The GFP expressing cells were selected, and SCNT and in vitro culture were performed by our standard procedure [Cho JK et al., *Theriogenology* 2002;57:1819–1828]. In Experiment 1, three types of transfected cells were randomly selected and the development rates and GFP expression of SCNT embryos were examined under microscope. As results, transgenic NT embryos derived from cumulus cells showed significantly higher development rates (28.7% versus 7.0, 15.2%) and GFP expression rates (54.2% versus 26.7, 28.3%) than those from fetal and ear fibroblast, respectively. In Experiment 2, trypsinized cumulus cells were divided into two groups according to size (<30  $\mu\text{m}$  as small cells and  $\geq 30 \mu\text{m}$  as large cells) and the development rates and GFP expression of SCNT embryos were examined. As results, there were no significant differences in fusion rates (75.6% versus 75.8%), cleavage rates (83.9% versus 79.1%) and GFP expression rates (63.9% versus 61.9%) in blastocyst stage between small and large cumulus cells ( $P < 0.05$ ). However, development rates to blastocyst stage were significantly higher in small cells than large cells (38.7% versus 23.1%). Our results suggest that cumulus cells in small size were the most efficient cells among used for the production of transgenic SCNT embryos.

## DEVELOPMENT OF NUCLEAR TRANSFER BOVINE EMBRYOS DERIVED FROM FETAL FIBROBLASTS

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Donor cell cycle and oocyte activation play a major role in the success of nuclear transfer (NT). Experiments were designed to examine the effects of serum-starvation of donor cell and of different activation treatments after fusion of donor nucleus and enucleated cytoplasm on subsequent development of bovine NT embryos. A primary culture of fibroblasts derived from 1 month gestation was established. Experiment 1 compared the effect of serum starvation of donor nuclei on the rates of cleavage and development into blastocysts of NT embryos. Following 6–8 passages, cells were cultured either in DMEM +10% FCS until confluent or in serum-starved condition (0.5% FCS) for 5 days prior to NT. Cumulus–oocyte-complexes derived from slaughterhouse ovaries were matured for approximately 20 h in TCM199 supplemented with 5% FCS and hormones (LH, FSH and estradiol 17). Expanded cumulus cells were removed from the oocytes by vortexing in 3% sodium citrate. After enucleation, the oocytes were injected with a fibroblast cell and fused using a single DC electric pulse (1.6 kV/cm, 60  $\mu$ s). The fused eggs were exposed to 5  $\mu$ M ionomycin for 5 min, followed by 10  $\mu$ g/ml cycloheximide (CHX) for 5 h, and cultured in CR1aa medium at 39 °C in an atmosphere of 5% CO<sub>2</sub> for up to 192 h post-activation. Data were analyzed by ANOVA program. There were no differences in the rates of cleavage and of blastocyst formation between two treatments of donor cells with (76 and 18%) and without serum-starved (81 and 21%). Experiment 2 compared the effect of different activation treatments on the rates of cleavage and development into blastocysts of NT embryos. Chemical activation were accomplished by ionomycin, followed by A) CHX, B) CHX +5  $\mu$ g/ml cytochalasin B, and C) 2 mM sodium pyrophosphate (cdc2 kinase inhibitor) for 5 h, respectively. There were no significant differences ( $P \leq 0.05$ ) among the treatments in the rates of cleavage and of development into blastocysts (A, 65 and 14%; B, 72 and 21%; C, 55 and 13%). The results suggest that both treatments of donor fibroblasts and those three activation treatments of NT eggs are similarly effective to development of NT embryos. (Funded by High Technology Development Project for Agriculture and Forestry, Korea, MAF-SGRP, 30012-5).

EFFECT OF ACTIVATION TIME ON THE NUCLEAR REMODELING AND IN VITRO DEVELOPMENT OF NUCLEAR TRANSFER EMBRYOS DERIVED FROM BOVINE SOMATIC CELLS

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This study was conducted to investigate the effect of recipient activation time on the chromatin structure and in vitro development of bovine nuclear transfer embryos derived from adult ear cells. Serum starved or confluent cells were transferred and electrofused to enucleated oocytes at 22–24 h of IVM. Fused eggs were activated with a combination of calcium ionophore (A23187, 10  $\mu$ M, for 5 min) and cycloheximide (10  $\mu$ g/ml, for 5 h) at 1, 1.5, 2, 2.5, 3, and 5 h after fusion treatment. Nuclear transfer embryos were cultured in CR1aa + 3 mg/ml BSA for 3 days and in CR1aa + 10% FBS for further 4–5 days. About 30 fused eggs were fixed by whole-mount method at each time point without activation, and premature chromosome condensation (PCC) state was examined. Some eggs were fixed at 3 and 7 h after A23187 treatment to examine their chromatin structures. Some 1-cell stage embryos were fixed by air-dry method at 32–34 h after activation to analyze their chromosome constitutions. The proportion of eggs with a morphologically normal PCC state increased at 1–2 h after fusion treatment. Whereas eggs with elongated chromosome plate increased as activation time prolonged to 3 h, and at 5 h after fusion, 58% (18/31) of eggs showed two or more small scattered chromosome sets. After 3 h of activation, the proportion of eggs with single chromatin clump (40.6 to 56.7%) was significantly increased when eggs were activated within 2.5 h after fusion compared to those of eggs were activated at 3 (22.3%, 7/31) and 5 h (0%, 0/31) after fusion ( $P < 0.05$ ). Development rate to the blastocyst stage significantly higher in eggs were activated within 2 h after fusion (17.3–21.7%) compared to those of eggs were activated at 2.5–5 h after fusion (0–8.6%,  $P < 0.05$ ). Proportion of embryos with normal chromosome constitutions (having 60 chromosome sets) decreased as activation time prolonged. In nuclear transfer embryos were activated at 5 h after fusion, only 50% (15/30) of embryos formed a PN, and 26.7% (8/30) of embryos extruded a polar body at 7 h after activation. From the present result, it is suggested that activation time can affect chromatin structure and in vitro development of bovine nuclear transfer embryos. Activation within 2 h after fusion can enhance the in vitro development of nuclear transfer bovine embryos. Supported by the GSRP-MAF (300012-5), South Korea.

SCREENING TRANSGENIC PRIMARY CELL LINES PRIOR TO USE FOR  
SOMATIC CELL NUCLEAR TRANSFER

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The generation of transgenic animals via somatic cell nuclear transfer requires the efficient characterization of a large number of candidate donor cell lines. Cell lines carrying very high copy numbers, rearranged transgenes, or multiple integration sites have to be eliminated. In addition, tetraploid and other cytologically abnormal cell lines must be removed. Frequently occurring mixed populations of transgenic and non-transgenic colonies must also be identified. Pre-selection is crucial to the success of a nuclear transfer program as the monetary and time costs associated with the production of non-transgenic or otherwise compromised animals are steep. Several methods are currently available for use to genotype the cell lines prior the initiation of nuclear transfer. The Polymerase Chain Reaction (PCR), Southern blotting, and Metaphase and Interphase Fluorescent In Situ Hybridization (FISH) analysis have been tested in our bovine and caprine transgenic founder programs. Data from several nuclear transfer programs, with respect to the clonal selection methodology used and the number of transgenic animals produced are summarized below. These data indicate that a combination modality screen is probably preferable for the identification of candidate cell lines for nuclear transfer. Respectively 40 and 18% non-transgenic offspring were generated when interphase FISH or Southern blotting only were used (Table 1). Southern blotting does not allow for the visualization of individual cells, and interphase FISH is not very sensitive and may lead to the elimination of cell lines with low-copy number integration. Although metaphase FISH has been a reliable method of preselection (96% accuracy), it is much more labor-intensive than interphase FISH and it does not provide the information on transgene integrity that can be obtained by Southern blotting. For these reasons, the following strategy is suggested to screen large number of transfected cell lines. Initially, use both PCR and interphase FISH on filters to quickly exclude a number of cell lines followed by the more cumbersome Southern blot analysis and metaphase FISH methods to further characterize the remaining candidates lines.

Table 1

Comparison of different cell line pre-selection methods for the generation of transgenic founder goats and cows by somatic cell nuclear transfer

Selection method	No. of cell lines used	No. of live offspring	No. of transgenic offspring (%)	No. of negative offspring (%)
Southern blot	5	28	23 (82)	5 (18)
FISH on slides	12	23	22 (96)	1 (4)
Interphase FISH on Filters	9	20	12 (60)	8 (40)

PRODUCTION OF CLONED CALVES FOLLOWING NUCLEAR TRANSFER  
WITH CULTURED FROZEN–THAWED SOMATIC CELLS USING  
SIMPLE PORTABLE C<sub>2</sub>O INCUBATOR

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The successful production of offspring from differentiated somatic cells by nuclear transfer techniques has demonstrated that the nuclei of these cells can be reprogrammed by the cytoplasm of recipients oocytes [Campbel et al., Nature 1996;38:64–66]. Cryopreservation techniques have been widely used to preserve cell lines or embryonic cells for research or breeding purposes. The ability of frozen-thawed fetal skin was examined to generate viable cell lines for nuclear transfer. Fetal skin (of approximately 8 months fetus) frozen at  $-20$ ,  $-30$  or  $-80$  °C in the presence of 5% DMSO used as tissue explants to generate somatic cells. The resultant confluent cells were then used as donors for nuclear transfer (NT). Oocytes were collected from slaughterhouse ovaries and in vitro matured in SOF supplemented with amino acids. Oocytes with the first polar body extruded were selected as recipient cytoplasm and were enucleated. Embryos were reconstructed by inserting the donor cell into the perivitelline space of a recipient oocyte through the slit in the zona pellucida made during the enucleation. Data were analyzed by Student's *t*-test and Duncan's multiple range tests using the General Linear Models procedure of the Statistical Analysis System. Probability of  $P < 0.05$  was considered to be statistically significant. Of the bovine NT embryos reconstructed from the somatic cells, 78, 81–78% showed cleavage 44, 43–48% reached the stage of morula formation and 29, 34–35% reached blastocyst formation. There were no significant differences in development when the NT embryos were compared with those reconstructed from fresh somatic cell derived skin tissues (75, 45, and 38%, for cleavage, and development to morula and blastocyst stage, respectively). During the whole process for culture of NT embryos, we used the simple portable incubator C<sub>2</sub>O [Varisanga et al., Cloning 2000;4:167–173] and carried to China from Japan by air. After reach to farm, each two NT embryos were transferred to five recipients. We obtained two female NT calves which birth weights is 30 and 36 kg, and gestation periods of 281 and 284 days, respectively. There were no abnormalities observed in those calves. The results indicated that cell lines derived from bovine fetal skin cryopreserved by a simple method could be used as donors in nuclear transfer using the portable C<sub>2</sub>O incubator.

## LECTIN-CELL AGGLUTINATION AGENT IMPROVES THE EFFICIENCY OF BOVINE SOMATIC CLONING

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During somatic nuclear transfer (NT), one of the impediments contributing to its low efficiency is the difficulty of cell fusion between a large recipient oocyte and a small donor cell. Lectin is a glycoprotein isolated from the kidney bean, which is proved to have the ability of lymphocyte agglutination. This experiment was designed to test lectin agglutination activity on fusion rate and subsequent developmental potential of cloned bovine embryos *in vitro*. Oocytes were aspirated from antral follicles of slaughterhouse ovaries, selected with at least four layers of cumulus cells, and then cultured in maturation medium for 20 h in 5% CO<sub>2</sub> and 95% humidified air at 39 °C. Cumulus cells were then denuded from the oocytes by incubation in 0.2% hyaluronidase and vigorous vortexing. Oocyte enucleation was conducted in 20% FBS PBS at 21 h after maturation culture, and confirmed by fluorescent microscopy. Skin fibroblast cells used for NT were derived from cultured ear explants taken from a 13-year-old dairy cow, and cumulus cells were cultured from the cumulus-oocytes complexes collected from the same animal by ultrasound guided transvaginal retrieval. Cells were cultured in 10% FBS DMEM at 37 °C in 5% CO<sub>2</sub> humidified air. Fibroblasts and cumulus cells at passage 5 or 6 were used as nuclear donors. Small donor cells with an approximate diameter of 12–15 µm were used for transfer into the perivitelline space of enucleated oocytes, subsequently somatic donor cell-cytoplasm pairs were allocated to a 2 × 2 factorial treatment, with lectin treated at the concentration of either 0 or 150 µg/ml in M199 7.5% FBS at 39 °C, 5% CO<sub>2</sub> for 15 min. Somatic cell-cytoplasm pairs were then fused by applying two direct current pulses at 2.0 kV/cm for a duration of 10 µs/pulse. Fusion rates were determined 90 min after electrical pulse. Fused embryos were activated in 10 µg/ml cycloheximide in M199 + 7.5% FBS for 4 h. Embryos were cultured in CR1aa plus 3 mg/ml BSA for 2 days (initiation of activation = Day 0) at 39 °C, 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>, then cultured in CR1aa medium supplemented with 7.5% FBS for successive 5 days on buffalo rat liver cell (BRLC) monolayers. Cleavage, morulae and blastocyst development rates were recorded on Days 2, 5 and 7, respectively. The results (Table) showed that the fusion rate, after lectin treatment, was significantly increased from 31 to 61% ( $P < 0.05$ ), for skin fibroblasts, and 58–87% ( $P < 0.05$ ) for cumulus cells as nuclear donors, respectively. There were no differences in subsequent cleavage and blastocyst development between the treatments. The total NT efficiency was improved in skin fibroblast (11% versus 20%,  $P = 0.05$ ) as well as cumulus cell NT group (17% versus 31%,  $P < 0.05$ ) by the lectin treatment. This study demonstrated that agglutination agent lectin improves the success of bovine cloning, and that lectin seems to have no detrimental effect on pre-implantation development of NT embryos *in vitro*. The potential of *in vivo* development in lectin derived NT embryos is under further investigation.

Treatment	Lectin (µg/ml)	Number	Fused (%)	Number (%) of embryos developed to			% Blastocysts/ oocytes
				2–8 cell	Morula	Blastocyst	
Skin fibroblast	0	232	73 (31) <sup>a</sup>	62 (85)	39 (53)	27 (37)	11 <sup>a</sup>
Skin fibroblast	150	200	121 (61) <sup>b</sup>	109 (90)	60 (55)	40 (33)	20 <sup>a,b</sup>
Cumulus cells	0	233	136 (58) <sup>b</sup>	115 (84)	72 (53)	39 (29)	17 <sup>a</sup>
Cumulus cells	150	242	210 (87) <sup>c</sup>	173 (82)	114 (54)	76 (36)	31 <sup>b</sup>

Values with different superscripts within columns are significantly different ( $P < 0.05$ ).

## DIFFERENT PATTERNS IN THE INCIDENCE OF APOPTOSIS IN BOVINE EMBRYOS DERIVED FROM IVF AND SOMATIC CELL NUCLEAR TRANSFER

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Compared with IVF embryos, decrease in developmental competence was found in embryos produced by somatic cell nuclear transfer (SCNT). Previous research strongly suggested that apoptosis induced by various causes might be one of major causes for such development retardation after embryo reconstruction. This experiment was subsequently conducted to evaluate the occurrence of apoptosis during preimplantation development of embryos produced by SCNT and IVF. IVM and IVF of oocytes, SCNT and activation, and IVC of embryos were performed by our standard procedure [Cho JK et al., *Theriogenology* 2002;57:1819–1828] and ear fibroblasts subcultured three to five times were employed as a donor somatic cell of SCNT. Apoptosis was detected by a terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling assay. As experimental parameters to compare apoptosis in IVF and SCNT embryos, number of embryos having more than one apoptosis blastomere and mean number of apoptosis blastomeres per embryo were screened. Data were analyzed by ANOVA using the generalized linear model (PROC-GLM) in SAS program and were represented as mean  $\pm$  S.D. As results, different patterns in the incidence of apoptosis were found between the SCNT and control IVF embryos, which made a statistical difference ( $P < 0.05$ ) in various developmental stages. Stage of the first incidence of apoptotic blastomere was different; from the 4- to 8-cell stage in the SCNT, while the 8–16-cell stage in the IVF. Regardless of embryo origin, number of embryos having more than one apoptotic blastomere gradually increased with the progress of embryo growth and all blastocysts being evaluated had apoptotic blastomeres. In blastocysts, apoptotic blastomeres were more found in the ICM than trophectoderm. Overall, the number was greater in the SCNT than in the IVF at every developmental stage and a significant ( $P < 0.05$ ) increase was found at the 4–8-cell [12/84 (14%) versus 0/81 (0%)] and 8–16-cell [53/95 (56%) versus 36/108 (33%)] stage. When we evaluated the mean number of apoptotic blastomere per embryo, a gradual increase with the progress of embryo development was found in both groups. Comparison showed greater number in the SCNT than in the IVF; a significant increase at the 4–8-cell (1.3 cells versus 0 cells) and blastocyst (8.9 cells versus 6.2 cells) stages in SCNT embryos. In conclusion, these results showed a good possibility that apoptosis is one of major causes for developmental retardation found in SCNT embryos.

## REPROGRAMMING OF SOMATIC CELL NUCLEI IN CLEAVAGE STAGE BLASTOMERES

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Somatic cell nuclei can support full development when transplanted into the enucleated metaphase II (MII) ooplasm of the recipient oocyte. In contrast, no development is observed upon transfer of the somatic cell nucleus into the zygotic cytoplasm. Apparently, transfer to the metaphase II ooplasm is critical for development of somatic cell clones. This, however, does not preclude a potential role for reprogramming events occurring in cytoplasm other than that of the MII oocyte. For example, fusion of somatic cells with ES cells has shown that reprogramming of genes can occur in an environment other than the enucleated MII oocyte. Furthermore, mosaicism of expression between blastomeres has been observed in blastocyst stage clones, indicating that some reprogramming events occur during cleavage stages. To assess the capacity of post MII embryonic stages to reprogram somatic cell nuclei, we monitored expression of the embryonic/germ line specific gene, *Pou5f1* (*Oct3/4*) from cumulus cell nuclei in cleavage stage blastomeres of mouse embryos. One blastomere of late 2-cell stage blastomeres was injected with cumulus cells containing a *Pou5f1*-GFP transgene. Of 67 injected hybrid embryos 58 survived the procedure and 48 of the injected blastomeres subsequently developed within the whole embryo. *Pou5f1*-GFP, normally expressed at the compacted morula stage, was never observed in similar stage hybrid embryos (24 h post injection). However, 18 h later, GFP was detected in 28 (58%) embryos with GFP present predominantly in the inner cell mass. The blastocysts were placed onto feeder layers to develop into outgrowths. Of 31 outgrowths forming from 31 embryos, GFP expression was detected in 12 at 1 day after seeding. At subsequent days, no GFP was detected although outgrowths continued to develop. This is consistent with the tetraploid status of the injected cells that would predictably preclude contribution to postimplantation embryonic lineages. From these results it appears that blastomeres can reprogram gene expression of somatic nuclei. The respective roles of the blastomere nucleus and cytoplasm in reprogramming cannot be delineated, however, diploid reconstructions with the ability to undergo cleavage will address this.

## DEVELOPMENT OF CLONES CONSTRUCTED WITH MATERNAL CYTOPLASM

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Cloning by somatic cell nuclear transfer involves the transfer of a somatic nucleus into a foreign oocyte cytoplasm. Resulting embryos have nuclear DNA identical to somatic nucleus but mitochondrial DNA originating from cytoplasm. Objective of this study was to examine consequences of constructing cloned embryos in a foreign versus maternal cytoplasm. Ovarian/granulosa cells were aspirated from adult Jersey cows ( $n = 6$ ) using an ultrasound-guided transvaginal probe. Primary cell lines were established for each cow and before nuclear transfer, cultured in the presence of 10% fetal bovine serum (passage = 3; confluence 70–90%). On the day before nuclear transfer, COC were collected from ovaries surgically removed from Holstein and Angus cows or Jersey cows from which the ovarian/granulosa cell lines were previously derived. COC were cultured separately according to origin and matured for 18–20 h. To construct cloned embryos in a foreign or maternal cytoplasm, ovarian/granulosa cells were fused (electrical pulse of 2.2 kV/cm for 40  $\mu$ s) with oocyte cytoplasm originating from Holstein or Angus cows (Foreign Cytoplasm) or from the Jersey cow from which the cells were derived (Maternal Cytoplasm). Cloned embryos were activated and then cultured in an atmosphere of 7% O<sub>2</sub> and 5.5% CO<sub>2</sub> in KSOMaa + BSA. Ability of cloned embryos to cleave and develop to morula or blastocyst was assessed on Days 4 and 7 post-activation, respectively. Compact morulae and blastocysts were transferred to synchronized recipient heifers. Establishment of pregnancy was confirmed 30–32 days post-estrus by presence of an embryonic heartbeat using ultrasound. Data were analyzed using Fisher's Exact Test. Proportion of oocytes recovered after denuding (94%) and extruded a polar body (58.6%) was similar regardless of origin. In addition, ability of ovarian/granulosa cells to fuse with foreign or maternal cytoplasm was similar (66.0% versus 63.1%, respectively). However, a higher proportion of foreign cytoplasm lysed after fusion (33.0% versus 13.1%, respectively  $P = 0.0001$ ). Development of cloned embryos to 8–16-cell by Day 4 was greater ( $P = 0.005$ ) for those constructed with foreign versus maternal cytoplasm (Table). Ability of cleaved embryos to continue in development did not differ (Table). Four pregnancies progressed beyond 70 days. However, at Days 208, 236 and 241 of pregnancy, fetal death occurred in three recipients because of placental insufficiency associated with hydrops allantois. Only one clone constructed with foreign cytoplasm progressed to term. Use of maternal cytoplasm for constructing cloned embryos did not improve developmental competence of clones to establish or maintain a pregnancy to term.

Cytoplasm	No. of clones	8–16 cell (%)	M/B <sup>a</sup> (%)	ET <sup>b</sup>	No. of recipients	Pregnant 30–32 days (%)	Pregnant 73–75 days (%)	Term
Foreign	68	52 (76.5)	16 (23.5)	10	10	2 (20)	2 (100)	1
Maternal	99	54 (54.6)	24 (24.2)	16	16	5 (31.3)	2 (40)	0
<i>P</i> -value	0.005	1.0				0.668	0.428	

<sup>a</sup> M/B: total number of morulae and blastocysts.

<sup>b</sup> Total number of clones transferred to recipients.

## EPIGENETIC CHARACTERISTICS OF NUCLEAR TRANSFER DONOR CELLS IN VITRO: EFFECT OF HISTONE ACETYLATION

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Skin fibroblasts and cumulus cells are the most frequently used donor cell types for somatic cloning by nuclear transfer. Different cloning efficiencies have been reported for these two cell types by several groups. The present study examined whether adult fibroblast cells are different from cumulus cells in terms of their histone acetylation patterns. Bovine fibroblast and cumulus cells were each grown to confluence at passages 5, 10 and 15 using routine techniques and subjected to analysis for histone acetylation levels. The acetylation status of histone H2B at lysine 20, H3 at lysine 18 and H4 at lysine 8 was determined by Western blot analysis [Liu L and Yang X, Biol Reprod 1999;61:1-7] and flow cytometry with antibodies generated against the above proteins (Upstate Biotechnologies, Lake Placid, NY; Cell Signaling Technologies, Inc., Boston, MA). Flow cytometry was conducted through measuring the fluorescent levels of cells after incubation in specific primary antibodies for acetylated histones and fluorescent conjugated secondary antibodies. Appropriate controls for auto fluorescence and non-specific binding by the secondary antibodies were included. Ten thousand cells were collected with a fluorescence activated cell sorter (FACS Caliber; Becton Dickinson, San Jose, CA) and were analyzed using CELL QUEST 3.1 software (Becton Dickinson). Statistical analysis was performed using the General Linear Model Procedure in the Statistical Analysis System (Cary, NC). The main effects were cell type (cumulus versus fibroblast), passage and cell cycle stage. After confirming the specificity of the antibodies to acetyl histones by Western blotting, we analyzed the relative amounts of acetyl histones in  $G_0/G_1$  fibroblast versus  $G_0/G_1$  cumulus cells at passages 5, 10 and 15. Acetyl histone H4 levels did not change in fibroblast cells between passage 5, 10 and 15. However, acetyl histone H4 and acetyl histone H3 levels in cumulus cells increased with passage number from 5 to 15 ( $P < 0.05$ ). Acetylation levels for histone H3 in fibroblast cells were significantly greater at passage 15 compared to earlier passages. For acetyl histone H2B, no passage effects for either cumulus or fibroblast cells were observed. However, cumulus cells had higher acetyl histone H2B fluorescent intensities than fibroblast cells at all passages examined ( $P < 0.05$ ). These results indicate that histone acetylation status is different between cumulus and fibroblast cells and is further re-modeled by in vitro cell culture. As histone hyperacetylation is associated with gene activation, these results may have implications for the selection of donor cells for nuclear transfer.

APOPTOSIS DERIVED FROM SENESCENT DONOR CELL IN  
BOVINE PREIMPLANTATION EMBRYO

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Regarding cloned animals, a variety of donor cell types supported birth of live offspring using somatic cell nuclear transfer (SCNT). This study compared the developmental competence of bovine SCNT embryos using various types of young or senescent somatic cells and embryo viability was examined by apoptosis resulted in the arrest of development competence. Bovine adult ear fibroblasts, fetal fibroblasts and cumulus cells were isolated, cultured in vitro [Cho JK et al., *Theriogenology* 2002;57:1819–1828] and categorized by young and senescent cells in terms of population doublings (PD); <20 PD as young cells, >20 as old cells [Poothapillai K., *Biol Reprod* 2001;64:1487–1493]. Bovine oocytes from slaughterhouse ovaries were matured in TCM199, enucleated and reconstructed by SCNT. The reconstructed oocytes were fused, chemically activated, and cultured in modified synthetic oviduct fluid (mSOF) at 39 °C in a humidified atmosphere of 5% CO<sub>2</sub> air for up to 7 days [Cho JK et al., *Theriogenology* 2002;57:1819–1828]. Morphogenetic evaluation monitoring preimplantation development, apoptosis detection using a terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling (TUNEL) assay, counting inner cell mass (ICM) cells and trophectodermal (TE) cells in blastocysts by differential staining were employed as experimental parameters. General linear model consisting of ANOVA and a least square method in SAS program was used for statical analysis. As results, the percentage of blastocysts formed, and the number of inner cell mass (ICM) and the trophectoderm (TE) were not significantly different in SCNT of all types of young or senescent somatic cells. However, preimplantation embryo derived from all types of senescent donor cells showed a significant increase in the apoptotic blastomeres per embryo ( $6.7 \pm 2.5$  (young adult fibroblast) versus  $9.8 \pm 2.6$  (senescent adult fibroblasts);  $7.4 \pm 2.3$  (young fetal fibroblasts) versus  $9.5 \pm 2.5$  (senescent fetal fibroblasts);  $5.3 \pm 2.6$  (young cumulus cells) versus  $8.7 \pm 2.2$  (senescent cumulus cells),  $P < 0.05$ ). In conclusion, these results indicated that various cell types in different cellular senescence did not affect the developmental competence and total cell number of SCNT embryo, while it lead to increased incidence of apoptosis in bovine SCNT embryo derived from senescence donor cells.

## EFFECT OF CELL CONFLUENCE USING EMBRYONIC STEM CELLS FOR THE PRODUCTION OF CLONED MICE

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In this study we provide evidence to show that varying donor cell confluence can influence development to blastocyst and term in both hybrid and inbred embryonic stem (ES) cell nuclear transfer. A hybrid ES cell line "RI", derived from 129/sv × 129/sv-CP, was compared to an HPRT-deficient [Selfridge J et al., *Somat Cell Mol Genet* 1992;18:325–336] ES cell line HM-1, derived from an inbred mouse strain 129/Ola. Cells were seeded at different densities to produce high (80–90%) and low (60–70%) confluence, assessed subjectively over the same period of culture and grown in 5% serum the day before nuclear transfer. For this experiment oocytes from B6D2F1 mice (8–10 weeks) were collected at 13–14 h post-hCG (5 IU) injection and the metaphase II spindle aspirated in the presence of HEPES-buffered medium containing 5 µg/ml of cytochalasin B. The oocytes were removed to CZB medium [Chatot CL et al., *J Reprod Fertil* 1989;86:679–688] and incubated at 37 °C in 5% CO<sub>2</sub> in air prior to injection. ES cells were mixed with 10% (w/v) PVP (polyvinylpyrrolidone, 360 kDa, ICN) and selecting only small cells (<10 µm), the cytoplasm was carefully removed from the nucleus. In HEPES-buffered medium, enucleated oocytes were then injected with ES donor nuclei, cultured in CZB for 1–3 h and activated in calcium-free CZB medium containing 10 mM strontium chloride and 5 µg/ml of cytochalasin B for 5–6 h. All micromanipulation procedures were carried out using a Piezo Micromanipulator Controller PMM150 (Prime Tech Ltd., Ibaraki, Japan). Activated oocytes were cultured for 72 h in either CZB or M16 medium (Sigma–Aldrich Company Ltd., Dorset, UK) and resultant morulae/blastocyst stage embryos were transferred into 2.5-day pseudo-pregnant female recipients. Data were analyzed using chi-square test or ANOVA test. When HM-1 inbred donor cells were used at 80–90% confluence, 84.7% of treated embryos cleaved and 49% developed to morulae/blastocysts. At term 18 (9.1%) pups were alive and five developed into healthy adult males. However, when 60–70% cell confluence for HM-1 cells was used only 59.3% of treated embryos cleaved and 22.2% developed to morulae/blastocysts. Only one (2.3%) pup developed to term and died 1 h after Caesarean section. A similar confluence effect was shown in R1 ES cell nuclear transfer, 80–90% cell confluence produced 66.0% cleavage for treated embryos, and 52.7% of these embryos developed to morulae/blastocyst stage with three (2.05%) pups alive at term. In contrast, when low cell confluence was used only 58.2% of treated embryos cleaved, 29.7% developed to morulae/blastocysts and these failed to produce any pups at term. In conclusion, these results show that different degrees of confluence in cell culture have a significant affect (all values no less than  $P \leq 0.05$ ) upon development of cloned embryos reconstructed with R1 and HM-1 ES cell lines. Cloning efficiency increased when donor cells were cultured at a high cell confluence. These data also demonstrated that inbred ES cell line HM-1 can produce viable cloned mice capable of surviving to adulthood.

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COMPARISON OF ENUCLEATION METHODS FOR THE PRODUCTION OF  
TRANSGENIC DAIRY GOATS BY SOMATIC CELL NUCLEAR TRANSFER

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The production of recombinant therapeutics using transgenic dairy goats offers an alternative method compared to traditional manufacturing methods. While pronuclear microinjection has been the primary technique used to produce founder transgenic goats, somatic cell nuclear transfer offers advantages for increasing the efficiency of generating transgenic goats. Typically, enucleation of mammalian oocytes is performed using ultraviolet (UV) light after staining the metaphase plate with Hoescht 33342. However, the deleterious effects of the DNA dye and UV light on the subsequent development of nuclear transfer embryos has been reported. Experiments were conducted to compare enucleation of caprine oocytes utilizing UV and Hoescht 33342 or polarized light (SpindleView™ Imaging System, CRi, Inc., USA). For each experiment, in vivo ovulated oocytes surgically collected from superovulated does were evenly distributed from each doe into two groups for enucleation. Enucleated oocytes were reconstructed utilizing transfected goat fetal or adult skin cells cultured in 0.5% FBS for 3 or 4 days, then re-fed with 10% FBS prior to use. Cell/cytoplasm couplets were simultaneously fused and activated with a single electrical pulse of 2.6 or 3.0 kV/cm for 20  $\mu$ s. Fused couplets received a second electrical pulse of 2.0 kV/cm for 20  $\mu$ s 1 h post-fusion treatment, and non-fused couplets were refused with a single electrical pulse of 2.8 or 3.2 kV/cm for 20  $\mu$ s. Nuclear transfer embryos were cultured undisturbed in SOF/BSA for 24 or 48 h at 38 °C in a humidified modular incubation chamber (Billups Rothenburg, USA) containing approximately 6% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N. Nuclear transfer embryos with age appropriate development (up to 2-cells at 24 h or 2–8-cells at 48 h post-fusion and activation) were surgically transferred into the oviduct of synchronized surrogate recipients. Pregnancies were determined by ultrasonography starting at approximately Day 28 post-fusion and activation, and then monitored weekly thereafter. There were no significant differences in oocyte survival following either enucleation or reconstruction (1310 versus 1243 enucleated oocytes survived, or 1223 versus 1176 reconstructed couplets survived), fusion (1029 versus 960 couplets fused) or cleavage (384 versus 334 couplets cleaved) when comparing enucleation by UV or polarized light, respectively  $P > 0.05$ . However, there was a tendency for twice as many ongoing pregnancies (Days 55–113 of gestation) to be established in recipient does that received nuclear transfer embryos generated using the polarized light compared to the UV method of enucleation. These results suggest that caprine oocytes enucleated using polarized light generate nuclear transfer embryos capable of establishing early fetal development. In addition, enucleation by polarized light may offer a method for increasing the efficiency of generating transgenic founder goats by somatic cell nuclear transfer by increasing the number of term pregnancies and the health of offspring produced (Table).

Method	Number of oocytes	Number of enucleated (% survival)	Number of couplets (% survival)	Number of fused (% fusion)	Number of cleaved at 24–48 h (% fused) <sup>1</sup>	Number of recip./number of transf.	Number of pregnant (%)
UV	1419	1310 <sup>a</sup> (92)	1223 <sup>a</sup> (93)	1029 <sup>a</sup> (84)	384 <sup>a</sup> (37)	101/687	3 (3.0)
Polarized	1348	1243 <sup>a</sup> (92)	1176 <sup>a</sup> (95)	960 <sup>a</sup> (82)	334 <sup>a</sup> (35)	93/646	7 (7.5)

Values are totals of 50 experiments. Data were analyzed by chi-square test, the (a, b) within columns differ significantly,  $P < 0.05$ .

<sup>1</sup> Includes experiments at 24 h post-fusion and activation prior to embryo cleavage.

IN VITRO DEVELOPMENT OF CROSS-SPECIES NUCLEAR TRANSFER EMBRYOS  
CONSTRUCTED WITH OVINE, BOVINE AND PORCINE DONOR  
CELLS AND OVINE CYTOPLASTS

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To determine differences in preimplantation embryo development between cross-species and intraspecies nuclear transfer embryos, ovine-, bovine-, and porcine-derived cumulus cells were cultured in Glasgow's medium and frozen at passage 1 to serve as donor cells. In vitro-matured ovine oocytes were enucleated by micromanipulation and embryos reconstructed by sub-zonal injection and electrofusion of donor cells that had been thawed, cultured for 5–7 days and serum-starved for 2 days. Reconstructed embryos were activated with Calcium-ionophore and 6-DMAP and cultured in SOF medium. In vitro-produced ovine embryos served as controls. Four experimental groups were defined: ovine IVF (ovIVF), ovine NT (ovNT), bovine-ovine cross-NT (boCNT) and porcine-ovine cross-NT (poCNT). Embryo development was determined after 24, 36, 48, 72, 96, 120 and 168 h (7 days) of culture by fixing in glycerol-Hoechst 33342 and counting nuclei under UV. An embryo was classified as developmentally normal at a given time point if it contained a minimum number of nuclei; >2 at 36 h, >4 at 72 h and so forth (see Table). The development of boCNT- and poCNT-embryos was apparently comparable with intraspecies NT embryos for the first 72 and 96 h, respectively after which development was greatly reduced. The comparable development between the groups over the first 72–96 h indicates that ovine cytoplasm can support cellular events essential to at least the first three cell cycles of embryo development. The subsequent developmental retardation of both cross-species combinations and their inability to develop far beyond the third cell cycle stage suggests that ovine cytoplasm reprogram bovine and especially porcine nuclei to a much lesser degree than those of ovine. The major activation of the embryonic genome occurs at the 4-cell stage in the pig embryo, one cell cycle earlier than in bovine and ovine embryogenesis. This may explain why the poCNT-embryos appear less developmentally competent than the boCNT-embryos. The ribosomal RNA (rRNA) genes are activated as part of the major gene activation resulting in the formation of nucleoli required for ribosome and thus, protein synthesis. Ultrastructural studies are currently underway to elucidate whether cross-species nuclear transfer hampers the complex process of nucleolus development.

Embryo	36 h <sup>a</sup> (n)	48 h <sup>b</sup> (n)	72 h <sup>c</sup> (n)	96 h <sup>d</sup> (n)	120 h <sup>e</sup> (n)	168 h <sup>f</sup> (n)
OvIVF	100 (42)	100 <sup>k</sup> (39)	80.5 <sup>k</sup> (41)	56.4 <sup>k</sup> (39)	57.5 <sup>k</sup> (40)	60.5 <sup>k</sup> (38)
OvNT	100 (19)	86.7 <sup>l</sup> (15)	61.9 <sup>l</sup> (21)	37.5 <sup>k,l</sup> (24)	16.7 <sup>l</sup> (18)	37.5 <sup>l</sup> (48)
BoCNT	100 (14)	87.5 <sup>l</sup> (24)	64.7 <sup>k,l</sup> (17)	40.9 <sup>k,l</sup> (22)	5.9 <sup>l</sup> (17)	0 <sup>m</sup> (12)
PoCNT	100 (23)	78.3 <sup>l</sup> (23)	65.5 <sup>k</sup> (29)	22.7 <sup>l</sup> (22)	0 <sup>l</sup> (20)	0 <sup>m</sup> (19) <sup>a,f</sup>

(a–f) Normal development defined as nuclei counts >1, 2, 4, 8, 16, and 64, respectively. (k–m) Percentages and numbers (n) of developmentally normal embryos within columns showing different superscripts are significantly different ( $P > 0.05$ ) as analyzed by chi-square.

EVIDENCE OF IMPAIRED PLACENTAL FUNCTION IN CLONED FETAL SHEEP DURING LATE GESTATION

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Late-gestation fetal loss is one characteristic of somatic cell clones in ruminant species. Although placental insufficiency has been implicated as a cause of early-gestation loss [Hill JR et al., Biol Reprod 2000;63:1787], the physiological status of the late-gestation sheep fetus has not previously been examined. Clone embryos were constructed from IVM-produced oocytes and a confluent early-passage granulosa cell line. Successfully fused couplets were cultured in SOF medium supplemented with 4 mg/ml BSA with resultant blastocysts transferred to synchronized recipient ewes on Day 6. Control fetuses were produced by natural mating. Fetal survival was monitored using ultrasonography (3.5 MHz transducer) prior to surgery. Between Days 103 and 108 of gestation, vascular catheters were implanted in a fetal carotid artery and jugular vein and the amniotic cavity in 8 control and 12 clone fetuses. Fetal arterial blood samples were collected throughout late gestation (control,  $n = 210$  samples; clone,  $n = 223$  samples) in order to determine pH,  $pO_2$ ,  $pCO_2$ , bicarbonate, hematocrit and haemoglobin content as well as oxygen saturation (ABL 520 analyzer, Radiometer, Copenhagen, Denmark). Arterial oxygen content per 100 ml blood was calculated using the formula:  $O_2 \text{ content} = (pO_2 \times 0.003) + [Hb] \times (O_2 \text{ saturation}/100) \times 1.39$ . Analysis of variance with repeated measures was used to determine differences between groups. Seven control fetuses (88%) and five clone fetuses (42%) survived to 140 days gestation. Arterial  $O_2$  content,  $pO_2$  and saturation were significantly ( $P < 0.001$ ) decreased in clone fetuses whereas arterial  $pCO_2$  and pH were not different between clone and control fetuses throughout late gestation (see Table). There was also no difference in arterial haemoglobin content, hematocrit or bicarbonate concentrations between control and clone fetuses ( $9.5 \pm 0.4$  and  $10.1 \pm 0.6$  g/dl;  $29.4 \pm 1.3$  and  $31.3 \pm 1.9\%$ ;  $26.4 \pm 0.8$  and  $27.3 \pm 0.7$  mmol/l for control and clone fetuses, respectively). Clone fetuses were moderately hypoxaemic but not acidotic. It is, therefore, possible that placental oxygen transfer capacity is restricted in clone fetuses; however, it appears unlikely that the excessive fetal loss that occurs in clone pregnancies is due solely to this degree of placental restriction.

	pH	$O_2$ content (ml/dl)	$pO_2$ (mmHg)	$pCO_2$ (mmHg)	$O_2$ sat. (%)
Controls	$7.4 \pm 0.01^a$	$9.2 \pm 0.5^a$	$22.5 \pm 1.2^a$	$48.2 \pm 1.7^a$	$69.6 \pm 3.2^a$
Clones	$7.4 \pm 0.01^a$	$7.1 \pm 0.5^b$	$17.4 \pm 0.8^b$	$50.5 \pm 1.4^a$	$50.6 \pm 3.6^b$

Values are mean  $\pm$  S.E.M. Values within columns with different superscripts differ significantly ( $P \leq 0.001$ ).

EVIDENCE FOR HYBRID VIGOR BY NUCLEAR-CYTOPLASMIC INTERACTION  
IN CULTURED CELLS FROM TRANSMITOCHONDRIAL CLONED  
BOVINE FETUSES

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Hybrid vigor or heterosis, i.e. an increase in performance of crossbred progeny relative to their purebred parents, is a well-known phenomenon in plants and animals that is generally attributed to nuclear gene interactions. However, it has been speculated almost a century ago that hybrid vigor could also be caused by nuclear-cytoplasmic interactions. A.F. Shull (1912) hypothesized that "If it were possible to remove from an egg its own nucleus, and substitute for it a nucleus slightly different, but not so different as to be 'incompatible', with a diploid set of chromosomes, and have it develop normally, it should . . . produce an individual more vigorous than its parent, even if the introduced nucleus were completely homozygous" [Shull, Biol Bull 1912;XXI:1–13]. We have carried out such a nuclear transfer experiment in cattle, and produced cloned transmitochondrial bovine fetuses with identical *Bos taurus* nuclear (*n*) DNA and either *B. taurus* or *B. indicus* mitochondrial (mt) DNA to study the role of nuclear-cytoplasmic interactions in cellular functions. We generated a transmitochondrial clone of somatic cell nuclear transfer embryos using *B. taurus* granulosa cells as nuclear donor and recipient cytoplasts derived from oocytes with defined *B. taurus* or *B. indicus* mtDNA as described [Brüggerhoff et al., Biol Reprod 2002;66:367–373; Hiendleder et al., Biol Reprod; in press]. After embryo transfer to recipient *B. taurus* cows, a clone of four normal fetuses with two individuals each having *B. taurus* or *B. indicus* mtDNA was recovered on Day 80. Primary cell cultures were established from skin, liver and heart tissue samples. Cells were passaged once (heart) or twice (skin, liver) in DMEM/10% FCS and seeded at  $7 \times 10^5$  cells in 28 cm<sup>2</sup> culture dishes (four to six dishes per tissue and fetus, two independent experiments). Cultures were trypsinized in late log phase after 48 to 72 h, counted, and used for polarographic oxygen readings with a Clark type electrode. Cultures of cells from all four fetuses were set up and processed in parallel for each experiment. ANOVA (SAS version 8.02) showed significant effects of mtDNA genotype on cell proliferation and cellular oxygen consumption in all three tissues investigated. Moreover, cells derived from fetuses with *B. taurus* nDNA and *B. indicus* mtDNA showed a marked increase in both cell proliferation (17.5–39.7%) and oxygen consumption (11.6–19.8%) as compared to cells with the same nDNA but *B. taurus* mtDNA. Thus, the more distant *B. indicus* mtDNA in combination with the *B. taurus* nucleus yielded a superior phenotype, providing experimental evidence for a role of nuclear-cytoplasmic interactions in heterosis. Nucleotide sequence differences between the *B. taurus* and *B. indicus* mtDNA of the investigated fetuses amount to 236 polymorphic sites in total, and could provide a molecular basis for the observed differences in cellular proliferation and metabolism. The data suggest that not only the extent, but also the direction of nDNA–mtDNA interaction effects on phenotype is variable. This could be important for crossbreeding and interspecific nuclear transfer cloning in mammals, such as the rescue of endangered species or the proposed production of human stem cells using non-human ova.

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## TROPHOBLAST MHC-I EXPRESSION AND APOPTOSIS IN BOVINE NUCLEAR TRANSFER EMBRYOS

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Although the morphology of somatic cell nuclear transfer (NT) blastocysts appears normal, pregnancy rates are far lower than for *in vivo* or *in vitro* fertilized embryos. This study investigated three aspects of NT blastocysts (total cell count, apoptosis and trophoblast Major Histocompatibility Complex I expression) under two different embryo culture conditions (G1/G2 with or without added serum). MHC-I expression was examined as we have previously shown trophoblast MHC-I expression to be abnormal in Day 35 NT pregnancies [Hill et al., 2002]. This raised the possibility that abnormal MHC-I expression in blastocysts could be useful in screening for abnormal MHC-I expression prior to embryo transfer. NT was performed using a single fetal cell line that was serum starved prior to NT. For analysis of total cell count and apoptosis, NT and IVF embryos were cultured for 156 h in either G1/G2 or G1/G2 + charcoal stripped fetal calf serum. The Day-7 blastocysts were fixed and stained using the TUNEL reaction (Roche) for total cell count and apoptosis. Total cell count means were not significantly different between groups NT or IVF blastocysts whether cultured with or without serum (NT 109; NT + FCS 107; IVF 94; IVF + FCS 105). NT embryos showed a significantly increased percentage of apoptotic cells compared with IVF embryos (NT 16%; NT + FCS 14%; IVF 12%; IVF + FCS 9%). For analysis of MHC-I expression, NT and IVF embryos were cultured for 9 days in G1/G2 then hatched blastocysts were fixed in acetone in preparation for immunohistochemistry. Neither IVF nor NT blastocysts stained positive for MHC-I. The cell line for NT used in this experiment was the same as that used in a previous study that reported 6/6 Day 35 NT placentas expressed MHC-I. Thus, we conclude that screening embryos at the blastocyst stage for MHC-I expression was not useful in predicting later aberrant expression.

## ATTEMPTS OF CLONING RATS BY SERIAL NUCLEAR TRANSPLANTATION

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Cloned rats have not yet been produced by somatic cell nuclear transplantation (NT). In the successful cloning of mice by Honolulu method, potential of recipient cytoplasm to support premature chromosome condensation (PCC) of donor nucleus has been considered to be critical. However, our preliminary study in rats has indicated that cumulus cell nuclei injected into enucleated oocytes did not exhibit any signs of the PCC. In the present study, two attempts of rat cloning were carried out by serial NT of cumulus cells. In Experiment 1, cumulus cell nuclei from F1 (Sprague–Dawley × Dark–Agouti) rats were injected into intact oocytes from Wistar rats (no. of oocytes injected = 576). One hour later, recipient metaphase-plate were aspirated from the NT oocytes in which PCC of injected nuclei were observed by staining with Hoechst 33342 under UV light. The oocytes were activated for 6 h with 1.25 mM SrCl<sub>2</sub>, and then karyoplasts containing pronucleus-like vesicles were fused by a dc pulse (20 V/mm, 20 μs) with cytoplasts derived from pronuclear-stage zygotes from Wistar rats. Next day, reconstructed zygotes were transferred into oviducts of Day-1 pseudopregnant Wistar females. Fetal development was examined by Caesarean section at Day 21. In Experiment 2, cumulus cell nuclei of rats were injected into enucleated oocytes from BDF 1 mice (no. of oocytes injected = 943), and the heterogeneous NT oocytes were activated for 6 h with 10 mM SrCl<sub>2</sub>. Karyoplasts containing two pronucleus-like vesicles were fused with rat cytoplasts. The reconstructed zygotes were transferred for fetal development as in Experiment 1. The results in Experiment 1 showed that 54% of oocytes surviving nuclear injection supported PCC of cumulus nuclei and 76% among them formed pronucleus-like vesicles. Fusion rate between the karyoplasts and cytoplasts was 65%, and finally a total of 69 reconstructed zygotes including 25 two-cell embryos were transferred. No live fetus were observed in four implantation sites. The results of Experiment 2 showed that 68% of enucleated mouse oocytes supported formation of two pronucleus-like vesicles. Fusion rate between the heterogeneous karyoplasts and rat cytoplasts was 95%. Thus, the higher support of mouse oocytes for PCC and subsequent vesicle formation and the higher fusion rate, compared with those in Experiment 1, resulted in egg transfer of 339 reconstructed zygotes including 212 two-cell embryos. However, only five implantation sites were detected. These results suggest that reconstructed rat zygotes can be prepared by serial NT procedures, but developmental competence of the zygotes remains to be clarified.

## IMPROVED BIRTH RATES OF CLONED MINIATURE PIG USING IN VITRO-MATURED PORCINE OOCYTES CO-CULTURED WITH FOLLICLE SHELLS

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Live birth of cloned pigs using in vitro-matured oocytes have been reported elsewhere. However, efficiency for producing cloned pigs was still extremely low. The objective of this study was to examine the effect of method preparing for in vitro-matured oocytes on the developmental ability of nuclear transferred (NT) embryos in miniature pig. Ovaries were collected from domestic gilts at a local slaughterhouse, and immature oocytes were isolated from follicles ranging from 3 to 6 mm in diameter and cultured by following three methods: (1) oocytes were isolated from follicles dissected from ovaries, and 20–50 oocytes and two pieces of inverted follicle shells were co-cultured in TCM199 supplemented with 10% FCS, sodium pyruvate and 0.1 IU/ml hMG (mTCM) with gentle agitation (CC group); (2) oocytes were aspirated from ovaries and cultured in mTCM supplemented with 0.5 mg/ml dibutyryl cAMP for 22 h, followed by culture in mTCM without hMG (cAMP group); (3) oocytes were aspirated from ovaries and cultured in mTCM (NC group). Oocytes were examined their meiotic progression at 28, 32, 36, 40 and 44 h of maturation culture. Each oocyte from these groups was enucleated and fused with fibroblast cells from Potbelly miniature pig. Fusion and activation were simultaneously induced by dc pulses of 30 for 30  $\mu$ s twice in 0.3 M mannitol solution containing 0.05 mM CaCl<sub>2</sub> and 0.1 mM MgSO<sub>4</sub> at 48 h of maturation. The NT embryos were cultured in Whitten & Biggers medium supplemented with 0.5 mg/ml hyaluronic acid sodium salt, and examined the developmental ability in vitro. Cleaved NT embryos at 48 h after nuclear transfer were transferred into the oviducts of synchronized recipient gilts. Göttingen miniature pigs and Meishan pigs were used as recipients. Most of oocytes reached metaphase-II 44 h after maturation in CC (94%) and cAMP (92%) groups, while only 71% of those in NC group. Transition of meiotic process from metaphase-I to metaphase-II synchronously occurred in CC and cAMP groups, but the progression of maturation in cAMP group (36–44 h) was slower than that in CC group (26–36 h). Developmental rates of NT embryos to the blastocyst stage in CC group (24%) were significantly higher than those of cAMP (11%) and NC (13%) groups ( $P < 0.05$ , ANOVA). NT embryos that were reconstructed from oocytes of CC group were transferred to 14 Göttingen miniature pigs and four Meishan pigs. All Göttingens were aborted at 20–31 days of pregnancy. However, two of four Meishan recipients became pregnant and resulted in birth of cloned miniature piglets, three piglets from 15 embryos (20%) and two piglets from 29 embryos (7%) after transfer. These results indicated that in vitro-matured oocytes co-cultured with follicle shells have high developmental ability after nuclear transfer, and result in full-term development of NT embryos after embryo transfer. The breed of recipients for embryo transfer seems to be another important factor for producing cloned miniature pigs.

## LIVE CLONED PIGS GENERATED FROM NUCLEAR TRANSFERRED EMBRYOS ENCAPSULATED WITH SODIUM ALGINATE

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The cloned embryos before blastocyst stage have the high possibility of being attacked by leukocytic invasion from the hole of zona pellucida, which was created by micro-manipulation, and the further embryonic development will be failed in reproductive tract. To avoid this, encapsulation of cloned embryos with sodium alginate seems to be beneficial after the embryo transfer. In this present study, we evaluated an advantage of encapsulation of cloned embryos that were derived from in vivo- and in vitro-matured oocytes on their development in the oviduct of surrogate mothers. In addition, considering a fact that a successful pregnancy can be maintained by at least four fetuses, we hypothesized that co-transfer of four helper (fertilized) embryos would assist full development to term of cloned embryos. In Experiment 1, somatic cell cloned embryos at the 2–8-cell stage, which were derived from in vivo-matured oocytes as nuclear recipients, were encapsulated with (438 embryos) or without (78 embryos) sodium alginate and were transferred to recipients on Day 2 and then collected from the oviduct at the 3rd day after the transfer. The recovery rate was significantly higher ( $P < 0.01$ ) for encapsulated embryos (92.7%) than that of embryos without treatment (76.8%). The encapsulated embryos showed a significantly higher ( $P < 0.05$ ) rate of development to the morula or blastocyst (MB) stages (25.4%) than that of embryos without treatment (15.5%). Then the cloned embryos (103 encapsulated and 18 no treatment) developed to MB stages were re-transferred to recipients on Day 5 with four early blastocysts for each transfer (10 and 4, respectively). Four out of 10 recipients in which encapsulated embryos were transferred became pregnant and three of them farrowed three viable normal cloned piglets; one recipient farrowed only piglets from helper embryos. In contrast, although three recipients, in which embryos without treatment were transferred, were pregnant, no cloned piglet was obtained. In Experiment 2, somatic cell cloned zygotes (1344) derived from in vitro-matured oocytes as nuclear recipients were encapsulated with sodium alginate and were transferred to four recipients on Day 1. The cloned embryos were collected from the oviduct at the 4th day after the transfer. The percentage of the encapsulated and collected embryos was 81.8, and 17% of them developed to the MB stage. The cloned embryos (187) at MB stages were re-transferred to recipients (4) on Day 5. Two recipients became pregnant and one of them farrowed one viable normal cloned piglet; another recipient remained pregnant. These results indicate that encapsulation with sodium alginate for cloned embryos is effective for surviving embryos in female reproductive tracts. Also suggested that the transfer of cloned embryos with helper embryos is useful method to complete term. In conclusion, both in vivo- and in vitro-matured recipient oocytes can be used for NT.

## NUCLEAR STATUS OF PORCINE CLONED EMBRYOS AND THEIR SUBSEQUENT DEVELOPMENT AFTER ACTIVATION

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Our objective was to evaluate the dynamics of the transferred nucleus after injection and if development of somatic nuclear transfer (NT) porcine embryos would be affected by the status of donor nucleus at activation. Porcine oocytes collected from ovaries were matured *in vitro*, enucleated and injected with single nucleus of fetal fibroblasts prepared from miniature pigs. First, we evaluated the status of the nucleus after the reconstitution, especially as to nuclear envelope breakdown (NEBD) and chromatin condensation (CC). Immediately after injection for 2 h, the rates of NEBD and CC were 8.3 and 13.9%, respectively, and reached to peak at 3 h post-injection (11.4% in NEBD, 34.3% in CC). At 4.5 h post-injection, NT embryos were spontaneously activated (33.3%). Second, to evaluate when the activation would be applied after injection, NT embryos were activated by combined treatment with ionomycin, electrical stimulation and cycloheximide at 0 or 3 h post-injection. When activated at 3 h post-injection, the cleaved rate and the developmental rate to the blastocyst stage were 41.5 and 2.9%, respectively, compared to the corresponding rates (30.1 and 0%) in those activated at 0 h post-injection. Third, we tested the effect of cytoskeletal inhibitors involved in the process of activation on development of NT embryos. When cultured with cytochalasin B (CB) or cytochalasin D (CD) at the same time as cycloheximide treatment, NT embryos showed pronuclear stage without pseudopolarbody at rates of 85.7 and 96.0% in CB and CD, respectively, whereas subsequent development to the blastocyst stage was observed at rate of 4.8% in CD, but not in CB. In the absence of CB or CD, the rate of NT embryos without pseudopolarbody was 48.2%. Our results suggest that in pig NT embryos the nucleus transferred may be modified during and/or so quickly after manipulation, and that the timing for activation concomitant with a cytoskeletal inhibitor may have effect on subsequent development of NT embryos. In conclusion, the activation to the reconstructed eggs at 3 h post-injection, when the donor nucleus was condensed, may enhance subsequent development.

## SOMATIC CELL NUCLEAR TRANSFER IN THE GOAT: COMPARISON OF TWO ACTIVATION PROTOCOLS

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Transgenic animals that express value added recombinant proteins can be produced using somatic cell nuclear transfer (NT). A critical step in the NT process is activation of the reconstructed oocyte. In this study, the developmental potential of caprine granulosa cell nuclei was evaluated after nuclear transfer using two modifications of the calcium ionomycin activation protocol employing either 6-dimethylaminopurine (DMAP) or cycloheximide to inhibit phosphorylation or protein synthesis, respectively. Immature oocytes were obtained by laparoscopic aspiration from preovulatory follicles of Kinder and Nigerian Dwarf goats primed with FSH and eCG. Following maturation, oocytes were enucleated and nuclear transfer performed [Keefer et al. BOR 2001;64:849–856]. Granulosa cells (GC) were obtained by laparoscopic follicular aspiration of a transgenic goat. All donor cells had a normal karyotype. Donor cells at passage 1 were cultured in DMEM plus 0.5% FBS for 4 days prior to use in NT. Briefly, individual donor cells were transferred beneath the zona pellucida. The oocyte-cell couplets were fused with a single electric pulse (15  $\mu$ s, 2.44 kV/cm). Two to 3 h after fusion, the reconstructed embryos were activated using a three-step process. In steps 1 and 2, embryos were incubated in 5  $\mu$ M ionomycin for 5-min followed by a 5-min incubation in 3% BSA in EmCare<sup>TM</sup> (ICP, Auckland, New Zealand). In step 3, embryos were then either incubated in 2 mM 6-dimethylaminopurine (DMAP treatment) for 4 h or in 35.5  $\mu$ M cycloheximide plus 15.6  $\mu$ M cytochalasin B (CHX treatment) for 5 hr. Four sessions of NT and embryo transfer were performed. Seventy-nine GC derived embryos were produced using the DMAP treatment and 89 GC derived embryos were produced using the CHX treatment. Twelve percent (1/8) of the recipients in the GC-DMAP treatment group and 33% (3/9) in the GC-CHX group had term pregnancies and delivered two (GC-DMAP) and three (GC-CHX) kids. All of the kids remain alive and healthy. Pregnancy rates and NT efficiencies (kids per reconstructed embryo transferred) were not significantly different between the DMAP and CHX treatment groups. Offspring's identity was confirmed by PCR, Southern blotting analyses, and fluorescent in situ hybridization. Similar efficiencies following nuclear transfer of somatic cells were achieved using either of the two activation protocols, DMAP or CHX.

PREIMPLANTATION DEVELOPMENT OF BOVINE EMBRYOS RECONSTRUCTED  
USING OOCYTES HAVING PRESUMPTIVELY INACTIVATED  
CHROMOSOME BY X-RAY IRRADIATION

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In somatic cell nuclear transfer (SCNT), *in vitro* manipulation renders suboptimal condition for further development of clone embryos and many studies on alternative enucleation methods have widely been conducted in order to minimize mechanical damages during reconstruction. The objective of this study was to evaluate whether X-ray irradiation of oocytes could replace mechanical enucleation procedure of SCNT. Bovine oocytes matured *in vitro* and fetal fibroblasts obtained from 40-day-old fetus were used as enucleated oocytes and donor somatic cells, respectively. The rate of reconstruction and preimplantation development was monitored for analyzing treatment effect by PROC-GLM in SAS program. In Experiment 1, embryos were produced by reconstruction of donor somatic cells with oocytes according to our standard procedure [Cho et al., *Theriogenology* 2002;57:1819–1828] in Group A, being exposed to 15 kV<sub>p</sub> X-ray for 60 s and subsequently enucleated in Group B, being exposed to X-ray alone in Group C. An X-ray irradiator (Softex Co., Kanazawa, Japan) was used for the irradiation undertaken before depositing donor cell in the perivitelline space through the zona slit. The afterward procedure following reconstruction was identical to our standard procedure [Cho et al., *Theriogenology* 2002;57:1819–1828]. As results, a total of 451 oocytes (178, 131 and 142 for Groups A–C, respectively) were provided for SCNT. A significant ( $P = 0.0035$ ) model effect on blastocyst formation was found and less clone embryos from Group B developed to the blastocyst stage (4%), while no significant difference was found between Groups A and C (15–20%). This suggests that mechanical manipulation retards further embryo development. In Experiment 2, minimum exposure time was investigated to prevent excessive cytoplasmic damage caused by X-ray irradiation. Clone embryos were produced using mechanically enucleated oocytes (Group A), oocytes neither irradiated nor enucleated (Group B) for negative control, oocytes exposed to 15 kV<sub>p</sub> X-ray alone for 10, 20, 40, 60 s for Groups C–F. As results, a total of 289 oocytes (53, 56, 45, 41, 49, 45 for Groups A–F) were provided for SCNT. With the highest blastocyst formation rate in Group A (25%), a significant ( $P = 0.042$ ) model effect was found on blastocyst formation. However, no difference was found when X-ray was irradiated for various time (10, 20, 40 or 60 s). Therefore, such an effect was from the difference between non-irradiated (Group B) and irradiated (Groups C–F) groups (4% versus 11–21%). In conclusion, the developmental competence of clone embryos using X-irradiated oocytes was normal, showing the possibility that this new procedure can be introduced in SCNT. Further experiments are being undertaken to evaluate cell number and chromosome status of blastocysts derived from SCNT with X-irradiation.

IN VITRO DEVELOPMENT OF RABBIT NUCLEAR TRANSFER EMBRYOS  
DERIVED FROM FETAL FIBROBLAST CELLS EXPOSED  
TO DIFFERENT TREATMENTS

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Besides species-specific differences, also the protocol used for nuclear transfer (NT) appear to have a significant impact on the efficiency of producing cloned offspring. There is currently only one published report of the production of term rabbits by using somatic cell NT, in that case using cumulus cells [Chesne et al., *Nat Biotech* 2002;20:366–369]. Fibroblast cell lines derived from fetal tissue are currently the favored cell type to use as nuclear donors in transgenic animal production involving NT, because of the ease of isolation, proliferation and transformation. Therefore, we investigated the use of fetal fibroblasts as nuclear donors for rabbit NT, specifically the in vitro development when donor cells were exposed to different treatments prior to NT. Fetal fibroblast cells were maintained in culture medium consisting of DMEM, supplemented with 10% FCS, 2 mM L-glutamine, 1 mM Na-pyruvate, antibiotics and 1% MEM-nonessential amino acids at 38 °C, 5% CO<sub>2</sub> and 100% humidity. All cells were derived from the same population of fetal fibroblasts, treatments were started at the same passage number (P2) and confluency (70%). Cycling cells (Cycl), serum-starved cells (0.5% serum for 6 days; SS) and demethylated cells obtained by exposure to 5-azacytidine (5 µM for 72 h, Aza) were frozen in small aliquots (~1000 cells) and used for NT directly after thawing. For NT, in vitro matured MII oocytes were enucleated 15 h post hCG and subsequently reconstructed with a fibroblast from one of the three treatment groups. Cytoplasmic fibroblast complexes were fused in Zimmermann medium by a double electric pulse at 18 h post-hCG. The fusion rate was determined after 30 min, and at that time another double pulse was applied to induce activation. The embryos were subsequently incubated in 6% ethanol for 4 min and cultured for 1 h in 5 µg/ml cycloheximide and 7.5 µg/ml cytochalasin B. Finally, fused embryos were cultured in MPM-medium supplemented with 10% FCS at 38 °C, 5% CO<sub>2</sub> and 100% humidity. Cleavage and blastocyst rates were determined on Days 1 and 4 after NT, respectively. The results represent data of 5 replicates performed for each treatment-group. A higher proportion of NT-embryos derived from the SS-group cleaved when compared to the Cycl- or Aza-group (74% versus 57 and 54%,  $P < 0.05$ ). In contrast, a higher percentage of both Cycl- and SS-derived embryos developed to the blastocyst stage compared to Aza-derived embryos (24 and 16% versus 5%,  $P < 0.05$ , with Cycl versus Aza  $P < 0.001$ ). In cattle, it was shown that NT-embryos lack the physiologically occurring de- and re-methylation processes in early development [Kang et al., *Nat Genet* 2001;28:173–177]. Hence, we expected an improvement of in vitro development of NT-embryos derived from 5-azacytidine-treated cells, which have been actively demethylated. However, the use of these cells for NT resulted in lower development when compared to cycling or serum-starved cells, which might be an indication that 5-azacytidine might have caused irreversible changes in chromatin-configuration, which subsequently hindered further development.

DEVELOPMENTAL BLOCK OF BOVINE–MURINE INTERSPECIES  
NUCLEAR TRANSFERRED EMBRYOS IN VITRO

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Even though success in birth of live offspring from nuclear transfer using somatic cells in many species, detailed information on processes or mechanisms of development are not well known. Cytoplasm of bovine oocyte has been known to support the development of nuclear transferred embryos using nuclear donor cells from different species. Therefore, interspecies nuclear transfer might be used to find answers of some questions in basic aspect of nuclear transfer. In this study, we examined the developmental potential, especially developmental block of nuclear transferred embryos in vitro when bovine oocyte as a cytoplasm recipient and mouse embryonic fibroblast as a nuclear donor were used. As a nuclear donor, mouse embryonic fibroblasts from ICR strain (having 2-cell block) were used. Bovine oocytes were matured in vitro and enucleated after 22 h. The reconstructed embryos were placed into the fusion chamber (3.2 mm gap; BTX2001) in a solution containing 0.28 M mannitol and aligned manually. A double pulse of 1.80 kV/cm for 15  $\mu$ s was used to fuse the cells and activate the embryo simultaneously. The fused embryos were cultured for 4 min in 5  $\mu$ M ionomycin, 4 h 2 mM 6-DMAP after fusion. Then, embryos were moved to culture media inducing the block or not, and cultured in 5% CO<sub>2</sub> and 39 in 100% humidity. The nuclear transfer (NT) units were allocated in Group 1: murine block media (CZB with glucose) and normal media (CZB without glucose) or Group 2: bovine block media (CR1aa without FBS) and normal media (CR1aa with FBS) (Table 1).

NT units were not blocked at 2-cell stage regardless of types of medium. However, in mouse media, poor development of interspecies NT units was observed compared to bovine media. In bovine block medium, NT units were blocked at 8-cell stages. However, NT units cultured in bovine normal medium, embryos developed over 8-cell stage. Therefore, development of NT units might be under the control of bovine cytoplasm until 8-cell stage. Moreover, even though low development, bovine–murine interspecies nuclear transferred embryos could develop to blastocysts. As a result, it is suggested that the bovine cytoplasm controls the early preimplantation development in interspecies NT. However, the development of later stages might require genomic control from transferred donor nucleus. Therefore, even though the involvement of several other factors such as mitochondrial incompatibility, effective development of embryos produced by interspecies NT requires proper genomic activation of donor nucleus after overcoming the cytoplasmic control of recipient oocytes.

Table 1  
Development of bovine–murine interspecies nuclear transferred embryos

Conditions	NT unit	Embryos cleaved (%)	4-Cell embryos (%)	8-Cell embryos (%)	Morula (%)	Blastocyst (%)
Mouse normal medium (CZB w/o glucose)	68	51 (75)	19 (28)	1 (2)		
Mouse block medium (CZB w/g glucose)	70	54 (77)	20 (29)	6 (9)		
Bovine normal medium (CR1aa w/FBS)	91	61 (67)	21 (23)	16 (18)	5 (5)	1 (1)
Bovine Block medium (CR1aa w/o FBS)	42	35 (83)	11 (26)	9 (21)		

## COMPARISON BETWEEN ELECTRO-FUSION AND INTRACYTOPLASMIC NUCLEAR INJECTION METHODS IN PIG CLONING

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Cloned pigs have been successfully produced by two types of somatic cell nuclear transfer (NT) methods: electro-fusion (EF) and intracytoplasmic nuclear injection (NI). The aim of the present study was to compare characteristics of the two methods. IVM oocytes were obtained from abattoir ovaries and matured in NCSU23. In vivo-matured oocytes were collected from superovulated gilts. Fetal fibroblast cells to be used as nuclear donors were cultured for 2 days under serum starvation. In the fusion method, donor cells were fused with the recipient ooplasts by a single DC pulse of 200 V/mm for 10  $\mu$ s in 0.3 M mannitol + 0.1 mM MgSO<sub>4</sub> with/without 0.05 mM CaCl<sub>2</sub> (EF + Ca and EF-Ca). NT by NI method was performed using a piezo-driven micropipette. IVM oocytes were used to examine nuclear remodeling and developmental ability of the NT embryos. Some of the reconstructed embryos were electrically activated at 0.5–1.5 h (EF) or 2–2.5 h (NI) after the NT, respectively, followed by cytochalasin B treatment for 3 h. Development of the NT embryos was assessed by culture for 7 days in NCSU23 or by transfer to the oviducts of recipient gilts. Premature chromosome condensation of the donor nuclei took place frequently ( $n = 26$ – $35$ , 80–100%) regardless of the type of NT method used. At 1 h after NT, more than half of the NT embryos reconstructed by EF possessed metaphase-like chromosomes, whereas embryos having condensed chromosomes were predominant (71%) in the NI group. At 3 h after NT ( $n = 24$ – $37$ ), many of the embryos reconstructed by NI and EF-Ca had metaphase-like or disarrayed chromosomes (77, 71%). In contrast, embryos having a swollen nucleus appeared (24%) in the EF + Ca group, indicating that fusion and activation occurred simultaneously in this method. Formation of pronucleus-like nuclei tended to be lower when NT was performed by NI ( $n = 30$ ; 85.97% versus 65%,  $P < 0.05$ ). Blastocyst formation rate of the NT embryos reconstructed with IVM oocytes was higher in the EF + Ca group compared to the NI group (19.2%, 22/114 versus 5.4%, 6/112,  $P < 0.05$ ). NT by EF-Ca yielded a similar blastocyst formation rate as EF + Ca. NT with in vivo-matured oocytes resulted in a lower (4.1% by EF + Ca) or similar (7.6% by NI) blastocyst formation rate compared to the NT with IVM oocytes. Transfer of NT embryos reconstructed by EF + Ca with IVM and in vivo matured oocytes resulted in the production of normal fetuses (laparotomized at 3 months). These data demonstrate that differences between nuclear transfer methods are reflected in the remodeling pattern of the donor nucleus. Use of in vivo-matured oocytes as recipient cytoplasts did not increase the developmental rate of the NT embryos by either method. The EF method may produce transferable NT embryos more efficiently.

## BIRTH OF A NORMAL CALF AFTER TRANSFER OF THE DEFECTIVE OOCYTE FROM OLD INFERTILE CATTLE AFTER GERMINAL VESICLE TRANSFER

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*Objectives:* Two experiments were conducted to examine if the developmental ability of the defective oocytes from old infertile cattle can be improved by whole ooplasm replacement using germinal vesicle transfer (GVT). *Materials and methods:* In Experiment 1, GV-stage oocytes were obtained by the standard ultrasound-guided ovum pick-up system from old infertile cattle (15–19-year-old). Donor oocytes were obtained from the ovaries (3-year-old cattle) collected at a local slaughterhouse. After centrifuge of oocytes at  $20,000 \times g$  for 10 min in TCM-199 containing 7.5  $\mu\text{g/ml}$  cytochalasin B to visualize the GVs, they were removed by micromanipulation. The GV from the old oocyte were transferred to enucleated young intact oocyte and fused by a repeat of electric impulse of 7.5 kV/cm for 50  $\mu\text{s}$ . After IVM culture for 20 h in TCM-199 containing 5% FCS, the GVT oocytes were fertilized with frozen–thawed spermatozoa capacitated by 5 mM caffeine and 10  $\mu\text{g/ml}$  heparin, and cultured in IVD101 under 5%  $\text{CO}_2$ , 5%  $\text{O}_2$  and 90%  $\text{N}_2$  at 37 °C for 7 days. All the experiments were repeated for four times and data were analyzed by Student's *t*-test. In Experiment 2, the resultant four blastocysts derived from the defective oocytes from old infertile cattle after GVT and IVM/IVF/IVC were transferred non-surgically to four synchronized recipient cattle. *Results:* Experiment 1, no oocytes of the old infertile cattle developed over than 8-cell stage, however, 11% of reconstructed oocytes by GVT (GVT: old GV to young ooplasm) developed to the blastocyst stage. Experiment 2, when four blastocysts were transferred to four recipients, two of them became pregnant, and one normal calf was delivered. *Conclusion:* The results in the present study indicate that developmental arrest of the oocytes from old infertile cattle can be released by ooplasm replacement with normal one by GVT, and the GVT oocytes have developmental ability to the term.

## NUCLEAR TRANSFER IN HORSES

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In this study we investigated the developmental ability of horse embryos after nuclear transfer (NT) with cumulus cells or adult skin fibroblasts. Equine oocytes with compact and expanded cumulus were matured for 20–22 h in TCM199 with 10% FCS, 0.1 IU/ml LH and 0.1 IU/ml FSH, decumulated and enucleated. Cumulus cells for NT were obtained by trypsinisation from maturing oocytes with compact cumulus. Fibroblasts of passage 1–6 were cultured in TCM199/DMEM with 10% FCS to establish a confluent monolayer or were serum starved for 1–4 days. NT-embryos were obtained: (1) by transfer of a cell under the zona pellucida and fusion in 0.3 M mannitol by double pulse of 30  $\mu$ s DC 2.4 kV/cm; or (2) by attachment of zona pellucida free (0.5% pronase) oocytes to a single cell in TCM199 with 200  $\mu$ g/ml of lectin (PHAP) and fusion by double pulse of 30  $\mu$ s DC 1.2 kV/cm. The fusion rate of zona-intact ooplasts with cumulus cells was 69%, while 100% of zona-free ooplasts fused with cumulus cells and 97% with fibroblasts ( $P \leq 0.05$ ). One 2 h post fusion embryos were activated by 5  $\mu$ M ionomycin for 4 min and incubated in the mixture of 5  $\mu$ g/ml cycloheximide and 1 mM 6-DMAP in SOFaa for 4 h. Embryos were cultured in SOFaa in 5% CO<sub>2</sub>, 5% O<sub>2</sub> at 38.5 °C. The data were compared by Chi-square test. The cleavage rate of zona-free NT-embryos (84–88%) did not depend on quality of oocyte cumulus or origin of nuclei and was significantly higher than of zona-intact NT-embryos with cumulus cell nuclei (69.2%,  $P \leq 0.05$ ). However, this difference could be due to technical difficulties. Further work is needed to confirm it. There were 1.3–1.9% of NT-embryos with cumulus cell nuclei and 3.8–4.4% of NT-embryos with fibroblast nuclei that formed blastocysts on Day 8. This study shows that the development of NT-embryos to the blastocyst stage is not affected by the morphology of the oocyte cumulus before maturation and by the presence/absence of zona pellucida.

Nuclear donor	Cumulus morphology	Zona pellucida	Number of fused embryos	Cleaved (%)	Blastocysts (%)
Cumulus cells	Compact	+	78	54 (69.2) <sup>a</sup>	1 (1.3)
	Expanded	–	104	88 (84.6) <sup>b</sup>	2 (1.9)
Adult fibroblasts	Compact	–	205	180 (87.8) <sup>b</sup>	9 (4.4)
	Expanded	–	52	45 (86.5) <sup>b</sup>	2 (3.8)

EXPRESSION OF NUCLEAR LAMINS A/C IN CLONED BOVINE EMBRYOS  
RECONSTITUTED WITH BOVINE FETAL FIBROBLASTS

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The aim of the present study was to examine the expression pattern of nuclear lamins A/C during preimplantation development in cloned bovine embryos after reconstitution with bovine fetal fibroblasts. Donor cells of bovine fetal fibroblasts were isolated from 30 to 40 days bovine fetuses of slaughtered cattle and cultured in  $\alpha$ -MEM with 10% FBS. Prior to nuclear transfer (NT), donor cells of 3–6 passages were starved for 3–8 days and disaggregated by trypsin treatment. Oocytes matured for 18–19 h in TC-199 medium were used as recipient cytoplasm. A single donor cell was deposited into the perivitelline space of each enucleated oocyte and fused by two electric pulses of 2.0–2.5 kV/cm for 20–25  $\mu$ s, 1 s apart. Two hours after fusion the NT couplets were activated using 5  $\mu$ M ionomycin and 1.9 mM 6-dimethyl-aminopurine. The NT couplets were then cultured in 50  $\mu$ l drops of CR1aa solution in a 5% CO<sub>2</sub> incubator at 38.5 °C. At Day 3, the medium was replaced with CR1aa containing 10% FBS. The donor cells, oocytes, IVF and NT embryos were fixed in ethanol and 2.5% formaldehyde solution, respectively. After permeabilization, samples were incubated with mouse monoclonal antibody to nuclear lamins A/C. Following extensive washing, the primary antibody was detected with a FITC-conjugated anti-mouse secondary antibody. Samples were also counter stained with DAPI (2  $\mu$ g/ml) for 10 min. Coverslips were mounted in Vectashield mounting medium. Nonspecific antibody binding reactions were prevented by incubating samples in PBS containing 150 mM glycine, 3 mg/ml BSA and 10% normal goat serum. The slides were examined by fluorescence microscopy at wave lengths of 365/420 and 485/520–560, respectively, and by confocal microscopy. Bovine fetal fibroblasts, germinal vesicle stage oocytes and early cleavage stage nuclei exhibited positive staining with nuclear lamins A/C antibodies. As the oocytes proceeded through maturation the nuclear envelope dissolved and nuclear epitopes were lost. As early as 1 h after fusion of fetal fibroblasts with enucleated oocytes, the envelope and lamin antibodies in fibroblasts began to disappear and at 6 h after fusion the nuclear lamins reappeared at the swollen nuclear peripheries. This expression persisted until the 16-cell stage of NT embryos as well as IVF embryos. Most of the morulae and blastocysts did not react with anti-lamin A/C, whereas hatched blastocysts reacted (Table). These results suggest that nuclear structure of somatic cells could be reprogrammed to an embryonic state through nuclear transfer, as compared to IVF control embryos.

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Type of embryos	Hours post fusion				In vitro developmental stages					
	MO	1	6	12	2-c	4-c	8-c	M	B	HB
NT (n: 89)	-	-	-	+	+	+	+	-	-	+
IVF (n: 52)					+	+	+	-	-	+

NT: nuclear transfer, MO: mature oocyte, M: morula, B: blastocyst, HB: hatched blastocyst. Couplets were activated 2 h post fusion. Replication number: >3.

DIFFERENTIAL DEVELOPMENT OF RABBIT EMBRYOS DERIVED  
FROM PARTHENOGENESIS AND NUCLEAR TRANSFER

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Parthenogenetic development (PA) is often used as a model to investigate activation protocols for nuclear transfer (NT) embryos. The objective of this study was to compare the development, as well as the dynamics of the nuclear materials and microtubules of PA and NT embryos following similar activation treatment. Our results demonstrate that, during parthenogenesis, activation through either electrical pulses or chemical stimulation alone resulted in low cleavage rates and compromised development. A combination of two sets of electrical pulses and a 2-h exposure to chemical activation medium (5  $\mu\text{g/ml}$  CHX and 2 mM 6-DMAP in KSOM + 0.1% BSA) could effectively activate rabbit oocytes, and resulted in a 99% ( $n = 73$ ) cleavage rate with greater than 60% ( $n = 73$ ) developing to blastocysts at Day 4. However, the same activation protocol following NT resulted in only 65–72% of oocytes cleaved (depending on donor cell type), with less than 20% developing to the blastocyst stage (Table 1). The differences observed between NT and PA embryos subjected to the same activation protocol were also evident in terms of the time required for their development to the blastocyst stage, as well as the cell numbers present in blastocysts at Day 6 (Table 1). Furthermore, laser confocal microscopy revealed that pronuclear formation in the NT embryos was delayed by comparison to that in the parthenotes. In conclusion, our study suggests that an effective protocol for parthenogenesis cannot promise a comparable outcome for nuclear transfer embryos.

Table 1  
Development of rabbit embryos derived from nuclear transfer and parthenogenesis following the same activation protocol

Embryo type	Number NT	Number (%) fused/PA number	Number (%) cleaved	Number (%) blastocysts (BL) at Day 4	Number (%) BL at Day 6 (>60 cells)	Cell number in BL at Day 6
NT—cumulus cells	79	37 (47)	24 (65) <sup>a</sup>	2 (5) <sup>a</sup>	3 (8) <sup>a</sup>	89 $\pm$ 3
NT—fibroblasts	183	99 (54)	71 (72) <sup>a</sup>	10 (10) <sup>a</sup>	18 (18) <sup>a</sup>	110 $\pm$ 17
PA	–	73	72 (99) <sup>b</sup>	48 (66) <sup>b</sup>	47 (64) <sup>b</sup>	162 $\pm$ 5

Data within the same columns with different superscripts (a and b) are significantly different ( $P < 0.05$ ).

## MONOGENIC PROCREATION OF A NATIVE COLOMBIAN PORCINE BREED

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The monogenic procreation of a pig (*casco de mula*) from adult somatic cells, pretends to protect this specie from endangerment. This kind of pig has anatomic and genetic characteristics of great value due to its resistance to foot and mouth disease, its rusticity and adaptability to the tropics. Cecolfes and its animal biotechnology department, modern institutes in scientific research in Colombia, South America, have given their first step and decided to apply techniques and knowledge in biology development to animal species. They are looking forward to obtain, in the future, genetically modified farm animals, most of all in porcines for xenotransplants and protein production in milk, which is of high interest to mankind. They are also starting the new concept of zoo-human sciences. Experimental Methods Porcine oocytes from slaughterhouse were used and transported in 0.9% of saline solution at 39 °C, in a lab retrieval was performed with a 18G 1-1/2 needle and left for maturation in TCM 199 supplemented with insulin, FCS 10%, EGF, cysteine, sodium pyruvate, FSH and porcine follicular fluid, during 40 h with 5% of CO<sub>2</sub>, at 39 °C and 90% of relative humidity. Once mature and denuded by vortexing in 0.1% hyaluronidase in TCM 199, they were then put in Hoechst 33342 (5 µM), and placed in a micromanipulator, with manipulation media containing 7 µM cytochalasin B. MII oocytes were enucleated by removing first polar body and metaphase plate using 10 µm glass pipettes, obtaining cytoplasts. Porcine karyoplasts, were obtained by cellular culture of small ear skin biopsies, and once they reached 80% of confluence in culture, the cells were put in a drop with NSCU-23 calcium-free salts. Then we proceeded to the reconstruction of the oocyte introducing one karyoplast per each cytoplast. After reconstruction, electric fusion was performed in an electrocell manipulator BTX-ECM2001<sup>®</sup> (San Diego, USA), using fusion buffer (mannitol, CaCl<sub>2</sub>, MgCl<sub>2</sub>), in a chamber fusion of 1 mm, and giving an alignment pulse of 2 V AC during 2 s, followed by DC pulses of 140 V for 50 µs. Reconstructed oocytes were activated by two pulses of 120 V for 60 µs inactivation buffer and remained during 3 h in NSCU-23 bicarbonate with calcium until the transfer. Reconstructed oocytes were transported from our labs to the farm in NSCU-23 salts with calcium, in portable incubator at 39 °C until transfer. Three recipients were transferred with an average of 100 kg, 6-month-old, on Day 0 of estrous cycle, under going laparotomy, starting with sedation first and then under general anesthesia inhaled, Sevoflurane<sup>®</sup> (Abbott Labs). Asepsis and antisepsis were performed, then a low longitudinal (10 cm approx.) incision was made and we proceeded to the bilateral identification of the oviducts, and their catheterization with a Frydman set curt/short rigid catheter<sup>®</sup> (Laboratoire CCD) and transfer of reconstructed oocytes: 80 (sow No. 1), 120 (sow No. 2), and 80 (sow No. 3), placing half of the duplets in one side and the other half on the other side. Closing the abdominal wall with non-absorbable sutures. *Results:* Sow No. 1—Started estrous cycle. Sow No. 2—By abdominal ultrasound nine gestational sacs were detected. Sow No. 3—Under observation. *Conclusions:* Applying techniques and knowledge of reproductive biomedicine will make easier and improve the efficiency of monogenic procreation procedures in farm animals, specially in transportation of biological materials, transfer and anesthesia techniques. With all these modifications we are presenting our first pregnancy.

POST-HATCHING DEVELOPMENT OF NUCLEAR TRANSFER,  
IN VITRO-CULTURED AND IN VIVO-GROWN OVINE EMBRYOS

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Placentation, which is compromised at nuclear transfer, depends on proper formation of allantois (endoderm), and associated mesoderm. Hence, this process was studied in nuclear transfer (NT), in vitro-cultured (IVC) and in vivo-grown (VIVO) ovine embryos. NT embryos were constructed from in vitro matured-oocytes and granulosa cells and cultured in BSA-supplemented SOF medium. IVC-embryos were produced by culture of in vivo-fertilized zygotes in SOF with 20% human serum. Both NT- and IVC-embryos were transferred to recipients at Day 6 and recovered on Days 9, 11, 13, 17 and 19. VIVO embryos were collected on Days 7, 9, 11 and 13 from superovulated ewes. To obtain Day 17 and 19 IVO-embryos, in vivo zygotes were grown the initial 6 days in an intermediate recipient, and the remaining days in a second recipient. Embryos were fixed in either 3% glutaraldehyde or 4% paraformaldehyde in 0.1 M PBS and analyzed by stereomicroscopy. In total, 66 NT-, 50 IVC- and 70 VIVO-embryos were analyzed. Means were compared by *t*-test applying a *P* level of 0.05. On Day 7, the diameter of VIVO- ( $318 \pm 55 \mu\text{m}$ ) and IVC-embryos ( $344 \pm 97 \mu\text{m}$ ) was significantly larger than of NT-embryos ( $160 \pm 7 \mu\text{m}$ ). On Day 9, the diameter of VIVO-embryos ( $950 \pm 314 \mu\text{m}$ ) was significantly larger than of NT- ( $175 \pm 50 \mu\text{m}$ ) and IVC-embryos ( $433 \pm 172 \mu\text{m}$ ). On Day 11, elongation and embryonic disc formation especially in VIVO- and IVC-embryos was evident. VIVO- ( $4.3 \pm 4.8 \text{ mm}$ ) and IVC-embryos ( $2.9 \pm 1.3 \text{ mm}$ ) were significantly longer than NT-embryos ( $0.7 \pm 0.2 \text{ mm}$ ). The embryonic disc was significantly longer in VIVO- ( $328 \pm 79 \mu\text{m}$ ) than in IVC-embryos ( $214 \pm 50 \mu\text{m}$ ) where it was significantly longer than in NT-embryos ( $120 \pm 17 \mu\text{m}$ ). On Day 13, VIVO- ( $49.2 \pm 14.6 \text{ mm}$ ) and IVC-embryos ( $38.1 \pm 14.3 \text{ mm}$ ) were significantly longer than NT-embryos ( $2.8 \pm 3.2 \text{ mm}$ ). The embryonic disc in VIVO- ( $962 \pm 236 \mu\text{m}$ ) and IVC-embryos ( $798 \pm 291 \mu\text{m}$ ) was significantly longer than in NT-embryos ( $293 \pm 172 \mu\text{m}$ ). On Day 17, the body of the embryo proper had shaped and formation of allantois, somites and organs was ongoing. No significant difference was found between the length of the embryo body between VIVO- ( $6.2 \pm 0.7 \text{ mm}$ ), IVC- ( $5.6 \pm 2.0 \text{ mm}$ ) and NT-embryos ( $5.6 \pm 1.0 \text{ mm}$ ). The allantois was significantly wider in VIVO- ( $9.3 \pm 5.2 \text{ mm}$ ) than in NT-embryos ( $3.8 \pm 1.7 \text{ mm}$ ) with IVC-embryos ( $6.7 \pm 7.0 \text{ mm}$ ) in between. Significantly more somites were found in VIVO- ( $19.1 \pm 3.9$ ) than in IVC- ( $14.2 \pm 3.6$ ) and NT-embryos ( $13.3 \pm 3.1$ ). On Day 19, the embryo body was significantly longer in VIVO- ( $11.4 \pm 1.8 \text{ mm}$ ) than in NT- ( $7.2 \pm 1.6 \text{ mm}$ ) and IVC-embryos ( $6.7 \pm 1.2 \text{ mm}$ ). The allantois was significantly wider in VIVO- ( $70.6 \pm 21.9 \text{ mm}$ ) than in NT- ( $13.7 \pm 7.8 \text{ mm}$ ) and IVC-embryos ( $12.3 \pm 5.0 \text{ mm}$ ). Significantly more somites were observed in VIVO ( $27.8 \pm 3.4$ ) than in NT- ( $22.5 \pm 4.2$ ) and IVC-embryos ( $21.4 \pm 0.9$ ). For Days 17 and 19 embryos, the width of allantois was plotted against first the length of the embryo body in the same graph for the three groups of embryos and second the somite numbers. In both cases the result was a practically straight line indicating that the relative intra-embryonic dimensions were not distorted in NT- and IVC-embryos. In conclusion, NT- and IVC-embryos develop slower than VIVO-embryos although they maintain their relative intra-embryonic dimensions.

SYNCHRONIZATION OF GOAT FIBROBLAST CELLS AT QUIESCENT STAGE  
AND DETERMINATION OF THEIR TRANSITION  
FROM G0 TO G1 BY DETECTION  
OF CYCLIN D1 MRNA

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Cell cycle stage of donor cells is an important factor in nuclear transfer (NT) studies. Even though donor cells that are either at quiescent (G0) or G1 stage of cell cycle have been used to generate viable offspring by NT, advantages or disadvantages of these donor cells have not been studied in molecular details. Objectives of this study were to analyze cell cycle of transgenic goat fibroblast cells, determine culture conditions giving the highest synchronization of cells at quiescent stage, and timing of transition from G0 to G1 for these synchronized quiescent cells in media supplemented with 10% serum. Cell growth (doubling times) was determined by culturing cells at subconfluency, seeding at 10% ( $15 \times 10^4$  cells/10-mm culture dish) and 30% ( $35 \times 10^4$  cells/10-mm culture dish) densities in three separate plates and counting the cell numbers at 6, 12 and 24 h later. Another set of cells were cultured to 80%, then cultured with media containing only 0.5% serum for 4 days, and finally they were trypsinized and seeded the same as the non-starved group above. These cells were counted 6, 12, 24, and 30 h later. Culture conditions giving the highest amount of cell cycle synchronization at quiescent stage was achieved by culturing cells to 80% confluency, then starving for 2 or 4 days, and finally processing for cell cycle analyses by fluorescent activated cell sorting (FACS). G0 to G1 transition was obtained by detecting mRNA of cyclin D1, which is induced specifically in G1 phase of the cell cycle, by northern blot. Total cellular RNA was isolated from non-starved cycling cells, starved cells, and from cells that were first starved and then cultured in media containing 10% serum for 3, 6 and 10 h. Total cellular RNA used for each sample was normalized according to the cell number. Signal intensities were measured by phosphoimager. Cell cycle analyses by FACS was performed using half of the cell samples used for northern. Results showed that 4 days of starvation gave better synchronization at the G0 stage than 2 days. Four days of starvation resulted in 96.38, 0.48 and 3.14% of cells in G0/G1, S and G2/M phases, respectively, as detected by FACS. Doubling times of the cell lines are about 12 h for non-starved and, between 12 and 24 h for starved cells during the first cell cycle. Northern results indicated a residual amount of cyclin D1 mRNA in starved cells while significantly higher levels in cells that were cultured in media supplemented with 10% serum for 10 h after starvation (Table). Amount of cells at G0/G1 cells as determined by FACS and levels of cyclin D1 mRNA as determined by northern blot are shown below. In conclusion, transgenic goat fibroblast cells: (1) had approximately 12 h of doubling times, (2) were better synchronized at quiescent stage by 4 days of serum starvation, and (3) exited G0 and entered G1 within 10 h after start of serum stimulation.

Culture conditions	Percent of cells at G0/G1 by FACS	Amount of cyclin D1 mRNA by northern blot
Starved	96.4	9,712.73
Starved and then serum stimulated for 10 h	95.9	159,959.1
Cycling, never starved	51.4	137,545.2

PRODUCTION OF NORMAL OFFSPRING FROM MOUSE OOCYTES  
RECONSTRUCTED BY GERMINAL VESICLE REPLACEMENT

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Reconstruction of oocytes by replacement of germinal vesicle (GV) has important implications for the production of offspring from valuable and/or scarce senescent females in domestic and wildlife species, and also for ART programs in humans. The aim of the present study was to develop efficient protocols for GV replacement and in vitro maturation and fertilization of the GV replaced oocytes in mice. GV stage oocytes were collected from 8–12-week-old B6D2F1 female mice 46–48 h after eCG injection. The G was removed from the oocytes by Piezo-micromanipulation in the presence of 10 µg/ml cytochalasin B (CB), 13 µM nocodazole and 200 µM db-cAMP. A karyoplast containing GV was electrically fused with an enucleated cytoplasm prepared from GV stage oocytes of the same strain. GV replaced oocytes were matured in vitro in  $\alpha$ MEM + 10% FCS or TYH + 4 mg/ml BSA for 16–18 h. The GV replaced oocytes matured in the two media were pooled together, and their first polar body and MII plate were removed in the presence of 5 µg/ml CB and electrically fused with enucleated cytoplasm prepared from ovulated MII stage oocytes. These reconstructed oocytes were then fertilized in vitro by epididymal sperm in TYH, cultured in vitro and transferred at the 2-cell stage to the oviducts of recipient females. Some of the GV replaced oocytes were subjected to IVF directly after in vitro maturation. Successful fusion rate of GV karyoplasts was 68.6% (256/373); 135 of 185 (73.0%) and 67 of 71 (94.4%) of the GV replaced oocytes matured in vitro to the MII stage in  $\alpha$ MEM and TYH, respectively. Fusion rates of the MII plate and first polar body obtained from the GV replaced oocytes with enucleated MII cytoplasm were 70.9% (141/199) and 88.0% (22/25), and cleavage rates of these reconstructed oocytes were 60.3% (85/141) and 55.0% (11/20), respectively. Eleven normal live offspring were obtained after transfer of 96 reconstructed embryos. Some of these offspring have already grown normally and showed normal fertility. It was also shown that GV replaced oocytes could be fertilized and develop to blastocysts (5/8, 62.5%) without cytoplasmic replacement at MII stage. These data demonstrate that normal offspring can be produced from GV replaced oocytes following IVM and IVF, provided that the cytoplasm of these oocytes is replaced following maturation with that of in vivo-matured oocytes.

**CYNOMOLGUS MONKEY BLASTOCYSTS PRODUCED BY NUCLEAR TRANSFER USING AMNIOTIC EPITHELIAL CELLS**

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Nonhuman primate embryonic stem (ES) cells provide adequate model for the development of cell transplantation and regenerative medicine. Generation of autologous ES cells by human therapeutic cloning (HTC) through somatic cell nuclear transfer (NT) has been proposed to circumvent rejection. However, a limited number of oocyte and ethical concerns are the major obstacle to perform HTC. On the other hand, amniotic epithelial cell has been used for allotransplantation treatment, as it did not express major histocompatibility complex (MHC) class II antigen which is responsible for the acute rejection. The objective of this study was to produce cynomolgus monkey blastocyst using monkey amniotic epithelial (MAE) cells as nuclear donor for the establishment of ES cell line with reduced immunogenicity. Adult cynomolgus monkeys were given FSH (75 IU/animal) for 9 days to induced follicle development. Forty hours after i.m. administration of hCG (400 IU/kg), matured oocytes with cumulus cells were aspirated by syringe with needle under laparoscopic observation. Collected MII oocytes were denuded of cumulus cells and enucleated after exposure to cytochalasin B. Enucleation was confirmed by Hoechst 33342 staining. MAE cells which were cultured in serum-deficient medium for 5 days were used as donor cells for NT. A single MAE cell was inserted into the perivitelline space of the enucleated oocyte. Then, fusion of NT pairs was induced by either two direct current (DC) pulses of 150 V/mm for 25  $\mu$ s with chamber electrode (experiment no. 1) or two DC pulses of 30 V/100  $\mu$ m for 50  $\mu$ s with needle electrode (experiment no. 2) in 0.3 M mannitol medium containing 0.05 mM calcium acetate, 0.1 mM magnesium acetate and 3 mg/ml BSA. Fused NT embryos were chemically activated by either cycloheximide (CHX) (5  $\mu$ g/ml) for 4 h or a combination of ionomycin (IA) (5  $\mu$ M) for 2 min and 6-dimethylaminopurine (DMAP) (1.9 mM) for 4 h. Reconstructed embryos were cultured in TALP-BSA for 24 h at 38 °C in 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. Cleaved embryos were transferred to CMRL-1066 medium supplemented with 20% BCS and cultured for a maximum of 9 days. Development of NT embryos was summarized in Table 1. These results suggested that fusion at 30 V/100  $\mu$ m followed by activation with IA and DMAP was most efficient for development to the blastocyst stage (66.7%) of NT embryos. In addition, the condition of fusion, chemical activation, and electrode affected the developmental abilities of NT embryos. We are currently trying to establish ES cell lines from these blastocysts to investigate whether these ES cells express MHC class II antigen.

Table 1  
Development of NT embryos derived from MAE cells

Experiment number	Pulse	Elelctrode	Fused (%)	Activation	Cleaved (%)	Blastocyst (%)
1	150 V/mm $\times$ 2 <sup>a</sup>	Chamber	4/6 (66.7)	CHX	3 (75)	0
2	30 V/100 $\mu$ $\times$ 2	Needle	13/24 (54.2)	CHX	7 (100)	1 (14.3)
				IA/DMAP	6 (100)	4 (66.7)

<sup>a</sup> Pulse was repeated three times at intervals of 30 min.

## CHROMATIN STRUCTURE IN CLONED MOUSE EMBRYOS

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When a nucleus is transferred from a differentiated adult cell into an enucleated oocyte with the aim of producing a viable offspring, the tissue specific organization must be erased before remodeling of chromatin to ensure normal development. Oocytes receive sperm chromosomes that are packed primarily in protamines, whereas chromosomes in the transferred nucleus are packed in somatic histones. Furthermore, there is no transcription in the sperm head, whereas a transferred somatic nucleus is active. Chromatin structure has been defined in cloned mouse embryos derived from T-cells and embryo stem (ES) cells. Reconstruction of embryos by nuclear transfer was performed according to the method reported elsewhere [Wakayama et al., *Proc Natl Acad Sci USA* 1999;96(26):14984–14989]. Oocytes were collected from 8–10-week-old female B6D2F1 mice 13–14 h after hCG (5 IU) injection and pre-cultured in CZB medium [Chatot et al., *J Reprod Fertil* 1989;86:679–688]. The reconstructed oocytes were cultured in CZB medium for 1–3 h before being activated for 5–6 h in calcium-free CZB medium containing 10 mM strontium chloride and 5 µg/ml CZB to inhibit polar body extrusion. The activated oocytes with pseudo-pronuclei were collected and cultured in CZB or M16 medium (Sigma-Aldrich Company Ltd., Dorset, UK). T-lymphocytes were isolated and selected in G1 and G0 according to procedures described earlier [Pallante et al., *Theriogenology* 2001;55(1):2822 (abstract)]. The HPRT-deficient ES cell line HM-1 was derived from an inbred 129/Ola and employed for nuclear transfer at passage 19. HM-1 ES cells were cultured in Glasgow MEM (GMEM) media (GIBCO, Life Technologies Ltd., Paisley, UK) supplemented with 15% heat-inactivated FCS, 1000 units of leukaemia inhibitory factor/ml and the following reagents: 2 mM L-glutamine, 1% MEM non-essential amino acid solution (GIBCO) and 1% β-mercaptoethanol. R1 ES cells were cultured in DMEM media (GIBCO) containing the same reagents as HM-1 cells. One night before the experiment, the serum concentration was reduced to 5% and small cells were selected as nuclear donors. In vivo fertilized control embryos were collected at the 1-cell stage and then cultured in vitro. Changes in chromatin associated with the onset of transcription were monitored by describing acetylation of histone H4 lysine 5 and 12 [Worrad et al., *Development* 1995;121(9):2949–2959] at the 2- and 4-cell stages. Less than 5% of embryos derived from T-lymphocytes developed to blastocysts compared with approximately 50% with ES cells. The great majority of control embryos (85–100%) exhibited the characteristic peripheral distribution of H4 acetylation during the 2- and 4-cell stages. Fewer cloned embryos had the same pattern (0–48%) and the proportion was influenced by donor cell type and cell cycle stage. The peripheral distribution was present for a shorter period in embryos cloned from T-lymphocytes. In embryos created from G1-cells this pattern was detected in 2-cell embryos whereas those derived from G0-cells had a peripheral distribution only in the 4-cell stage. In embryos derived from ES cells, 48% had the peripheral pattern at the 2-cell stage. In conclusion, only a proportion of cloned embryos had a normal H4 acetylation pattern; the proportion was affected by differentiation state of donor cells, and donor cell cycle stage affects H4 acetylation patterns.

## INTERSPECIES NUCLEAR TRANSFER USING CAPRINE SOMATIC CELLS AND BOVINE OR PORCINE CYTOPLASTS

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Recently, cloned animals of several species have been produced by somatic cell nuclear transfer (NT). However, studies on interspecies NT using caprine somatic cells and other cytoplasts of different species are scarce. Thus, we designed this study to evaluate the possibility of interspecies nuclear transfer using caprine somatic cells as donor nuclei and bovine or porcine recipient cytoplasts. Prepared ear skin cells from Korean native goats were cultured in TCM-199 supplemented with 10% FBS at 39 °C, 5% CO<sub>2</sub> in air, and confluent monolayers were obtained. Bovine and porcine oocytes collected from slaughterhouse-derived ovaries were matured in TCM-199 (bovine) and NCSU-23 (porcine) supplemented with 10% FBS and hormones, respectively. The matured MII oocytes were enucleated and donor cells from serum starvation (0.5%) culture were fused through one or two electric pulses (DC 1.96 kV/cm, 30 μs each), then activated by a single electric pulse (AC 5 V/mm, 5 s +DC 1.56 kV/cm, 30 μs). Reconstructed oocytes with bovine cytoplasts were cultured in TCM-199 with 10% FBS and bovine oviductal monolayer for 7–9 days, and NT oocytes with porcine cytoplasts were cultured in NCSU-23 supplemented with 10% FBS for 6–8 days at 39 °C, 5% CO<sub>2</sub> in air for the observation of in vitro development. Data were analyzed by chi-square test. In the first experiment, we examined the effect of pulse frequency of electric stimulation on fusion and in vitro development rates of NT embryos. There were no significant differences in the fusion and cleavage rates by the pulse frequency of electric stimulation. The fusion rate of NT oocytes was 44.8% (13/29, one electric pulse) and 31.3% (15/48, two electric pulses) in caprine–bovine NT, and 45.7% (16/35) and 45.0% (18/40) in caprine–porcine NT oocytes, respectively. Cleavage rate of fused oocytes was 23.0% (3/13, one electric pulse) and 53.3% (8/15, two electric pulses) in caprine–bovine NT, and 75.0% (12/16) and 55.6% (10/18) in caprine–porcine NT embryos, respectively. In in vitro development, however, no NT embryos developed to the morula or blastocyst stage except for caprine–bovine NT embryos which had received two electrical pulses (13.3%, 2/15). In the second experiment, we compared in vitro development rate of NT embryos with IVF embryos. Cleavage rate of NT embryos was not different from IVF embryos (52.2%, 12/23 in caprine–bovine NT and 69.9%, 121/173 in bovine IVF embryos; 68.4%, 26/38 in caprine–porcine NT and 80.1%, 145/181 in porcine IVF embryos). Although development rate was lower ( $P < 0.05$ ) in NT embryos (8.3%, 1/12 in caprine–bovine NT and 0%, 0/26 in caprine–porcine NT embryos) than IVF embryos (29.8%, 36/121 in bovine IVF and 12.4%, 18/145 in porcine IVF embryos), three caprine–bovine and one caprine–porcine NT embryos developed to the morula (two caprine–bovine, 16.7%, 2/12 and one caprine–porcine NT embryos, 3.8%, 1/26) or blastocyst stage (one caprine–bovine NT embryo, 8.3%, 1/12). Our results imply that bovine oocyte cytoplast may be more suitable host for development of NT embryos to the blastocyst stage than porcine cytoplast after interspecies transfer using caprine somatic nuclei.

EFFECTS OF DIFFERENT ACTIVATION PROTOCOLS ON THE DEVELOPMENT  
OF CLONED SWAMP BUFFALO EMBRYOS DERIVED  
FROM GRANULOSA CELLS

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The activation protocols is one of the major step to achieve the success rate of producing embryos from nuclear transfer technique. We report here the activation protocols which have not yet been examined in swamp buffalo species. Granulosa cells from adults were cultured in alpha MEM + 10% fetal calf serum (FCS) and frozen at the third cell culture passage. These cells were thawed and cultured in alpha MEM + 10% FCS for 3–4 days before being used as donor cells. Oocytes were matured in vitro and enucleated at 20 h post-maturation. After 3–8 passages, individual cell (diameter 14–16  $\mu\text{m}$ ) were inserted into perivitelline spaces of enucleated oocytes. Fusion was performed at 24–26 h post-maturation by placing a cell–oocytes couplet between the both tips of electrode and electrostimulating with 2 DC pulses (30, 15  $\mu\text{s}$ ) in Zimmerman fusion medium. Activation was performed 1–2 h after starting fusion by incubation in 7% ethanol at room temperature for 5 min followed by culture in 5%  $\text{CO}_2$  in either (1) 1.9 mM 6-DMAP + 1.25  $\mu\text{g}/\text{ml}$  cytochalasin D (CD) for 4 h; (2) 10  $\mu\text{g}/\text{ml}$  cycloheximide (CHX) + CD for 5 h or (3) 6-DMAP + CHX + CD for 4 h. Reconstructed embryos were cultured in 100  $\mu\text{l}$  droplets of SOFaa + 1% FCS in 5%  $\text{O}_2$ , 5%  $\text{CO}_2$  in air for 2 days then co-cultured with frozen–thawed swamp buffalo oviduct epithelial cells in 100  $\mu\text{l}$  of SOFaa + 5% FCS in 5%  $\text{CO}_2$  for 3 days. Half of the volume of this medium was replaced twice with SOFaa + 10% FCS at 12-h intervals, then cultured for another 2 days. The results are summarized in Table 1. Our study showed that 6-DMAP + CHX + CD gave a higher morula and blastocysts yield than 6-DMAP + CD or CHX + CD.

Table 1  
In vitro development of cloned swamp buffalo embryos derived from granulosa cells after various activation protocols

Treatment	No. of couplet treated	Cleaved (%)	8-Cell (%)	Morula (%)	Blastocysts (%)
6-DMAP + CD	40	32 (80)	24 (60)	8 (20) <sup>a</sup>	6 (15) <sup>a</sup>
CHX + CD	40	33 (82)	25 (62)	9 (22) <sup>a</sup>	7 (17) <sup>a</sup>
6-DMAP + CHX + CD	40	32 (80)	26 (65)	12 (30) <sup>b</sup>	10 (25) <sup>b</sup>

<sup>a,b</sup> $P < 0.05$  (chi-square).

INCREASED IN VITRO DEVELOPMENT RATES OF SHEEP SOMATIC  
CELL NUCLEAR TRANSFER EMBRYOS PRODUCED BY  
'REVERSE-ORDER' ZONA-FREE METHOD

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Zona-free methods originally developed for cattle embryonic cloning [Peura et al., *Mol Reprod Dev* 1998;50:185–191] have since been modified for somatic cloning in cattle [Vajta et al., *Cloning* 2001;3:89–95; Booth et al., *Cloning* 2001;3:139–150] and pigs [Booth et al., *Cloning Stem Cells* 2002;3:191–197]. The aim of this study was to utilise a 'reverse-order' zona-free cloning method to determine if the introduction of donor nucleus prior to recipient oocyte enucleation has effect on an in vitro development of ovine somatic cell cloned embryos. In Fusion/Enucleation-group, in vitro-matured sheep oocytes were enucleated by a traditional aspiration method using a micromanipulator, followed by removal of zona pellucidae in 0.5% pronase. Cytoplasts and intact but likewise zona-free oocytes (Fusion/Enucleation-group) were attached with donor cells after brief 200 µg/ml phytohemagglutinin exposure and couplets fused electrically. Donor cells were passage 3 sheep granulosa cells frozen in 10% DMSO and thawed on the day of cloning. After 2 h in 10 µg/ml cycloheximide, oocytes in the latter group were enucleated using a micromanipulator by aspirating the second polar body and the telophase II chromosomes. The reconstructed embryos were cultured in small wells made into solidified 2% agarose in modified SOF–HCO<sub>3</sub> medium for 6 days. Differences in development rates between the groups were analyzed by unpaired *t*-test using GraphPad Software. The Fusion/Enucleation-group yielded significantly more blastocyst as shown in the table. Despite the overall clone production efficiency (reconstructed embryos produced per oocytes used) being lower in this group (63.6% versus 72.5%), the blastocyst production efficiency (cloned blastocysts produced per oocytes used) was higher (21.0% versus 14.8%), although neither of these values differed significantly. These results suggest that fusion before enucleation is beneficial for in vitro development and that the utilized zona-free 'reverse-order' cloning method is applicable to ovine somatic cell nuclear transfer. The method has since been modified further by excluding the use of micromanipulator completely by changing the enucleation method from micromanipulator-assisted aspiration to manual bisection.

Group	Fused/pulsed (%)	No. of cultured	Cleavage (%)	Blastocysts (%)
Enucleation-Fusion	295/342 (86.3) <sup>a</sup>	290	257 (88.6) <sup>a</sup>	59 (20.3) <sup>a</sup>
Fusion-Enucleation	316/371 (85.2) <sup>a</sup>	236	221 (93.6) <sup>a</sup>	78 (33.1) <sup>b</sup>

Different superscripts (a, b) within a column denote significant differences ( $P < 0.05$ ).

SOURCE OF FETUS DETERMINES EFFICIENCY OF PRODUCTION OF  
NUCLEAR TRANSFER JERSEY CATTLE GENETICALLY ENGINEERED  
TO RESIST MASTITIS

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To assess the influence of nuclear donor cells on efficiency of nuclear transfer, six cell lines from D 100 Jersey bovine fetal fibroblasts (BFF) were compared. These cells were aseptically collected from the biceps femoris muscle. One gram of tissue was minced in 5 ml of 0.05% trypsin in HBSS with EDTA and incubated at 38 °C for 1 h. Cells were washed and plated in 3 T75 in 10% DMEM. Cultures were split 1:3 on Day 4 and 1:2 on Day 6 before freezing on Day 7 in 92% FCS, 8% DMSO. Prior to nuclear transfer, BFFs were thawed and cultured for 5 days before being transfected with a multigenic transgene consisting of lysostaphin driven by ovine beta-lactoglobulin and two selectable markers, the neo-resistance gene and nuclear localized enhanced green fluorescent protein (GFP) gene. Transfected cells were cultured in the presence of G418 (400 µg), and surviving colonies were evaluated for GFP expression on Day 21 for use that week. Abattoir-derived oocytes matured for 18–21 h were enucleated and fused with selected fibroblasts 1–3 h after enucleation. Couplets were activated 2–4 h after electrofusion with ionomycin (5 µM) for 4 min followed by 6-DMAP (1.9 mM) for 4 h. Embryos were cultured in BARC media at 38.5 °C with 5% CO<sub>2</sub> + 5% O<sub>2</sub> + 90% N<sub>2</sub> until Day 8 when two blastocysts were transferred into each synchronized 14–17-month-old Holstein recipient. Return to estrous was detected with a computer monitored transponder system and all recipients were examined by ultrasound on Day 39. Animals who remained pregnant were examined on Day 53 and Day 67 by ultrasound and continued to be monitored for estrous throughout the pregnancy. During the past 3 year a total of 4007 fused couplets were placed in culture. Overall fusion rate was 80%. Nine hundred and twenty (23%) embryos developed into blastocysts, and 650 were transferred to 329 recipients. Percentage of fused couplets did not differ between cell lines. BFF8 and BFF9 cells produced the smallest proportion of blastocysts (18%) and BFF11 cells the highest (29%). Overall 51% of recipients returned to heat within 30 days. Non-return rates varied among cell lines from 29–58%. Eighty-two percent of pregnancies were lost by 56 days. Only two cell lines, one male (BFF7) and one female (BFF10), maintained pregnancies beyond 210 days at a rate of 9.3 and 7.1%, respectively. Four live calves were produced from BFF10 cells and three from BFF7 cells. More transfers will be required to determine statistical differences; however, the proportion of pregnant recipients at Day 55 for BFF7 and BFF10 cells was at least double that of the pregnancy rate of other cells and may provide a fitness indicator of BFF cell lines for nuclear transfer.

## EVALUATION OF BOVINE BLASTOCYSTS CLONED USING DIFFERENT ACTIVATION PROTOCOLS

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The aim of this study was to compare two different oocyte activation protocols with regard to subsequent rates of development, cell number, levels of apoptosis and ploidy of cloned bovine embryos derived from female ear fibroblast cell lines. The activation procedures used either a protein synthesis inhibitor (cycloheximide, CHX) or cdc2 kinase inhibitor (sodium pyrophosphate, SPP) in combination with ionomycin. Cumulus–oocyte complexes, derived from slaughterhouse ovaries, were matured for 18–19 h in TCM 199 supplemented with 2% serum, 10 µg/ml FSH, 10 µg/ml LH and 1 µg/ml estradiol-17β. Expanded cumulus cells were then removed by vortexing for 2 min in 3% sodium citrate. Oocytes having a first polar body and dense cytoplasm were selected for enucleation. Embryos reconstituted with donor fibroblast cells were exposed to a single electrical pulse (1.4 kV/cm DC, 20 µs) with fusion rates of approximately 80% (1953 in 25 replicates). Following fusion, embryos were exposed to 5 µM ionomycin for 5 min and were then either exposed to 10 µg/ml CHX (group 1) or 1.9 mM SPP (group 2) for 5 h. Cloned embryos and control IVF embryos were then cultured in modified SOF medium with BSA, essential and nonessential amino acids at 39 °C in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> (5/5/90) for 72 h post-activation (hpi) or post-insemination (hpi). At 72 hpa/hpi, embryos were transferred to SOF medium supplemented with glucose for up to 192 hpa/hpi. Differences between groups were analyzed using one-way ANOVA after arc–sine transformation of proportional data. The rates of cleavage within group 1 (CHX) were significantly higher ( $P \leq 0.05$ ) than in group 2 (SPP) or IVF controls (92% versus 63 and 75%, respectively). However, there was no difference ( $P \leq 0.05$ ) in blastocyst development rates among groups 1, 2 and IVF controls (33, 26 and 22%, respectively). Cell numbers as determined on Giemsa-stained embryo spreads at 192 hpa/hpi were  $103 \pm 22$  ( $n = 18$ ),  $112 \pm 33$  ( $n = 12$ ), and  $140 \pm 13$  ( $n = 17$ ) for groups 1, 2 and IVF controls, respectively. Chromosomal analysis showed that most blastocysts in group 1 (80%, 15/19) and group 2 (90%, 9/10) were diploid. The proportional incidence of TUNEL-positive blastomeres from 192 hpa blastocysts examined in groups 1 ( $8 \pm 4\%$ ,  $n = 10$ ) and 2 ( $7 \pm 4\%$ ,  $n = 8$ ) were similar to that in IVF controls ( $11 \pm 4\%$ ,  $n = 15$ ). In combination, these results suggest that both sodium pyrophosphate and cycloheximide can be used with ionomycin in activation protocols for bovine embryo cloning.

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## BIRTH OF RATS BY NUCLEAR TRANSPLANTATION USING 2-CELL STAGE EMBRYO AS DONOR NUCLEUS AND RECIPIENT CYTOPLASM

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Enucleated 2-cell embryos have been widely used as a recipient cytoplasm for mice embryonic nuclear transplantation (NT), [Smith and Wilmut, *J Reprod Fertil* 1994;100:323–329]. However, there is only one report about birth of rat by nuclear transplantation using zygote as donor nucleus and recipient cytoplasm in rats [Kono et al., *J Exp Zool* 1988;248:303–305]. In this study, the nuclei from 2-cell rat embryos were transferred into enucleated 2-cell embryos and developed to term after transferred to foster mother (NT2). Zygote to zygote NT was also tested as comparison group (NT1). Zygotes and 2-cell embryos were harvested from 4-week-old Sprague–Dawley female rats super-ovulated with eCG and hCG injection and mated with the same strain males, and manipulated between 20–22 and 42–44 h after hCG injection, respectively. Enucleation was carried out by slitting the zona pellucida using microneedle and subsequent removing the pronuclei of zygotes or karyoplast from both blastomeres of the donor 2-cell embryos using a pipette with an approximate inner diameter of 15  $\mu\text{m}$ . NT was performed by transferring a karyoplast from a different embryo into the perivitelline space of another. The reconstructed embryos were fused by electrofusion method (1.5 kV/cm, 60  $\mu\text{s}$ , double pulses). The procedure was repeated once when fusion was not successful. The fused eggs were cultured *in vitro* for 4 or 24 h before transferred into Day 1 pseudopregnant recipients (Hooded Wistar) at 1- or 2-cell stage with 10–20 embryos in each oviduct. All the media used in this study were based on mR1ECM [Miyoshi et al., *Biol Reprod* 1997;56:180–185]. *In vitro* culture investigation was also carried out to check the developmental competence of the reconstructed embryos. The differences of the results between groups were tested by chi-square test. *In vitro* development to the blastocyst stage was not significantly different between the two groups (NT1: 34.3%, 34/99; NT2: 45.0%, 18/40). Two of the three recipients of NT1 and two of the five recipients of NT2 were pregnant. Six pups (three from NT1, three from NT2) were delivered from the four foster mothers. Two pups from NT1 and one pup from NT2 were alive on the next day of delivery, whereas other three pups died when we checked. These three pups are so far 3-week-old and still alive healthy although the pups from both groups have low dense hair around their head. It is not clear that this low dense hair resulted from NT procedure itself or not. Our results suggest that by using nuclear karyoplasts from the 2-cell embryos as the nuclear donors and reconstructing them with enucleated 2-cell embryos, health animals can be developed in the rat. With our knowledge, it is the first report of birth of rats using 2-cell stage embryo as recipient cytoplasm.

## REPROGRAMMING OF TELOMERASE ACTIVITY IN NUCLEAR TRANSFER BUFFALO EMBRYOS USING QUIESCENT OR NON-QUIESCENT SOMATIC CELLS AS DONOR NUCLEI

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Telomerase is a ribonucleoprotein that synthesizes telomeric DNA from its own RNA template to restore telomere length during cell replication. Reprogramming of telomerase activity has been detected as early as the blastocyst stage in nuclear transfer (NT) bovine embryos reconstructed with either quiescent or senescent somatic cells that display low/non-detectable levels of telomerase. The objective of this study was to examine the telomerase activity in cloned buffalo embryos using quiescent or non-quiescent somatic cells as donor nuclei. Slaughterhouse-derived oocytes were matured *in vitro* by routine methods for 22 h. Denuded MII oocytes with the presence of the first polar body were selected for enucleation. Adult fibroblasts recovered from biopsied ear skin of 6-year-old female were cultured and used as donor nuclei between passages 2–8 of culture. Donor cells were induced into quiescence by culturing in DMEM supplemented with 0.5% FBS for 3–5 days or additional culture of confluent cells for 5 days with 10% FBS and subjected to flow cytometric cell cycle analysis. Non-quiescent donor cells were prepared by culturing fibroblasts with 10% FBS until reaching 60–80% confluency. Donor cells were fused with enucleated oocytes by two electrical pulses of 2.1 kV/cm for 30  $\mu$ s, activated by 5  $\mu$ M calcium ionophore followed by incubation in 2 mM 6-DMAP for 4 h. The reconstructed embryos were cultured in TCM 199 supplemented with 10% FBS at 39 °C in 5% CO<sub>2</sub> for 7 days. Nuclear transfer embryos at 2-cell ( $n = 5$ ) and blastocyst stage ( $n = 5$ ) were collected at 1 and 7 days of culture and subjected to telomerase activity assay (three replicates). Quiescent or non-quiescent somatic cells and cloned embryos were lysed in ice-cold lysed buffer and telomerase activity was assayed using the PCR ELISA kit. Telomerase activity in quiescent or non-quiescent cells collected before NT was undetectable (negative). Relative telomerase activity in NT embryos was low in 2-cell stage and reached highest level in the blastocyst stage. Blastocyst derived from nuclei of quiescent cells induced by serum starvation displayed a significantly ( $P < 0.05$ , one-way ANOVA) higher telomerase activity compared with those derived from confluent or non-quiescent cells. In conclusion, reprogramming of telomerase activity was demonstrated in buffalo NT embryos reconstructed using adult fibroblasts that had displayed negligible levels prior to reconstruction.

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## EFFECT OF DIFFERENT CULTURE SYSTEMS, DONOR CELL ORIGIN AND ROSCOVITIN TREATMENT OF RECIPIENT OOCYTES IN BOVINE CLONING

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Different variables were tested in an experiment aimed at simplifying procedures and increasing embryo survival rate in a bovine cloning program. Three culture systems for reconstructed embryos were used when adult fibroblasts were used as donor cells: TCM-199 + 5% FCS, Menezo + 5% FCS (both with VERO cells as co-culture) and SOF without co-culture but with lower O<sub>2</sub> concentration. SOF medium was also used to culture reconstructed embryos when donor cell were genetically and non-genetically modified fetal fibroblasts. Finally, when genetically modified fetal fibroblasts were used as donor cells, recipient oocytes were previously treated with roscovitine (R), to suspend meiosis and optimize recipient usability. Oocytes were aspirated from slaughterhouse ovaries and matured in TCM-199 + 5% FCS at 39 °C for 24 h. For R-treated group, oocytes were incubated with 25 uM R in TCM 199 + 5% FCS for 24 h at 39 °C prior to the maturation. Matured oocytes were denuded by vortexing for 3 min in TL-HEPES with 1 mg/ml bovine testis hyaluronidase. Metaphases were assessed and oocytes were enucleated by visualization with Hoechst 33342 (5 ug/ml) under UV light (<6 s). Adult fibroblast from an Angus bull and fetal fibroblast from a 45-day-old Jersey female fetus were used as donor cells. Transfection with constructs containing a neomycin resistance gene was performed using liposomes. After selection with genetic in for 10–15 days, donor cells at G0/G1 stages were fused to enucleated oocytes by an electrical pulse. After 3 h, activation was induced by incubation in TL-HEPES with 5 uM ionomycin for 4 min and 2 mM 6-DMAP for 3 h. The oocytes were then washed with TL-HEPES and co-cultured in either TCM-199 + 5% FCS + 10 g/l albumin or Menezo + 2% FCS both with VERO cells, or in SOF medium and atmosphere of 5% CO<sub>2</sub> + 5% O<sub>2</sub> + 90% N<sub>2</sub>. Generally, two blastocysts were transferred non-surgically per recipient cow, and pregnancies at 30–35 days determined by ultrasonography. Cleavage (48 h), development to blastocysts (Day 7–9) were recorded and analyzed by chi-square. Cleavage rates and development to blastocysts were higher when embryos were cultured in SOF. However, no differences were observed in pregnancy rates due to different culture conditions or source of donor cells. Suspension of meiotic maturation for 24 h did not compromise the developmental competence of recipient oocytes. Therefore, treatment with R might be used to increase the availability of oocytes for NT procedures.

Treatment	<i>n</i>	Cleavage (%)	Blastocyst (%)	IR	PR (%)
Adult fibroblast TCM 199 + VERO	294	156 (53.9) <sup>a</sup>	22 (8) <sup>a</sup>	13	5 (38)
Adult fibroblast Menezo + VERO	324	236 (72.3) <sup>b,c</sup>	29 (9) <sup>a</sup>	17	5 (29)
Adult fibroblast SOF	108	81 (75.0) <sup>b,c</sup>	24 (22) <sup>b</sup>	11	5 (45)
Fetal fibroblast SOF	197	122 (61.9) <sup>a,b</sup>	33 (17) <sup>a,b</sup>	16	5 (32)
Transfected fetal fibroblast SOF	646	476 (73.7) <sup>b,c</sup>	128 (20) <sup>b</sup>	56	25 (45)
Transfected fetal fibroblast SOF-R	228	191 (84) <sup>c</sup>	51 (22) <sup>b</sup>	30	16 (53)
Total	1797	1262 (70)	287 (16)	143	61 (45)

IR: implanted recipients, PR: pregnant recipients. Percentages within columns with different superscripts are different ( $P < 0.05$ ).

## SOMATIC CLONING IN PIGS; EFFECT OF ACTIVATION TREATMENTS

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At the present stage of studies the efficiency of somatic cloning technology in pigs is still lower than in other species of farm animals and as a rule do not overstep 5.0% obtained blastocysts and 1.5% born piglets towards number of the reconstructed oocytes.

The aim of our study was to determine the *in vitro* developmental potential of porcine embryos reconstructed by the direct micro injection of cumulus cell nuclei into a cytoplasm of enucleated oocytes. Within the framework of the generally accepted aim effect of nuclei microsurgical transfer on a viability of reconstituted embryos according to the used activating agents: either chemical (ionomycin and cycloheximide; group A) or physical (electrical pulses; group B) was examined. The estimation criterion of *in vitro* cultured embryos developmental competences was the percentage of obtained morulae and blastocysts. The quality of reconstructed porcine blastocysts was evaluated on the base of mean cell number.

Good quality cumulus–oocyte complexes (COCs) were collected from ovaries of slaughtered gilts and sows. They were matured in 50  $\mu$ l of HEPES-buffered TCM-199 medium (Gibco) supplemented with 20% estrus porcine serum (EPS), 10% porcine follicular fluid (pFF), 1 mM L-glutamine and 75  $\mu$ g/ml kanamycin monosulfate at 38.5 °C in humidified air with 5% CO<sub>2</sub>. The matured oocytes were separated from cumulus cells and Met II oocytes were enucleated in the presence of cytochalasin B (CB; Sigma). To prepare karyoplasts, the cells were dissociated from COCs by vortexing matured COCs for 1 min in TCM-199–HEPES containing 4 mg/ml BSA or by centrifugation in the same medium. The karyoplasts were injected into a cytoplasm of the enucleated oocytes. Afterwards, reconstituted pre-embryos were incubated in NCSU-23 containing 4 mg/ml BSA at 38.5 °C in atmosphere of 5% CO<sub>2</sub> in air for 1–1.5 h before activation. After preincubation reconstructed oocytes were artificially activated. In group A, cytoplasmic hybrids were activated by incubation in 15  $\mu$ M ionomycin for 5–6 min followed by incubation with 10  $\mu$ g/ml cycloheximide (CHXM) and 5  $\mu$ g/ml CB for 1.5–2 h in NCSU-23 medium containing BSA. After this period reconstructed zygotes were transferred to the same medium supplemented only with CHXM for additional 2–2.5 h. In group B, cytoplasmic hybrids were activated electrically in mannitol medium by application of two DC pulses of 1.2 kV/cm for 60  $\mu$ s each. Then, cloned zygotes were transferred to NCSU-23 containing 5  $\mu$ g/ml CB for 2 h. After activation treatments, reconstructed embryos were cultured in 50  $\mu$ l NCSU-23 containing 0.4% BSA at 38.5 °C in humidified air with 5% CO<sub>2</sub>. Three to four days later, developing embryos were placed in 50  $\mu$ l of NCSU-23 supplemented with 10% FBS for additional 72 h. After 6–7 days of culture, the blastocyst formation rate was evaluated and cell numbers of blastocysts were counted following Hoechst 33342 staining.

The percentage of cleaved embryos were 61.4 and 51.1% in group A and B, respectively. In group A, the percentage of development to the morula and blastocyst stages were 35.4 and 3.8%, and in group B, 28.1 and 3.0%, respectively. The cell numbers in blastocysts were comparable (approx. 26.0).

In conclusion, porcine cloned zygotes exposed to two-grade chemical postactivation treatment with ionomycin and CHXM showed slightly higher *in vitro* developmental competences to morula and blastocyst stages compared to these ones exposed to electroporation.

PRODUCTION OF INTERSPECIES NUCLEAR TRANSFER EMBRYOS USING  
MALE AND FEMALE LLAMA (*LAMA GLAMA*) CELL LINES

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Interspecies nuclear transfer can be used as a tool to produce embryos in endangered species and also in species where oocyte availability is limited. In this laboratory, we have previously used the interspecies model to study the effect of different horse cell lines on in vitro embryonic development [Sansinena M. et al., *Theriogenology* 2002;58:775–777]. Due to the limited availability of llama oocytes, we used the interspecies approach to produce nuclear transfer llama embryos, and to compare female and male adult llama fibroblast cell lines by monitoring in vitro embryo development. Two male and female (Treatments A and B) adult llama fibroblast cell lines were evaluated in this study. All cell lines used were between 2nd and 3rd passage and serum starved for at least 3 days prior to their use. Cell line identity was not known during the experiment. Of a total of 585 bovine metaphase II stage oocytes, 130 were randomly assigned to activation only control group (Control, Treatment D). Of the remaining 455 oocytes, 438 (96%) survived enucleation. Of these, 124 were assigned to an enucleation/activation control group (Control, Treatment C). The remaining enucleated oocytes were assigned to the following cell treatments: 164 were reconstructed with female llama fibroblasts (Treatment A), 150 were reconstructed with male llama fibroblasts (Treatment B), and all reconstructed couplets were fused with two pulses of direct current (1.9–2.3 kV/cm, 30  $\mu$ s each). After electrofusion, activation was performed by a 5-min exposure to 5  $\mu$ M ionomycin followed by 3-h incubation in cycloheximide (10  $\mu$ g/ml) in CR1aa medium. All embryos were cultured in 35- $\mu$ l drops of CR1aa under mineral oil at 38.5 °C in 90% N<sub>2</sub>, 5% O<sub>2</sub> and 5% CO<sub>2</sub> for 7 days. There was no significant difference in either fusion or cleavage rates among treatments. However, there was a significant difference in the number of lysed couplets post-activation between the llama cell lines (Treatments A and B) and the control groups (Treatments C and D) (Table 1). From fused couplets, the proportions of those completing the first mitotic cycle were: 53, 51, 62 and 67% for Treatments A, B, C and D, respectively. Embryos from the llama cell lines (Treatments A and B) progressed to the 8- and 16-cell stages, but did not develop further in vitro. Presence of llama nuclei was confirmed using Hoechst-33258. Results indicate that the cow oocyte is capable of activation of mRNA after transfer of a llama donor karyoplast, and supporting the first mitotic cycles in vitro. Although embryos developed beyond the early mitotic cycles, an in vitro developmental block occurred between the 8- and 16-cell stages. Further research is needed to overcome the developmental block in llama interspecies nuclear transfer.

Table 1  
Interspecies nuclear transfer with two llama (male and female) cell lines

Treatment group	Cell type	<i>n</i>	Fused <sup>1</sup>	Lysed <sup>2</sup>	Cleaved Day 2	BLST Day 7
A	Llama female	164	104 (63%)	26 (25%) <sup>a</sup>	55 (33%)	0 (0%)
B	Llama male	150	76 (51%)	19 (25%) <sup>a</sup>	39 (51%)	0 (0%)
C (Control)	Enucleation	124	124 (100%)	0 (0%) <sup>b</sup>	77 (62%)	0 (0%)
D (Control)	Activation only	130	130 (100%)	0 (0%) <sup>b</sup>	87 (67%)	15 (11%)

Groups with different superscripts within a column are significantly different, ANOVA ( $P < 0.05$ ).

<sup>1</sup> Exposed to fusion buffer and electrofusion under same conditions.

<sup>2</sup> Lysed post-activation.

COMPARISON OF DEVELOPMENTAL POTENTIAL OF KERATINOCYTES  
AND FIBROBLASTS AFTER NUCLEAR TRANSFER IN BOVINE

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In most nuclear transfer experiments, the somatic cells used as donors are issued from a pool of cultured cells where their differentiation status is undefined. At the moment, evidence of the genetic totipotency of nuclei from terminally differentiated somatic cells is lacking. The epidermis is a tissue that is maintained through the proliferation of stem cells which produce daughter cells that undergo terminal differentiation. We established primary cultures of ear epidermal cells from adult animals in the bovine species to evaluate the developmental potential of nuclei from fully differentiated cells, and we compared it with the potential of fibroblasts derived from the dermis of the same animal, these cells having previously proved to be fully reprogrammed when used as donor cells in nuclear transfer experiments. Outgrowth from ear skin biopsies were subcultured in a medium for the proliferation of keratinocytes [adapted from Reynolds A.J., Jahoda C.A.B., J Cell Sci 1991;99:373–385]. Fibroblasts were selectively harvested by a cell detachment solution (Accutase, PAA Laboratories). Keratinocytes and fibroblasts were suspended by trypsinisation. Cells suspensions were kept in complete medium overnight at 4 °C to restore cytoplasmic membrane. Four hours before nuclear transfer, keratinocytes and fibroblasts suspensions were incubated at room temperature for 1 h with primary antibodies anti-desmoplakin I & II (Boehringer-Manheim) then for 1 h with biotinylated donkey anti-mouse antibodies, and finally for 15 min with fluorescein-avidin. Cells were observed under attenuated UV light and selected according to their peripheral immuno-labelling intensity with a micropipette under the microscope before being fused with an enucleated IVM oocyte. Reconstructed embryos were activated in 10 µg/ml cycloheximide and 5 µg/ml cytochalasin B for 5 h after fusion, then cocultured on Vero cells for 7 days in microdrops of B2 medium supplemented with 2.5% FCS. The reconstructed embryos cleaved at the same rate with both type of cells, but the development to the blastocyst stage was significantly lower with keratinocyte type as compared to the fibroblast type (Table 1).

We are using this model to assess whether nuclei issued from differentiated keratinocytes present differences in the remodeling and in the cell cycle characteristics during the early development after nuclear transfer into an enucleated bovine oocyte as compared to the fibroblast.

Table 1

In vitro development of embryos reconstructed with bovine keratinocytes and fibroblasts

Cell type	Couplets	Fused (%)	Cleaved (%)	Blastocysts (%)
Fibroblasts	82	71 (86.6)	49 (69.0)	32 (45.0) <sup>a</sup>
Keratinocytes	111	85 (76.6)	51 (60.0)	8 (9.4) <sup>b</sup>

(a, b) Values differ significantly ( $P < 0.05$ ; chi-square test).

THE EFFECT OF ELECTRICAL FIELD STRENGTH FOR ACTIVATION  
ON DEVELOPMENT OF CAPRINE NUCLEAR TRANSFER EMBRYOS  
CLONED FROM ADULT EAR CELLS

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Activation conditions used in nuclear transfer (NT) procedure is one of the many critical factors affecting the efficiency of animal cloning. The purpose of this study was to investigate the effect of electrical field strength used for activation on the developmental capacity of caprine NT embryos that cloned from the fibroblast cells derived from an adult Alpine doe. Reconstituted embryos were obtained by transfer and electrical fusion of the quiescent ear-derived fibroblast cells at their 4th passage to the enucleated metaphase II (M II) oocytes. Activation of those NT embryos with either electrical field strength of 1.67 or 2.33 kV/cm was then performed at 4–5 h after fusion. Cleavage rate of the NT embryos activated with 2.33 kV/cm following incubation by 6-DMAP was significantly higher than those with 1.67 kV/cm following incubation by 6-DMAP after in vitro culture for 18 h (65.6% versus 19.6%,  $P < 0.001$ ). No pregnancy was found after transfer of 51 NT embryos activated with 1.67 kV/cm to 14 recipient does. In contrast, two of seven recipients became pregnant and gave birth to three kids after embryo transfer of 61 NT embryos activated with 2.33 kV/cm. Body weight of these three kids at birth were within the normal range of typical Alpine goats. However, one kid died at 1 h after birth, the remaining two appeared normal and healthy so far. DNA analysis by polymerase chain reaction single-strand conformation polymorphism confirmed that these three NT kids obtained were genetically identical to their nuclear donor. The results demonstrated that activation induced by an increased electrical field could enhance the cleavage and the subsequent development capacity to term of reconstituted caprine embryos.

PRELIMINARY RESULTS OF CELL CRYOBANKING AND EMBRYO  
PRODUCTION OF BLACK BEAR (*URSUS THIBETANUS*) BY  
INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER

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Cell cryobanking and embryo production by interspecies somatic cell nuclear transfer (SCNT) have been considered as a possible approach to meet the urgent need of the protection of endangered mammalian species in Vietnam [Uoc et al., *Theriogenology* 2002;57:437]. In this study, we present the preliminary results of the application of this approach to black bear (*Ursus thibetanus*)—a wild specie facing extinction due to the massive illegal hunting for medical use. The skin biopsies were taken from two male and three female adult animals and were transported to the laboratory in 4 °C PBS solution. The culture was carried out in four-well dish containing DMEM medium (Gibco) supplemented with 10% heat-treated fetal bovine serum (FBS; Gibco) at 39 °C under 5% CO<sub>2</sub> in air. The confluent cells were treated with trypsin (Sigma) and replicated with split ration 1:8 for further subculture. The karyotype estimation by staining with Giesma was carried out with the cells collected at 2nd and 10th passages. The cryo-conservation was performed from passage 6. Cells were lodged in 0.5 ml straws (IVM, France) containing the mixture of 10% DMSO-sucrose 0.5 M then frozen with step-wise equilibration at 4 °C, -20 °C and over nitrogen vapor (0.5 h) before plunging into liquid nitrogen. Prior to nuclear transfer, quiescent cells were obtained by the culture of frozen-thawed cells in FBS-free medium for 3–4 days. SCNT was undertaken using the bovine enucleated oocytes as host ooplats. A single cell was transferred into an enucleated cytoplasm and the reconstructed oocytes were treated with two direct current (DC) pulses of 150 V/mm for 25 μs in Zimmerman medium, following by two DC pulses at 20 V/mm for 20 μs. The fused oocytes were cultured in CR1aa medium containing cyclohexamide for 6 h then in B2 (Menezo) supplemented with 10% FBS on Vero cell layer. The results show that the cell culture with normal morphology and karyotype ( $2n = 74$ ) was obtained from all five collected biopsies. The first subculture took place at Days 10–12 after the collection and the interval for next subculture to get confluent monolayer was varied from 2 to 3 days. Primary record from 270 reconstructed oocytes showed that the percentages of oocytes successfully fused-activated, cleaved, developed to morula stage and to early blastocyst with the formation of blastocoelic cavity were 74.4, 57.9, 10.4 and 4.1%, respectively. The similar diploid chromosome number of 74 was observed in reconstructed embryos developed to morula stage (16–32 cells). In conclusion, these results show that interspecies SCNT using bovine enucleated oocytes can be applied to produce embryo from black bear.

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CHIMAERAS FROM HAND-MADE CLONED (HMC) AND IVP BOVINE EMBRYOS:  
AN EFFICIENT SYSTEM FOR PRODUCTION AND CELL  
ALLOCATION TRACKING

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Placental malformations are common causes of intrauterine developmental abnormalities of embryos produced by nuclear transfer. A possible way to overcome this problem is to establish chimaeras where the fetus develops from the cloned embryo, and the placenta is formed from an embryo of different origin (e.g. IVP, in vivo-derived) more capable of healthy placentation. This principle was successfully applied previously in mouse using embryonic stem cell and tetraploid aggregates [Nagy et al., PNAS 1993;90:8424–8428]. However, in spite of numerous attempts, bovine application is hampered by the lack of simple and reliable methods for chimaera production, blastomer labelling and, consequently, establishing developmentally biased blastomere allocation. The aim of this work was to find a solution for the first two problems. Hand-made cloned (HMC) and in vitro produced (IVP) embryos were produced as described previously [Vajta et al., Cloning 2001;3:89–95, Theriogenology 1996;45:683–689, respectively] and were used on Day 4 after embryo reconstruction or fertilization, respectively. The zona pellucida of the IVP embryos was removed with pronase digestion, and one HMC and one IVP compacted morula were placed into a single microwell of the Well of the Well (WOW) system [Vajta et al., Mol Reprod Dev 2000;55:256–264] and cultured together until Day 7, when their morphology was evaluated with both a stereomicroscope and an inverted microscope. For control, a single zona digested IVP compacted morula was cultured in a WOW from Day 4 to Day 7. Chimaera formation was regarded accomplished if one blastocoel and one ICM was developed from the two embryos and apart from this blastocyst, no significant cell mass was found in the WOW. In three replicates, a total of 64 attempts resulted in 52 chimaeras (81%). There was no difference in the developmental rate compared to controls (68 blastocysts from 80 zona-free IVP morulae, 85%). For blastomere allocation detection, one Day 4 compacted morula was stained with 10 µg/ml Hoechst 33342 for 5 min, then aggregated with the unstained counterpart in a WOW. According to our preliminary experiments, Hoechst staining without UV exposition did not hamper embryo development. On Day 7, chimaeras were placed in 2 µl glycerol on a microscopic slide, alongside with another 2 µl drop of glycerol containing 1 mg/ml propidium iodide. The coverslip flattened the embryo and unified the two drops. Blue UV fluorescence of nuclei of the Hoechst stained embryo was detectable immediately, while 2 days later all nuclei of the chimaera have shown an intensive pink fluorescence as the result of the propidium iodide diffusion. In 14 of 15 attempted stainings, blastomere allocation was successfully detected. These methods for bovine chimaera formation and allocation detection may open the way to construct chimaeras with the required allocation, and to resolve placentation problems of bovine embryos produced by nuclear transfer.

## EFFECTS OF PROCEDURES AND MEDIUM ON INDUCED ENUCLEATION AND DEVELOPMENT OF CLONED CF1 MOUSE EMBRYOS

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Cytoplasts prepared by induced enucleation (IE) of mouse oocytes by the antimetabolic drug demecolcine are competent to support term development after reconstructed with somatic cells [Baguisi and Overstrom, 2000]. The IE method also supports preparation of goat, sheep and cow cytoplasts [Ibanez et al., 2002; Gasparrini et al., 2002; Fischer et al., 2002]. Previous experiments have revealed strain-specific differences in IE rates between different strains of mice [Ibanez et al., 2002]. The study assesses modifications of IE in efforts to optimize its application to the outbred CF1 strain mouse. Metaphase II oocytes were recovered from superovulated (5 IU eCG, 5 IU hCG) CF-1 female mice (8–12 weeks) 18–20 h post-hCG. Denuded oocytes were washed in H-KSOM or M2<sup>-</sup> medium (M2 devoid of phosphate and glucose) and then immediately activated (Act I) by 2 min exposure to 10 mM ionomycin. Four treatments were used: A, Ca<sup>2+</sup> free KSOM with 10 mM Sr<sup>2+</sup>; B, KSOM containing 10 mM Sr<sup>2+</sup>, 2 mg/ml CD (cytochalasin D) and 0.4 mg/ml demecolcine; C, KSOM containing 10 mM Sr<sup>2+</sup>, 0.8 mg/ml demecolcine, 2% DMSO and 8.5 mM CaCl<sub>2</sub>; D, KSOM containing 10 mM Sr<sup>2+</sup> and 5 mg/ml CB (cytochalasin B). In the control (parthenotes) group, Act I oocytes were placed into medium A for 10–15 min then cultured in medium D for up to 5 h. In the double activation group, oocytes were activated a second time (Act II, same as Act I) after culture for 10–15 min in medium A before transfer into medium D. Control development (parthenotes) was compared between Act I and Act II oocytes after 96 h culture in KSOM. In the IE group, Act I oocytes were treated 10–15 min in medium B before Act II, then transferred to medium A for 10–15 min prior to IE culture in medium C for up to 4 h. PB2 extrusion and chromatid segregation was assessed by Hoechst staining (H33342, 5 mg/ml). Some control and IE treated oocytes were triple labeled for chromatin, microtubules and microfilaments and analyzed). To observe further development, zona pellucidae of IE oocytes were removed and reconstructed with CF1 cumulus cells or ICR fibroblasts by electrical fusion (DC 150 V/mm, 10 ms, 1 pulse) in 0.3 M glucose, 0.1 mM MgCl<sub>2</sub>, 0.05 mM CaCl<sub>2</sub>. Fused embryos were cultured (KSOM or CZB) for 96 h after brief culture (2–4 h) in medium D (Tables 1–3).

These results suggest that double activation and modification of activation treatments can increase blastocyst development rates of parthenogenetic embryos, and the IE rate in CF1 strain oocytes. Successful IE of CF1 mouse oocytes displayed two phenotypes: either all chromatids extruded with PB2, or PB2 and chromatids extruded separately. Development of somatic cell reconstructed CF1 embryos was poor in KSOM medium suggesting that KSOM or the CF1 strain of mice cannot support further development of somatic cell-cloned embryos.

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Table 1  
Activation treatment and the development (%) of CF1 parthenogenetic embryos

Groups	No. of experiments	No. oocytes	1 cell	2–8 cell	Morula	Blastocyst	Fragment
Act I	14	129	15 (11.6)	56 (43.4)	11 (8.5)	33 (25.5)	19 (14.7)
Act II	13	147	14 (9.5)	34 (23.1)	6 (4.1)	54 (36.7)	42 (28.6)

Table 2  
Activation treatment and the rate of induced enucleation of CF1 oocytes

Groups	No. of experiments	No. of oocytes	IE (%)
Act I	5	148	38 (25.7)
Act II	6	239	122 (51.0)

Table 3  
Development of CF1 cloned embryos reconstructed with somatic cells

Nt. Exp.	No. of reconstructed embryos	1-cell (%)	2–4 cell (%)	Fragment (%)
15	185	64 (34.5)	76 (41.1)	45 (24.3)

## NORMAL OFFSPRING PRODUCED BY SOMATIC CELL NUCLEAR TRANSFER IN BOVINE USING STRONTIUM AND IONOMYCIN AS ACTIVATING AGENTS

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Strontium has been successfully used in nuclear transfer (NT) procedures to activate reconstructed oocytes in mice and to produce normal pups. In bovine, however, viable offspring has not been yet obtained using strontium as the activating agent. The aim of this study was to evaluate the developmental capacity in vitro and after transfer to recipient cows of bovine embryos produced by somatic cell nuclear transfer, using strontium or ionomycin combined with strontium to induce activation. Bovine fibroblasts were isolated from one 16-year-old Nelore cow (*Bos indicus*) and cultured in DMEM supplemented with 10% FBS. It was used as source of nuclear donor cell lines cultured between passages 3 and 9. Donor cells were synchronized in G<sub>0</sub>–G<sub>1</sub> stage by applying serum starvation for 5–12 days before NT. Recipients oocytes were obtained from slaughter cows ovaries and matured in vitro for 21 h. Oocytes presenting an extruded first polar body were enucleated between 22 and 24 h. After enucleation, one fibroblast was introduced into the perivitelline space of each enucleated oocyte, and cell-cytoplasm couplings were fused in 0.28 M mannitol, 0.05 mM CaCl<sub>2</sub>, 0.01 mM MgSO<sub>4</sub>, 0.05 mM HEPES and 0.05% BSA, using two DC pulses of 2.25 kV/cm for 20 μs. At 30 h post-maturation, successfully reconstructed oocytes were activated in strontium (20 mM SrCl<sub>2</sub> and 10 μg/ml cytochalasin B) for 6 h, or in ionomycin (5 μM for 5 min) followed by incubation in strontium and cytochalasin B for 6 h. Embryos were cultured in SOF supplemented with FCS (2.5%) at 38.5 °C in 5% CO<sub>2</sub> in air for 7 days. Overall, 134 and 129 reconstructed embryos activated, respectively, in strontium (Sr<sup>2+</sup>) or in ionomycin and strontium (Ion/Sr<sup>2+</sup>) were cultured. Development to the blastocyst stage of NT embryos activated in strontium (20.15%) was significantly lower ( $P < 0.05$  by chi-square test) when compared with NT embryos activated in ionomycin combined with strontium (34.88%). In the second part of this experiment, 12 (NT embryos activated in Sr<sup>2+</sup>) and seven blastocysts (NT embryos activated in Ion/Sr<sup>2+</sup>) were inactivated, respectively to six and five recipient cows. In some cases, two blastocysts were transferred to one recipient cow. On Day 50 after embryo transfer, one pregnancy in NT group activated in Sr<sup>2+</sup> and two pregnancies in NT group activated in Ion/Sr<sup>2+</sup> were confirmed by ultrasonography. One pregnancy was lost in each NT group between Days 60 and 90, and the remaining gestation from NT group activated in Ion/Sr<sup>2+</sup> developed to term on Day 290 of pregnancy. The newborn, delivered by Cesarean, appears normal and healthy. These results indicate that strontium, combined or not with ionomycin, can be used in the activation of nuclear transfer bovine embryos reconstructed with adult somatic cells allowing the establishment of pregnancies and development to term. To our knowledge, this is the first description of viable offspring production using strontium as an activating agent of NT bovine embryos. Embryo development in vitro and development to term showed reasonable results, specially when strontium was used in combination with ionomycin. It is also the first report of a calf produced by adult somatic cell nuclear transfer in Latin America.

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PRODUCTION OF CLONED PIGLETS BY TRANSFER OF FETAL  
FIBROBLASTS INTO ENUCLEATED OOCYTES

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Very recently, Yin Xi-Jun et al. reported that electronically stimulated porcine eggs following by culture in colcemid supplied with NCSU-37 for 1 h after fusion of enucleated porcine cytoplasts with somatic cells can support development until terms [BOR 2002;67:442–446]. The objective of this study was to examine the delayed versus simultaneous activation (DA versus SA) strategies on in vitro and in vivo developmental ability of nuclear transfer embryos in the pig. Maturation of porcine COC's was accomplished by incubation in NCSU-37 medium supplemented with 0.6 mM cysteine, 10% porcine follicular fluid, 1 mM dibutyryl cyclic adenosine monophosphate (dbc-AMP, Sigma), and 0.1 IU/ml human menopausal gonadotrophin (hMG, Teikokuzoki, Tokyo, Japan) for 20 h and then cultured without db-cAMP and hMG for another 18–24 h. Fetal cells were isolated from a fetus, cultured in ES-DMEM medium containing 10% FCS. Enucleated oocytes were fused with fetal fibroblasts (passage 4–15). The fused oocytes were activated simultaneously (SA) or activated after culture in NCSU-37 medium with 0.4  $\mu$ /ml colcemid for 1 h. Reconstructed embryos were cultured in NCSU-37 with 4 mg/ml BSA under mineral oil at 39 °C in 5% CO<sub>2</sub>, in air for 6 days. The development of the nuclear transferred embryos to the blastocyst stage following delayed activation (DA) method yielded blastocysts more reliable than did SA (8 and 3%, respectively,  $P < 0.05$ ). However, the mean cell numbers of blastocysts developed from two NT method was similar ( $20.3 \pm 13.1$  and  $20.4 \pm 11.1$ , respectively,  $P > 0.05$ ). A total of 875 embryos (1–2, 4-cell stage) derived from DA method were transferred to six synchronized females and pregnant was determined by ultrasound. One recipient aborted two piglets at Day 46, one recipient gave a birth to three offspring, the other recipient is ongoing. In SA method group, 704 embryos transferred into four recipients, but no pregnant observed. This study demonstrates that efficient reprogramming of porcine donor nuclei by combination of culture in colcemid 1 h after fusing cells with activation was reflected in development to blastocyst and pregnancy initiation leading to full term development.

DNA FRAGMENTATION AND DEVELOPMENTAL COMPETENCE OF  
BOVINE OOCYTES RECONSTRUCTED FROM THE ADULT CELLS  
BEFORE AND AFTER SERUM STARVATION

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Since the first cloned sheep from a somatic cell was produced, serum starvation (SS) has been widely used to synchronize the cell cycle of donor cells for NT to the quiescent G<sub>0</sub>. It is known that SS can induce DNA fragmentation in porcine and sheep fetal fibroblasts, however, whether SS can also induce DNA fragmentation in adult somatic cells such as bovine granulosa cells is still unclear. The objective of this study was to examine the frequency of DNA fragmentation in adult donor cells before and after SS, and to evaluate the developmental competence of embryos after NT of the donor cells before and after SS. Granulosa cells, aspirated from a Holstein cow using an ultrasound-guided transvaginal technique, were cultured for several passages and allowed to be 80% confluent in DMEM containing 10% FCS. Subsequently for SS, these cells were washed and cultured in DMEM containing 0.5% FCS for up to 5 days. As donor cells, the granulosa cells before or after SS were fused (DC pulse of 30 V, 200  $\mu$ m for 10  $\mu$ s in Zimmerman's cell fusion medium) with cytoplasts from in vitro-matured oocytes. Oocyte activation was performed by incubation in cytochalasin D (2.5  $\mu$ g/ml) and cycloheximide (10  $\mu$ g/ml) for 1 h and subsequently in cycloheximide alone for 4 h. At Days 4 and 7 of post-activation culture, frequencies of cleavage and blastocyst formation were recorded, respectively. The total cell number of blastocyst were counted at Day 8. For in situ detection of DNA fragmentation, the donor cells and the reconstructed embryos were fixed in 4% paraformaldehyde for 30 min. Using an apoptosis detection kit (Takara, Japan), DNA breaks were labeled by the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay. Unlabeled cells were visualized by propidium iodide staining. Some of the blastocysts were transferred to synchronized recipients, and survival of embryos was confirmed at Days 40 and 90 post estrus by ultrasound technique. Data were analyzed by chi-square test and ANOVA. The frequency of DNA fragmentation in granulosa cells after SS (8.2%) was significantly ( $P < 0.01$ ) higher than that before SS (0.2%). There were no significant differences between before and after SS in fusion rate (mean  $\pm$  S.E.M.; 59.1  $\pm$  5.5 and 70.2  $\pm$  6.5%), cleavage rate (74.7  $\pm$  5.1 and 84.6  $\pm$  12.3%), blastocyst formation rate (30.0  $\pm$  7.5 and 27.2  $\pm$  4.5%), total cell number of blastocysts (98.3  $\pm$  8.9 and 96.2  $\pm$  9.2), and frequency of DNA fragmentation in blastomeres (3.2  $\pm$  1.4 and 2.4  $\pm$  0.9%). After ET, survival rates of embryos derived from the donor cells before and after SS were 38 (5/13) and 33% (5/15) at Day 40, and 38 (5/13) and 20% (2/15) at Day 90, respectively. Finally, three (23%) and one (7%) cloned calves were produced from the donor cells before and after SS, respectively. In conclusion, SS could induce DNA fragmentation in bovine granulosa cells as in fetal cells, however, the damages in DNA of donor cells might not affect on the early embryonic developmental competence of the reconstructed embryos.

LONG-LASTING PHYSIOLOGICAL DISORDERS EXHIBITED BY  
MOUSE FROM NUCLEAR TRANSFER

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Several reports have shown that mice obtained from nuclear transfer often die prematurely just after birth or rapidly develop physiological disorders such as severe pneumonia, hepatic failure or obesity. Since little is known on the etiology of these disorders, we have evaluated the long-term viability of mice derived from ES nuclei. ES cells from the R1 cell line harvested from semi-confluent cultures at passages 16–20 were used as donor cells in metaphase or in interphase. In this case, large nuclei were chosen for injection. Recipient oocytes were collected 13 h after hCG injection from the oviducts of 8-week-old F1 females (C57Bl/6 × CBA/J). Embryos were reconstructed according to our previously described procedure [Zhou et al., *Biol Reprod* 2001;65:412–419]. Caesarean sections were performed at Day 19 of gestation, body and placenta were weighted before adoption by a lactating mother. In vivo fertilized embryos transferred into foster recipients at the one- or two-cell stage were used as controls. The weight of placentas of all cloned pups obtained was about twice higher than control (*t*-test,  $P < 0.05$ ). More than half of the pups (14 out of 25) suffered from respiratory failure and general weakness and died only few hours after delivering (Table 1). The weight curves of seven cloned mice followed during 12–19 months were similar to that of controls. Five of them were shown to be fertile but the two remaining ones were not, although they could mate and give vaginal plugs normally. Two mice died at 1 year, suffering from multiple necrotic wounds that did not heal for several weeks. The five others were sacrificed at different ages (1, 8, 19 months old) and compared to four control animals of the same ages. Blood count and formula were determined, and several organs (lung, liver, spleen, testis, kidney) were processed for histology. No marked differences could be detected between normal and cloned animals. Our data, together with the fact that one of our first cloned mice of proven fertility obtained from somatic nuclei (cumulus) was still physiologically normal before mercy killing at 2.5 years after birth, provide evidence that nuclear transfer, despite multiple disorders, can also result in physiologically normal and fertile animals.

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Table 1  
Full term development of embryos reconstructed from ES nuclei

No. of embryos transferred	No. of fosters	No. of pregnant fosters (%)	No. of implantation sites	No. of pups			Survival after 1 week	
				Total	% from implanted	% from transferred	Total	% of born pups
1166	54	19	276 <sup>a</sup>	25	8.3	2.0	11	44.0

<sup>a</sup> Two pups were delivered naturally, for the two fosters we do not know the number of implantations.

## Cryopreservation/Cryobiology

### COMPARISON OF SURVIVAL OF IN VITRO-DERIVED BOVINE EMBRYOS CRYOPRESERVED BY SLOW FREEZING, QUICK-FREEZING OR VITRIFICATION METHODS

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The aim of this study was to compare the in vitro survival rate of bovine embryos cryopreserved with different protocols. A total of 3486 oocytes were used for in vitro maturation, fertilization and co-culture. At the expanded blastocyst stage, the embryos were cryopreserved by either of two slow-freezing (0.5 and 1.2 °C/min), one quick-freezing and two vitrification (EFS and ethylene + glycerol) protocols. In the two slow-freezing procedures, embryos were exposed to 10% ethylene glycol (EG) for 10 min. After loading, the straws were placed into methanol at  $-7^{\circ}\text{C}$  for 5 min, seeded and after 5 min cooled at 0.5 or 1.2 °C/min. After 10 min at  $-31^{\circ}\text{C}$ , the straws were plunged and stored in liquid nitrogen. In the quick-freezing method, the embryos were exposed to 10% glycerol (Gly) for 5 min and then to 10% Gly + 10% EG + 10% sucrose for 5 min. The straws were held in nitrogen vapor for 5 min and then plunged and stored in liquid nitrogen. For both vitrification methods, a three-step procedure was followed. With the EFS method, embryos were exposed to 10% EG for 5 min and then to 20% EG for 5 min. With the Gly + EG method, embryos were exposed to 10% Gly for 5 min and then to 10% Gly + 20% EG for 5 min. In both vitrification methods, embryos were exposed to vitrification solutions (EFS or Gly + EG) for 30 s, and then plunged and stored in liquid nitrogen. After warming, cryoprotectants were diluted using 10% sucrose and isotonic solution, both for 5 min. Embryos were co-cultured on a granulosa cell monolayer and evaluated after 24 h for re-expansion and at 48, 72 and 96 h of co-culture for hatching rates. The in vitro survival rates of embryos cryopreserved by the slow-freezing methods (43.3 and 32.7%, for 0.5 and 1.2 °C/min, respectively) were higher than those of embryos cryopreserved by the quick-freezing (6.4%) or EFS vitrification protocol (9.8%). But vitrification using Gly + EG yielded a similar survival rate when compared to slow-freezing at 1.2 °C/min (23 and 32.7%, respectively). However, since the in vitro development rate of the control group was much higher (78.4%), new studies are needed to maximize cryopreservation protocols of in vitro-derived embryos.

## CHANGES IN MOTION PARAMETERS AND SUBPOPULATION DISTRIBUTION OF BOAR SPERMATOZOA DURING THE CRYOPRESERVATION PROCESS

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Changes in sperm motion reflect physiological events within the sperm cell. Therefore, studies of sperm movement are relevant in the diagnosis of sperm function. During cryopreservation, spermatozoa are subjected to chemical, osmotic, thermal, and mechanical stresses that affect their motion pattern. The present study was designed to evaluate potential changes in the motion pattern of boar spermatozoa at different stages of the cryopreservation process.

Ejaculate-rich fractions from five mature Iberian boars were diluted in Beltsville Thaw Solution (BTS) extender (stage 1), cooled to 17 °C over 3 h, centrifuged and diluted in lactose/egg-yolk extender to a concentration of  $1.5 \times 10^9$  sperm/ml (stage 2), cooled to 5 °C over 2 h and diluted in lactose/egg-yolk/glycerol/Equex Stem medium at a final concentration of  $1 \times 10^9$  sperm/ml (stage 3), dispensed into 0.5-ml straws, and frozen in a programmable cell freezer at 20 °C/min before plunging into liquid nitrogen. After 1 week at -196 °C, straws were thawed at 37 °C for 20 s, diluted in BTS (stage 4) and held at 37 °C for 150 min (stage 5). At the end of each stage, sperm motion parameters were assessed using a computer assisted semen analyser (Sperm Class Analyser, S.C.A.®). Total sperm motility (TSM), curvilinear (VCL) and straight (VSL) velocity, linearity (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and dance (DNC) were recorded.

ANOVA analysis revealed that the stages of cryopreservation affected ( $P < 0.01$ ) all sperm motion parameters. Whereas the percentage of TSM did not differ in the stages before freezing ( $79.86 \pm 2.64$ ,  $83.74 \pm 1.67$  and  $91.49 \pm 1.15$  on stages 1, 2 and 3, respectively), it decreased ( $P < 0.05$ ) after thawing ( $56.01 \pm 3.73$  and  $38.14 \pm 6.52$ ) at stages 4 and 5, respectively. Moreover, cooling to 5 °C increased ( $P < 0.05$ ) the VCL, VSL, ALH and DNC and thawing increased ( $P < 0.05$ ) the LIN and BCF values. Sperm motion parameters of the 7, 558 spermatozoa analysed at the five stages of the cryopreservation process were used to detect and quantify sperm subpopulations (SSP) using a pattern analysis technique [Abaigar et al., Biol Reprod 1999;60:32–41]. Three different ( $P < 0.05$ ) SSPs characterized by high VCL, VSL and BCF (SSP 1), high LIN and low BCF (SSP 2) and low VCL, VSL and LIN and high ALH (SSP 3) were detected. Although SSP 1 was predominant ( $P < 0.05$ ) in the five stages of cryopreservation (ranging from 95.42 to 78.65%), chi-square test showed differences ( $P < 0.05$ ) in the distribution of three subpopulations among the five stages. Stage 3 showed the highest percentage of spermatozoa of SSP 1 (95.42) and the lowest of SSP 2 (4.58). Conversely, stages 1 and 5 showed the lowest percentages of spermatozoa of SSP 1 (78.65 and 80.83, respectively) and the highest of SSP 2 (21.35 and 18.64, respectively). No spermatozoa of SSP 3 were found in stages 1, 2 and 3. In conclusion, the various stages of cryopreservation affect the motion parameters and the distribution of subpopulations of boar spermatozoa. Changes during cooling at 5 °C and after thawing are particularly important.

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## SUCCESSFUL CRYOPRESERVATION OF IN VITRO-PRODUCED PIG EMBRYOS BY THE SOLID SURFACE VITRIFICATION (SSV) METHOD

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Recent technical improvements resulted in a high cryosurvival of in vivo-produced porcine embryos. However, no reports have been published on the successful cryopreservation of in vitro produced (IVP) pig embryos. Our goal was to test a vitrification method for porcine embryos produced in a serum-free in vitro culture system. Porcine cumulus-oocyte complexes were matured and fertilized in vitro, and cultured under 5% O<sub>2</sub>, 95% air in glucose-free NCSU-37 containing 4 mg/ml bovine serum albumin and 50 µM β-mercaptoethanol as basic medium supplemented with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate (IVC-PyrLac) from Days 0 to 2 (Day 0 = IVF), and then in basic medium with 5.55 mM D-glucose (IVC-Glu) from Days 2 to 6 [Kikuchi et al., Biol Reprod 2002;66:1033–1041]. Day 6 blastocysts classified as (a) expanded (>160 µm diameter); or (b) expanding (120–160 µm) and were cryopreserved by solid surface vitrification [SS; Dinnyes et al., Biol Reprod 2000;63:513–518]. The vitrification solutions studied contained (1) 35% ethylene glycol (EG), 5% polyvinylpyrrolidone, and 0.4 M trehalose (EPT) versus (2) 20% EG, 20% DMSO, and 0.4 M trehalose (EDT) in NCSU-37 medium with 20 mM HEPES added (IVC-Glu-HEPES). Embryos were equilibrated in 4% EG or 4% EG and 4% DMSO at 38 °C for 3 min before EPT or EDT, respectively and then exposed to the vitrification solutions at 38 °C for about 20 s. Embryos were dropped onto an approximate –180 °C metal surface in 2-µl droplets of vitrification solution. Warming was performed by moving the vitrified droplets into 0.4 M trehalose at 38 °C. Recovered embryos were exposed to 0.2, 0.1, and 0.05 M trehalose for 1 min each, and then rinsed three times in IVC-Glu-HEPES. Warmed embryos were cultured for an additional 24 h in IVC-Glu, as described above. Non-cryopreserved embryos were used as controls. Recovery of full blastocoeles and further development were recorded. Data of 5–6 replicate experiments were analyzed by chi-square test. Results are presented in the table. The results showed that the two vitrification solutions are equally suitable to cryopreserve various blastocyst-stages, and cryopreservation significantly reduced survival compared to the controls ( $P < 0.05$ ). The developmental stage had no significant ( $P > 0.1$ ) effect on cryosurvival. In conclusion, vitrification of IVP pig embryos resulted in relatively high rates of survival for the first time. The main contributing factors for success might have been the novel vitrification method and the serum-free IVP system. Further experiments are needed to increase survival, and to demonstrate full developmental competence of the vitrified/warmed embryos.

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Vitrification solution	Surviving expanding blastocysts	Surviving expanded blastocysts
EPT	10/68 (15%) <sup>a</sup>	13/54 (24%) <sup>a</sup>
EDT	11/69 (16%) <sup>a</sup>	11/47 (23%) <sup>a</sup>
Control	17/41 (41%) <sup>b</sup>	22/26 (85%) <sup>b</sup>

Groups with different letters (a, b) within columns differ,  $P < 0.05$  (chi-square test).

## SUCCESSFUL CRYOPRESERVATION OF PORCINE IVM-DERIVED EMBRYOS

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The use of in vitro-matured (IVM) oocytes is becoming common for the production of IVF, transgenic and cloned embryos in the pig. The aim of the present study was to develop an efficient cryopreservation protocol for porcine in vitro-produced (IVP) embryos, using parthenogenetic embryos derived from IVM oocytes as a model. Oocytes collected from abattoir ovaries were matured in NCSU23 medium. Parthenogenesis of the IVM oocytes was induced by electric stimulation (DV 150 V/mm, 100  $\mu$ s) followed by 5  $\mu$ g/ml cytochalasin B treatment for 3 h. Cryopreservation of the embryos was carried out by vitrification using the minimum volume cooling (MVC) method (Kuwayama & Kato, 2000). Embryos were equilibrated with 7.5% ethylene glycol (EG) and 7.5% DMSO for 4 min, followed by exposure to the vitrification solution consisting of 15% EG, 15% DMSO, 0.5 M sucrose and 10% FCS for 1 min. Embryos were then loaded onto a MVC plate (Cryotop, Kitazato Supply, Tokyo) and plunged into liquid nitrogen. The vitrified solution was warmed and liquefied by immersing the MVC plate directly in 1 M sucrose at 37 °C, followed by stepwise dilution of the cryoprotectants. Survival of the vitrified embryos was assessed by culture in NCSU23 for 2 days. Exp. 1: Parthenogenetic Day 6 blastocysts were vitrified and their survival was compared with non-vitrified control blastocysts. Exp. 2: Cytoplasmic lipid droplets of the parthenogenetic morulae (Day 4) were removed by micromanipulation [delipation; Nagashima et al., 1995], and then allowed to develop to the late blastocyst stage (Day 6) and vitrified. As shown in Table 1, IVP Day 6 blastocysts (Exp. 1) survived vitrification, although their post-warm developmental rate was significantly lower than the control. Post-warm development of the delipated blastocysts (Exp. 2) was comparable to that of the control non-vitrified blastocysts. These data demonstrate that vitrification using the MVC method allows cryopreservation of porcine blastocysts derived from IVM, and that survival of the cryopreserved IVP embryos can be increased by using the MVC method in combination with delipation. It is hypothesized that the improved cryo-tolerance of embryos by delipation, the innate cryo-tolerance of the Day-6 blastocyst and the effectiveness of the MVC method synergistically enabled the efficient cryopreservation of IVP porcine embryos.

Table 1  
Survival of in vitro produced parthenogenetic porcine blastocysts after vitrification

Embryos	Vitrification	No. of embryos cultured	No. of embryos developed (%)	Cell numbers (mean $\pm$ S.E.)
Day-6 blastocysts	+	34	14 (41.2) <sup>a</sup>	38.4 $\pm$ 5.1 <sup>a</sup>
Day-6 blastocysts	-	25	22 (88.0) <sup>b</sup>	61.9 $\pm$ 5.8 <sup>b</sup>
Delipated blastocysts	+	26	23 (88.5) <sup>b</sup>	52.8 $\pm$ 4.3 <sup>bc</sup>
Delipated blastocysts	-	23	21 (91.3) <sup>b</sup>	46.7 $\pm$ 4.1 <sup>ac</sup>

(a, b, c)  $P < 0.05$ .

EFFECT OF AGE AND STAGE OF EMBRYO DEVELOPMENT ON  
POST-CRYOPRESERVATION EMBRYO SURVIVAL OF IN VITRO  
BOVINE EMBRYOS

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The objective of this experiment was to determinate the effect of age and stage of embryo development on the survival of in vitro-derived embryos after cryopreservation. For production of morulae (MOR), blastocysts (BL) and expanded blastocysts (EBL), 2–6 mm follicles were aspirated from slaughterhouse ovaries. Cumulus–oocyte complexes were selected (compact and complete cumulus) and cultured for maturation (IVM) in groups of 40–50 COCs in 400 µl of TCM199 medium, 5 mM Hepes, 36 mM NaHCO<sub>3</sub>, 2.3 mM Na pyruvate, 5.5 mM Ca lactate, 50 µg/ml gentamicin, 10% estrous cow serum (ECS), 0.05 IU of r-hFSH and 100 µM of cysteamine for 20–22 h. Fertilization (IVF, Day 0) was carried out in TALP medium with frozen/thawed semen selected on a discontinuous Percoll gradient (30–60–90%, 1 × 10<sup>6</sup> sperm/ml) and 50 µg/ml heparin. After 22–24 h, presumptive zygotes were vortexed to remove granulosa cells and spermatozoa, and placed in 400 µl of CR1aa medium and co-cultured with cumulus/granulosa cells and 5% ECS under paraffin oil (IVC). Atmospheric conditions for IVM–IVF were 38.5 °C and 5% CO<sub>2</sub> in air with high humidity; IVC was in 5% O<sub>2</sub>, 5% CO<sub>2</sub>, 90% N<sub>2</sub> at 38.5 °C with high humidity. For each stage of development, two embryo ages (15 embryos in each group), and three stages of embryo development (80 embryos in each group) were examined. Embryos were frozen slowly in 1.5 M ethylene glycol (EG). The embryos were thawed and cultured with fresh BL and EBL for culture control. Data were compared by chi-square analysis.

The age of embryos at the time of freezing did not affect the post-thaw viability. More advanced stages exhibited better freezability and post-thaw survival than early stages of embryo development (Tables 1 and 2).

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Table 1  
Effect of age on post-thaw survival of in vitro bovine produced embryos

Age of development	Post-thaw expansion (%)	BL + EBL 24–48 h (%)	HBL 48–72 h (%)
Morula			
Day 6.5	11/15 (73.3)	7/15 (46.6)	5/15 (33.3)
Day 7	9/15 (60)	4/15 (26.6)	1/15 (6.6)
Blastocyst			
Day 7	13/15 (86.6)	13/15 (86.6)	11/15 (73.3)
Day 7.5	14/15 (93.3)	11/15 (73.3)	8/15 (53.3)
Expanded blastocyst			
Day 7.5	14/15 (93.3)	13/15 (86.6)	12/15 (80)
Day 8	12/15 (80)	12/15 (80)	10/15 (66.6)
Culture control			23/25 (92)

Table 2  
Effect of stage on post-thaw survival of in vitro bovine produced embryos

Stage of development	Post-thaw expansion (%)	BL + EBL 24–48 h (%)	HBL 48–72 h (%)
Morula	50/80 (62.5) <sup>a</sup>	34/80 (42.5) <sup>a</sup>	25/80 (31.2) <sup>a</sup>
Blastocyst	65/80 (81.2) <sup>ab</sup>	63/80 (78.7) <sup>b</sup>	44/80 (55) <sup>ab</sup>
Expanded blastocyst	71/80 (88.7) <sup>b</sup>	70/80 (87.5) <sup>b</sup>	55/80 (68.7) <sup>b</sup>

Values with different superscripts differ ( $P < 0.05$ ).

## CRYOTOLERANCE OF PORCINE EMBRYOS—THE NEED FOR BATCH-WISE ASSESSMENT

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We investigated the relationship between the viability of cryopreserved porcine embryos after (1) *in vitro* culture and (2) transfer to recipients without *in vitro* culture. On the basis of the hypothesis that sibling embryos from the same batch, defined as embryos taken from one donor gilt at one time, possess similar cryotolerance, these developmental parameters *in vitro* and *in vivo* were compared using embryos from the same batch. Using batches of embryos with medium or low cryotolerance, we also studied whether the survival of the frozen-thawed embryos *in utero* would improve if helper embryos were transferred with them. Expanded blastocysts (EBs:  $\geq 225 \mu\text{m}$ ) and hatched blastocysts (HBs:  $\leq 300 \mu\text{m}$ ) collected on Day 6 (day of AI = Day 0) from superovulated gilts were placed batch-wise in a freezing medium (1.8 M ethylene glycol, 20% fetal calf serum and 10% chicken egg yolk in phosphate-buffered saline). From each gilt, two EBs and two HBs, to be used for *in vitro* culture after freezing, were separated during 5 min of equilibration in the medium. The remaining embryos were also frozen-thawed and later used for transfer to recipients without *in vitro* culture. All the embryos were frozen by a slow freezing method (0.4 °C/min from -7 to -30 °C, and then into liquid nitrogen). The viability of the frozen-thawed embryos cultured *in vitro* was assessed using 42 batches prepared as above. After culture, there was a clear difference in the viability of EBs among the batches, whereas HBs did not show such a difference. On the basis of the results of the *in vitro* assessment of the EBs (2/2 EBs survived = 100% viability, 1/2 EBs survived = 50% viability and 0/2 = 0% viability), the remaining cryopreserved embryos from the same batches were classified into three categories: a total of 249 embryos from 14 batches with a High cryotolerance (100% viability), 248 embryos from 14 batches with a Medium cryotolerance (50% viability) and 251 embryos from 14 batches with a Low cryotolerance (0% viability). Fourteen batches of embryos in each group were then transferred into nine surrogate mothers, maintaining batch traceability through coat color. The pregnancy rates clearly differed depending on the cryotolerance of the embryos, being 78% (7/9), 22% (2/9) and 0% (0/9), respectively, for the High, Medium and Low cryotolerance embryos. The nine sows that conceived eventually farrowed a total of 46 normal piglets. The development rate of transferred embryos into piglets was 15.3% (38/249) in the High cryotolerance group and 3.2% (8/248) in the Medium cryotolerance group. Furthermore, frozen-thawed HBs from the batches classified as Medium or Low cryotolerance were transferred batch-wise into each of nine recipients together with four unfrozen fresh embryos (helper embryos) taken from other sows. The development rate of transferred frozen-thawed embryos into piglets was 12.7% (20/157) in the Medium group and 1.9% (3/161) in the Low group, showing a clear improvement in survival of cryopreserved embryos by the use of helper embryos. The results suggest that it is possible to use *in vitro* culture of frozen-thawed expanded blastocysts for evaluating the cryotolerance of porcine embryos. But it is necessary to carry out such assessment batch-wise. It also became clear that the transfer of four unfrozen embryos with the frozen-thawed embryos is an effective technique of increasing the chances of obtaining piglets from cryopreserved embryos.

## VITRIFICATION OF PRONUCLEAR-STAGE RABBIT ZYGOTES BY DIFFERENT ULTRA-RAPID COOLING PROCEDURES

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Cryopreservation of pronuclear-stage zygotes contributes to systematic production of transgenic rabbits, but rabbit zygotes at this stage are highly sensitive to cryopreservation. In the present study, pronuclear-stage rabbit zygotes were vitrified by three different ultra-rapid cooling procedures, and their post-warm survival was compared by *in vitro* culture. Zygotes from Japanese White rabbits were cryopreserved by vitrification using one of three devices: Gel-loading tip [Tominaga and Hamada, *J Reprod Dev* 2001;47:267–273], Cryoloop [Lane et al., *Nat Biotech* 1999;17:1234–1236] or Cryotop [Kuwayama and Kato, *J Assist Reprod Genet* 2000;17:477 (abstract)] as their containers. In the Gel-loading tip and Cryoloop methods, zygotes were first exposed to 10% ethylene glycol (EG) + 10% DMSO in TCM199 + 20% fetal bovine serum (FBS) for 2 min, and then equilibrated for 30 s in a vitrification solution composed of 20% EG + 20% DMSO + 0.6 M sucrose (Suc) in TCM199 + 20% FBS. In the Cryotop method, zygotes were first exposed to 7.5% EG + 7.5% DMSO + 20% FBS in TCM199 for 3 min, and then equilibrated for 1 min in a vitrification solution composed of 15% EG + 15% DMSO + 0.5 M Suc + 20% FBS in TCM199. The zygotes loaded in each container were directly plunged into liquid nitrogen at the end of equilibration time. Post-warm zygotes were recovered in a diluent containing 0.25 M Suc (Gel-loading tip and Cryoloop methods) or 0.5 M Suc (Cryotop method), and were washed three times with TCM199 + 20% FBS. The proportions of zygotes cleaving and developing beyond the compacted morula-stage were examined, respectively, at 24 and 72 h of culture at 38.5 °C in 5% CO<sub>2</sub> in air. In the groups of Gel-loading tip ( $n = 52$ ) and Cryoloop ( $n = 38$ ), culture of vitrified-warmed zygotes resulted in low cleavage rates (2 and 5%, respectively) and no development into morulae/blastocysts. In contrast, zygotes vitrified using the Cryotop ( $n = 55$ ) exhibited the higher proportions of cleavage (58%) and development into morulae/blastocysts (33%). The proportion of fresh zygotes developing beyond morulae was 84% ( $n = 75$ ). When morulae/blastocysts derived from culture of the zygotes were vitrified-warmed by the three procedures, 80–93% of them ( $n = 29–30$  each) resulted in blastocoele expansion or zona hatching. These results suggest that pronuclear-stage rabbit zygotes can be cryopreserved by vitrification using the Cryotop as the container.

## VIABILITY AND TELOMERASE ACTIVITY OF PORCINE EMBRYOS VITRIFIED BY ULTRA-RAPID COOLING METHOD USING THE OPEN PULLED STRAW

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We have reported the birth of piglets derived from embryos vitrified in straws containing 8 M ethylene glycol (EG) as a cryoprotective additive (CPA) [Kobayashi et al., *Cryobiology* 1998;36:20–31], and the confirmation by others [Kobayashi et al., *Cryobiology* 1998;37:436 (abstract)]. In this study, using the Open Pulled Straw (OPS) method of Vajta et al. [*Mol Reprod Dev* 1998;51:53–58], we vitrified porcine embryos in lower concentrations of EG than in our previous study, and measured the functional survival and telomerase activity of these embryos after they had been warmed and cultured. Porcine embryos were collected surgically or at slaughter from superovulated gilts at Day 6 after artificial insemination. They were classified by developmental stages as blastocysts (BL), expanded blastocysts (ExB) or hatched blastocysts (HB), and then were vitrified by the OPS method. Two CPA solutions were used for vitrification: 6 M EG + 1 M galactose (Gal) + 7% polyvinyl-pyrrolidone (PVP); 7.2 M EG + 1 M Gal + 7% PVP. First, embryos were exposed to 2 M EG solution for 5 min, then pipetted into small droplets of CPAs, and finally aspirated into the tip of OPS within 2  $\mu$ l of CPAs. Within 50 s of their exposure to the vitrification solutions, the embryos were plunged in liquid nitrogen. To warm and liquefy the CPAs, the straw was held in air for 3 s, and then the tip was submersed directly into a 1.7 M Gal solution, causing the embryos to flow out of the straw into the diluent. After 1 min, embryos were rinsed for 2 min each in 1 M EG, and 0.5 M EG and finally in PBS containing 10% FCS. Functional survival of embryos was assayed by culturing them for 48 h in a modified CZB medium in an atmosphere of 5% CO<sub>2</sub> in air at 38 °C. The telomerase activity of embryos that had been cultured for 48 h was assayed by the telomerase repeat amplification protocol (TRAP) with the Telomerase Detection Kit (Intergen, NY). Extracted sample solutions were amplified by polymerase chain reaction (PCR), and then run on a DNA sequencer (Applied Biosystems), and analyzed using GeneScan and Genotyper software. The values of telomerase activity were analyzed to measure the signal of the TRAP product ladder bands from all samples. Based on their development after 48 h in culture, survival of embryos vitrified in the 6 M EG CPA solution as BL, ExB and HB were 27% (8/30), 68% (34/50) and 69% (22/32), and were 63% (17/27), 80% (40/50) and 76% (26/34) for those vitrified in the 7.2 M EG CPA solution, respectively. The corresponding hatching rates of embryos vitrified as BL and ExB in 6 M EG were 0% (0/30) and 42% (21/50), and the rates of those vitrified in 7.2 M EG were 37% (10/27) and 68% (34/50). These results suggest that 6 M EG may be too low a CPA concentration to vitrify porcine embryos reliably, since more embryos vitrified in 6 M EG seemed to suffer physical or functional injury compared to those vitrified in 7.2 M EG. Of those embryos that had survived for 48 h in culture, the telomerase activity of embryos that grew from ExB to HB or from HB to enlarged HB was significantly higher than that of the under-grown ExB- or HB-stage embryos ( $P < 0.05$ ). This activity seemed to be correlated with developmental potential of vitrified embryos. A total of 87 ExB- or HB-stage embryos vitrified in 7.2 M EG by the OPS method was transferred surgically to four recipient pigs as groups of 20–23 embryos/recipient. Three of the four pigs became pregnant and two pigs farrowed four or eight piglets; the third pig spontaneously aborted her pregnancy.

## DEVELOPMENT OF TRANSGENIC EMBRYOS FOLLOWING INJECTION OF FREEZE-DRIED SPERM IN PIGS

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There have been no reports of successful fertilization of pig oocytes and embryo development using freeze-dried sperm. The objective of this study was to determine whether freeze-dried boar semen could fertilize in vitro-matured porcine oocytes and mediate transgenesis following intracytoplasmic sperm injection (ICSI). Oocytes were aspirated from antral follicles (3–7 mm in diameter) and cultured in a 100 µl droplet of maturation medium (NCSU23 with 10% porcine follicular fluid, 0.1 mg/ml cysteine, 1% MEM non-essential amino acid and 0.2 mM pyruvate) with hormonal supplementation (2 µg/ml Folltropin V, Vetrepharm, Ontario, Canada) at 39 °C under 5% CO<sub>2</sub> in air for 44 h. Frozen-thawed motile spermatozoa were recovered from frozen boar semen (Swine Genetics International, Cambridge, IA) by Percoll gradient centrifugation. Spermatozoa were washed and resuspended in injection medium: TL-HEPES: 10% polyvinyl-pyrrolidone (1:1) before ICSI. For freeze-drying, ejaculated boar spermatozoa were first suspended in TL-HEPES medium, centrifuged and resuspended in TL-HEPES + 10% FBS. An aliquot (100 µl) of the sperm suspension was put in a 2 ml ampule and then placed in a precooled (4 °C) freezing-flask attached to a freeze-dry system. About 19 h later, the flask was removed from the system after it had been filled with argon supplied by way of a gas-drying jar. Each ampule was connected to a vacuum pump and flame-sealed after >99% of the gas was pumped out of it. Ampules were individually wrapped with aluminum foil and stored at room temperature. Prior to ICSI, 100 µl of distilled water was added to the dried spermatozoa in the ampule. After rehydration, freeze-dried spermatozoa were diagnosed as dead by staining with a sperm viability kit (Live/dead; Molecular Probes, Eugene, OR). Frozen-thawed or freeze-dried spermatozoa ( $1 \times 10^6/10 \mu\text{l}$ ) were mixed with 500 ng (1 µl) of plasmid DNA and incubated at 39 °C for 30 min and then co-injected into each oocyte. The plasmid DNA (pGeneGrip) included GFP cDNA under the control of cytomegalovirus promoter (Gene Therapy System, San Diego, CA, USA). In the control group, the GFP gene alone (1–2 pl) without sperm was injected into the cytoplasm of oocytes. All oocytes were subjected to electrical activation (a 10-s pulse at 0.05 kV/cm AC followed by a 30 µs pulse at 2.2 kV/cm DC at room temperature) in 0.25 M Mannitol solution supplemented with 100 µM CaCl<sub>2</sub>·H<sub>2</sub>O and MgCl<sub>2</sub>·6H<sub>2</sub>O. After ICSI and activation, oocytes were cultured in 50 µl drops of culture medium (NCSU23) supplemented with 1% MEM non-essential amino acid and 0.4 mg/ml BSA. One to 7 days after ICSI, *GFP* gene expression in embryos was examined with a Nikon fluorescence microscope equipped with excitation at 488 nm and detection at 500- to 530-nm filters. The rate of development to blastocyst did not differ after ICSI with frozen-thawed (21/82, 26%), or freeze-dried sperm (23/83, 28%) or from GFP-injection controls (27/85, 32%). The *GFP* gene expression, however, was only detected in blastocysts from the ICSI groups with frozen-thawed (17/21, 81%) or freeze-dried sperm (18/23, 78%), but not in the control group with injection of *GFP* gene alone (0/27, 0%). In summary, these results demonstrate that cytologically dead boar spermatozoa, which have been freeze-dried, still have the potential to fertilize oocytes and to deliver exogenous genetic material for production of transgenic embryos.

EFFECT OF COOLING LARGE AND SMALL EQUINE EMBRYOS PRIOR TO CRYOPRESERVATION ON PREGNANCY RATES AFTER TRANSFER

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The sensitivity of equine embryos to cooling and cryopreservation varies with the stage of development. Currently, only small ( $\leq 300 \mu\text{m}$ ) equine embryos can be frozen and thawed without reduced developmental rates. Embryos from 300 to 600  $\mu\text{m}$  have moderate success in embryonic vesicle formation. One major improvement in equine embryo transfer is the ability to store embryos at 5 °C. Cooled embryos can be shipped in a passive cooling unit to a centralized station for transfer to recipient mares without a major decrease in fertility. The potential, therefore, exists to transport cooled embryos to a central facility for cryopreservation. Objectives of this study were to compare embryo developmental rates after transfer of small ( $\leq 300 \mu\text{m}$ ) and large (300–600  $\mu\text{m}$ ) cooled or non-cooled cryopreserved equine embryos. Light-horse mares ( $n = 50$ ) between 3 and 12 years of age were used for the study. Mares were inseminated with fresh semen from one of four stallions. Embryos were recovered 6.5–7.5 days after ovulation by flushing the mare's uterus with 4 l of pre-warmed (30–32 °C) Emcare<sup>TM</sup> complete flush solution (ICP Bio, Auckland, New Zealand). Upon recovery, embryos were measured and washed through six drops of Vigro<sup>®</sup> holding solution (AB Tech, Pullman, US) before cooling or cryopreservation. Embryos were cooled in Ham's F-10 (Sigma, St. Louis, US) containing 10% FCS and gassed with 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> and placed in a Hamilton Thorne Equitainer for 22 h. All embryos were placed into 5% glycerol in Vigro<sup>®</sup> holding solution for 10 min and then into 10% glycerol in Vigro<sup>®</sup> holding solution for 20 min. Embryos were loaded into 0.25-ml plastic straws, placed in a programmable cell freezer and cooled from room temperature (22 °C) to –6 °C at 4 °C/min, seeded, held at –6 °C for 10 min and then cooled at 0.3 °C/min to –30 °C, and then at 0.1 °C/min to –33 °C before being plunged in liquid nitrogen. Straws were thawed in air for 10 s, and then immersed in a 38 °C water bath for 30 s. Cryoprotectant was removed in six steps, 8, 6, 4, 2, and 1% glycerol in Vigro<sup>®</sup> holding solution for 10 min per step and finally into Vigro<sup>®</sup> holding solution for transfer to synchronized recipient mares. A total of 48 embryos were collected. Ten control embryos (5  $\leq 300 \mu\text{m}$  and 5 from 300 to 600  $\mu\text{m}$ ) were cryopreserved immediately. Thirty-eight embryos were cooled for 22 h at 5 °C prior to cryopreservation. Fishers Exact Test was used to analyze the data. Results are shown in Table 1. There was no significant difference in the formation of embryonic vesicles among groups. Therefore, equine embryos  $\leq 600 \mu\text{m}$  can be cooled for 22 h and then cryopreserved and transferred to recipient mares without a reduction in viability. Research was funded by the Benefactors of the Preservation of Equine Genetics Program.

Table 1  
Pregnancy rates at Day 16 after transfer of frozen and thawed embryos

Treatment	Embryonic vesicles on Day 16 (%)
Control $\leq 300 \mu\text{m}$	4/5 (80)
Cooled $\leq 300 \mu\text{m}$	7/20 (35)
Control 300–600 $\mu\text{m}$	2/5 (40)
Cooled 300–600 $\mu\text{m}$	6/18 (33)
Total control	6/10 (60)
Total cooled	13/38 (34)

## VITRIFICATION METHODS FOR CRYOPRESERVATION OF IN VITRO PRODUCED CATTLE EMBRYOS

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In vitro-derived bovine embryos are often less resistant to injury from cryopreservation than their in vivo counterparts. The purpose of this study was to compare two vitrification methods to ascertain the best one for cryopreservation of bovine embryos produced in vitro in a combined KSOM/SOFaaci culture system. A total of 720 cumulus–oocyte complexes (COCs) was matured in vitro [Park et al., *Theriogenology* 1997;48:1127–1135] and fertilized in modified M199 (mM199) with 26.19 mM NaHCO<sub>3</sub>, 3.9 mM glucose, 1.25 mM Na-pyruvate, 6 mg/ml FAF-BSA and 10 µg/ml heparin, buffered with 25 mM Hepes. Cumulus–oocyte complexes (15–20) were transferred into 50 µl fertilization droplets and incubated with sperm (1 × 10<sup>7</sup> sperm/ml) for 6 h at 5% CO<sub>2</sub> in air at 39 °C. Presumptive zygotes were cultured in KSOM with 0.1% FAF-BSA for 4 days, and then the medium was changed to SOFaaci with 5% FCS until Day 7 [Nedambale et al., *Theriogenology* 2002;57:523] at 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub> at 39 °C. The cryopreservation methods tested in this study were: (1) modified solid surface vitrification [mSS: 35% ethylene glycol (EG) + 0.5 M sucrose + 5% PVP, Dinnyes et al., *Biol Reprod* 2001;63:513–518]; (2) vitrification Solution-EG in straw (VS-EG: 6.5 M EG + 6% BSA in PB1; modified from VS3a by Rall (*Anim Reprod Sci* 1992;28:237–245). The modification to the vitrification solution consisted of substituting ethylene glycol for the glycerol in the standard VS3a. Day 7 embryos (*n* = 230) were equilibrated 5 min in 10% EG or in 25% VS-EG for treatment 1 and 2, respectively, and then vitrified as described in detail in the above publications. Non-vitrified embryos (*n* = 77) served as controls. Data were analyzed by chi-square test. Results are presented in [Table 1](#). Among the cleaved zygotes, 47% developed into blastocysts with a well-defined ICM. Embryos that were vitrified/warmed in mSS re-expanded, developed, and more of them hatched (*P* < 0.05) by 6 and 48 h after warming than those in the VS-EG group. Cryopreservation reduced survival compared to controls; however, hatching rates of the mSSV group were not different from those of the controls. Furthermore, preliminary observations indicate that mSSV-vitrified embryos re-expanded earlier and developed faster than those vitrified in VS-EG. In conclusion, mSSV was found to be an efficient method to vitrify bovine embryos produced by the KSOM-SOFaaci in vitro culture system.

Table 1  
Development of vitrified/warmed IVP bovine embryos

Treatment	Embryos ( <i>n</i> )	Post-warming development at			Hatched
		6 h	24 h	48 h	
1. mSSV	118	96 (81%) <sup>a</sup>	92 (78%) <sup>a</sup>	87 (74%) <sup>a</sup>	82 (69%) <sup>a</sup>
2. VS-EG	112	73 (65%) <sup>b</sup>	70 (63%) <sup>a</sup>	60 (54%) <sup>b</sup>	56 (50%) <sup>b</sup>
3. Control	77	77 (100%) <sup>c</sup>	76 (99%) <sup>b</sup>	75 (97%) <sup>c</sup>	62 (81%) <sup>a</sup>

Values with different superscripts within columns are significantly different (*P* < 0.05).

**EMBRYOS PRODUCED IN VITRO AFTER RECOVERY OF OOCYTES FROM  
CAT OVARIES STORED AT 4 °C FOR 24 TO 28 HOURS RETAIN THE  
COMPETENCE TO DEVELOP INTO LIVE KITTENS AFTER  
TRANSFER TO RECIPIENTS**

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In vitro development of cat embryos derived by IVM/IVF from ovaries stored for 24–28 h at 4 °C is not different from that of embryos created from ovaries held at ~22 °C for 2–10 h [Freistedt et al., *Theriogenology* 1999;51:285 (abstract)]. Accordingly, we did two experiments to determine developmental competence after transfer to recipient females of in vitro-derived cat embryos produced by retrieval of oocytes from ovaries stored at 4 °C for 24–28 h. In Experiment 1, “fresh” IVM/IVF embryos were transferred to synchronous gonadotropin-treated recipients. In Experiment 2, IVM/IVF embryos were cryopreserved by a controlled-rate technique before being thawed and transferred to recipients. Excised cat ovaries were held in Hepes Saline + gentamicin for 24–28 h at 4 °C before mincing and retrieval of cumulus–oocyte complexes (COCs). For comparison of in vitro development, non-stored ovaries were held at ~22 °C for 3–6 h before recovery of COC. Oocytes with uniformly dark, spherical ooplasm surrounded by multiple layers of cumulus cells were cultured in 500 µl of modified TCM199 + BSA + eCG/hCG + EGF at 38.5 °C in 5% O<sub>2</sub>, 5% CO<sub>2</sub>, 90% N<sub>2</sub> for 24 h [Gomez et al., *Reprod Fert Dev* 2000;12:423–433]. Oocytes were inseminated with ejaculated spermatozoa (by artificial vagina) in modified Tyrodes (mTy) + 6 mg/ml BSA, and after ~15 h, rinsed (4×) before culture in mTy + 3 mg/ml BSA + MEM NEAA [IVC-1, Gomez et al., *Reprod Fert Dev* 2000;12:423–433]. On Day 2 or 3, embryos were placed in fresh IVC-1 + 1% MEM EAA. Non-frozen Day 5 embryos were cultured in mTy + 10% FBS + NEAA and EAA (IVC-2) until visual evaluation of blastocyst development on Day 7. Cleavage frequency and blastocyst development of embryos after IVF of oocytes from stored ovaries was 49% (364/737, 18 replicates) and 46%, respectively, as compared to frequencies of 49% (184/379, nine replicates) and 46%, respectively, for oocytes from fresh ovaries ( $P > 0.05$ ). Some embryos were frozen on Day 5 at a slow, controlled rate in Hepes Tyrodes + 1.4 M propylene glycol/0.125 M sucrose + 10% dextran [Pope et al., *Theriogenology* 2002;57:464 (abstract)] after three-step equilibration at 22 °C. Frozen embryos were thawed in air and, after five-step cryoprotectant removal, cultured in IVC-2 until uterine transfer to gonadotropin treated recipients 1 or 2 days later (Day 6 or 7). In Experiment 1, 10 or 11 blastocysts were transferred to each of two recipients on Day 7 and a third recipient received nine Day 6 embryos (three blastocysts). All three recipients became pregnant, but one had resorbing fetuses by Day 21. The other two recipients delivered, one by Caesarean section, a total of three live kittens. In Experiment 2, 10 Day 7 embryos (five blastocysts) and 10 Day 6 embryos (morulae) were transferred to each of two recipients 2 days or 1 days, respectively, after thawing. The recipient of the Day 7 embryos was not pregnant when examined by ultrasonography on Day 21. The Day 6 recipient delivered a kitten that died during labor on Day 68. In summary, the results of the present study clearly demonstrate that in vitro derived cat embryos produced after recovery of oocytes from ovaries stored for 24–28 h at 4 °C retain competency to develop into live offspring after transfer to recipients. In addition, term offspring can be delivered from such embryos following cryopreservation at the morula stage and uterine transfer 1 day after thawing. Supported by a grant from the John and Shirley Davies Foundation.

## RAPID FREEZING OF HATCHED ZONA-FREE MOUSE BLASTOCYSTS

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The viability of zona-free mouse blastocysts frozen rapidly was investigated in this study. The aim of the experiment was to examine how the absence of the zona pellucida affects the equilibration time and the optimal concentration of ethylene glycol (EG) and glycerol (Gly) in the freezing medium. Morula-stage mouse embryos were collected from superovulated 4- to 6-week-old CB6F1 donors approximately 85 h after the hCG injection. Embryos were cultured in G2.2 medium (Vitrolife, Göteborg) at 37 °C with 5% CO<sub>2</sub> and maximal humidity in air to the hatched blastocyst stage (Day 5; 120–125 h after the hCG injection). Hatched blastocysts were equilibrated in a freezing medium containing DPBS + 0.25 M sucrose + 10% fetal bovine serum + 1.5 or 3 M EG or 3 M Gly for 2 or 10 min at room temperature. The embryos were frozen in 0.25 ml plastic straws (7–10 embryos/straw) by keeping the straws 2 cm above the liquid nitrogen (LN<sub>2</sub>) level for 2 min in a closed polystyrene box before plunging them into LN<sub>2</sub>. Straws were kept at least for 1 week in LN<sub>2</sub> before being thawed. For thawing, straws were held in air for 20–30 min and then dipped into a 22–25 °C water bath for 2 min. Embryos were directly rehydrated and washed three times in G2.2 medium and then cultured as above. In vitro embryo survival was judged by morphology (reexpansion of the blastocoel cavity). The embryos were examined at thawing, 12 and 24 h later. Ten to 14 embryos were used in each group, and each experiment was repeated three times. For in vivo evaluation of the viability of rapidly frozen zona-free blastocysts, embryos were cultured for 2–3 h in G2.2 medium after thawing and then transferred into Day 3 pseudopregnant recipients. Ten to fourteen re-expanded embryos were transferred per mouse and each transfer was repeated twice. Untreated hatched blastocysts were used as controls. Pregnant females were examined at 18 days of pregnancy. The results show that EG penetrates quickly into the zona-free hatched mouse blastocysts. There was no significant difference in vitro and in the implantation rates of embryos equilibrated for 2 or 10 min prior to freezing. A 1.5 M concentration of EG provides sufficient protection against cryo-injuries. No significant difference was found between implantation rates of control and cryopreserved embryos. The embryos equilibrated with Gly showed lower in vitro (55%, 61%) and in vivo survival (0%, 12%) indicating that Gly, probably due to its higher molecular weight, penetrates more slowly into the cells. The 2 and 10 min of exposure to Gly did not provide enough time for optimal cryopreservation with this rapid freezing protocol.

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Freezing medium	Concentration (M)	Equilibration time (min)	<i>n</i>	Survival at thawing (%)	12 h after (%)	24 h after (%)	In vivo (offspring/ transferred)
EG	3	2	35	97	91	94	6/20 (30%)
EG	3	10	31	96	96	96	5/21 (24%)
EG	1.5	2	41	100	90	90	7/23 (30%)
EG	1.5	10	36	89	83	86	5/21 (24%)
Gly	3	2	40	67	60	55	0/26 (0%)
Gly	3	10	36	83	61	61	3/25 (12%)
Control	–	–	34	–	97	97	7/24 (29%)

**OLEIC ACID AMELIORATES THE SERUM-INDUCED REDUCTION IN  
RE-EXPANSION OF BOVINE EMBRYOS FOLLOWING  
CYTOCHALASIN-INDUCED COLLAPSE**

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Compared to their *in vivo*-derived counterparts, *in vitro*-produced (IVP) embryos have a greater sensitivity to cryopreservation procedures. Moreover, fewer IVP embryos that were developed in serum-containing media survive freezing compared to those developed in its absence. This survival may be improved by the addition of fatty acids to the culture medium [Ozake et al., *Theriogenology* 1997;47:354]. After thawing, the blastocyst must re-expand its cavity, through the activity of Na, K-ATPase located on the cell membrane. Since serum and fatty acids may modify this membrane function [Yeagle, *PL FASEB J* 1989;3:1832–1842], we examined whether the fatty acid, oleic acid affected the re-expansion of blastocysts following collapse and culture with ouabain, an inhibitor of Na, K-ATPase. Cumulus-enclosed oocytes were recovered from abattoir-derived ovaries matured and fertilized [Thompson et al., *Theriogenology* 1998;49:1239–1249]. Presumptive zygotes were allocated randomly (30–50 per well) to 500  $\mu$ l of one of the following treatments and cultured (39 °C, humidified 5% CO<sub>2</sub>, 7% O<sub>2</sub> and 88% N<sub>2</sub>) for 7 days. The treatment groups were SOFaaBSA [Gardner et al., *Biol Reprod* 1994;50:390–400]  $\pm$ 10% FCS and further supplemented with oleic acid-albumin (Sigma-OAA) to a final fatty acid concentration of 9.4  $\mu$ g/ml. The ability of resultant blastocysts to re-expand following cytochalasin D induced collapse was investigated [Betts et al., *Mol Reprod Dev* 1997;46:114–126]. Initial volume recordings were made of video images of blastocysts prior to exposure to 20  $\mu$ g/ml cytochalasin D (Sigma, USA) for 4 h at 39 °C. Embryos were then incubated overnight in SOFaaBSA with 0, 1 pM, 1 nM or 1  $\mu$ M ouabain (Sigma, USA), graded and their volumes again recorded. The relative volume change (RVC = final/initial volumes) was calculated. Between 20 and 30 blastocysts were exposed to each ouabain concentration for each culture treatment. Culture in 1  $\mu$ M ouabain significantly reduced RVC of blastocysts compared to 0, 1 pM or 1 nM ouabain ( $0.85 \pm 0.1$  versus  $1.49 \pm 0.1$ ,  $1.59 \pm 0.1$ ,  $1.42 \pm 0.1$ , respectively;  $P < 0.001$ ). RVC of blastocysts developed in FCS medium alone was reduced compared to those cultured in SOF alone ( $1.10 \pm 0.08$  versus  $1.51 \pm 0.1$ ;  $P < 0.05$ ) or compared to SOF + OAA ( $1.59 \pm 0.1$ ;  $P < 0.001$ ). Addition of OAA to FCS medium resulted in a RVC of  $1.26 \pm 0.08$  which only differed ( $P < 0.05$ ) from the SOF + OAA treatment. The percentage of blastocysts that regained at least their initial volume (i.e. RVC  $\geq$  1.0) was affected by IVC treatment (Table 1). Fewer blastocysts that developed in FCS-supplemented medium regained their initial volume ( $P < 0.001$ ) compared to any other treatment. These results demonstrate that the addition of serum to IVC medium affects the embryos' ability to re-expand after collapse and may explain, in part, the poor survival of serum-derived IVP embryos following freezing. Serum, which is sequestered by the developing embryo, may be modifying membrane properties or inducing lipid peroxidation events. How OAA ameliorates this effect is not known. However, as OAA is resistant to oxidation, it may inhibit lipid peroxidation [Vossen et al., *Prostaglandins, Leukotrienes and Essential Fatty Acids* 1995;52:341–347].

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Table 1  
Percent ( $\pm$ S.E.M.) blastocysts regaining initial volume

IVC treatment			
FCS	FCS + OAA	SOF	SOF + OAA
34.8 5.9	61.1 5.9	70.35 5.9	76.35 5.9

## CHILLING SENSITIVITY OF BOVINE OOCYTES FOLLOWING MATURATION IN THE PRESENCE OF FATTY ACIDS OR PUTRESCINE

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Bovine oocytes are susceptible to low temperatures and exhibit a high incidence of lysis following cooling to 0 °C [Martino et al., *Mol Reprod Dev* 1996;45:503–512]. Fatty acids, either fed in the animal's diet [Kojima et al., *J Reprod Dev* 1996;42:67–72] or added during IVC [Hochi et al., *Theriogenology* 1999;52:497–504] can improve the survival of embryos following cryopreservation; the addition of polyamines to the holding medium can improve the developmental ability of bovine IVF zygotes following exposure to 0 °C for 5 min [Pugh and Tervit, 14th ICAR 2000;17:20]. Therefore, we investigated whether IVM in the presence of putrescine (PUT-5 mg/ml) or fatty acids affected the chilling sensitivity of bovine oocytes. The experiment examined the effect of IVM (TCM199 + 10% FCS + 10 µg/ml FSH, LH + 1 µg/ml E<sub>2</sub>), in the presence or absence of PUT and/or linoleic acid (LAA) or oleic acid (OAA) on the developmental competence of oocytes following cooling for 1 h to 0 °C. Fatty acids, as albumin conjugates (Sigma) were used at a final concentration of 9.4 µg/ml. Abattoir-derived oocytes were matured in 50 µl wells (10 per well), without oil overlay, at 39 °C for 22 h in 5% CO<sub>2</sub> in air, and then loaded into 0.25 ml insemination straws in HEPES-buffered 199 + 10% FCS and held at 39 °C or cooled to 0 °C for 1 h. Following warming to 39 °C, oocytes were fertilized and cultured using standard laboratory procedures [Thompson et al., *Theriogenology* 1998;49:1239–1249] for 7 days. Cleavage and cytoplasmic integrity were evaluated following 24 h culture and development to compact morula–blastocyst stages was assessed after a further 6 days. The experiment was replicated four times using 1500 oocytes. Results were analyzed using GLM (Genstat; Lawes Agricultural Trust, UK). There was an interaction between fatty acids and temperature on the proportions of oocytes that lysed. Few oocytes held at 39 °C lysed (<1%) in any treatment. However, IVM in LAA or OAA reduced lysis of cooled oocytes (12.9 ± 2.3% versus 11.6 ± 2.3% versus 41.2 ± 2.6%, LAA versus OAA versus none; *P* < 0.001). More oocytes held at 39 °C cleaved than cooled oocytes (77.5 ± 2.6% versus 62.9 ± 1.8%; *P* < 0.001). In addition, there was a tendency for LAA and OAA to increase cleavage of cooled oocytes (*P* = 0.07) but had no effect on those held at 39 °C. There was a significant effect of temperature on development to blastocysts (35.7 ± 2.2% versus 19.6 ± 1.6%, 39 °C versus 0 °C; *P* < 0.005). While fatty acids did not affect development of oocytes held at 39 °C, there was an effect of fatty acids on cooled oocytes. Compared to no fatty acid in IVM, OAA increased the development rate of cooled oocytes (27.4 ± 3.8% versus 11.9 ± 3.7%; *P* < 0.05) while LAA was not significantly different to either treatment (18.5 ± 3.8%). There was no effect of PUT. The chilling sensitivity of oocytes can be altered by their exposure to fatty acids during IVM indicating that these fatty acids are taken up into the oocyte. As LAA and OAA reduced the degree of lysis of cooled oocytes, these fatty acids may be incorporated into cytoplasmic lipid rendering this more resilient to direct chilling injury. The absence of a beneficial effect of PUT on cooled oocytes compared to zygotes, as we have previously found, may indicate that this polyamine protects the nuclear rather than cytoplasmic components. It remains to be determined whether the improvements in chilling sensitivity of oocytes by fatty acids during IVM will also improve their survival following cryopreservation.

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EFFECT OF CULTURE OF BOVINE IVP EMBRYOS WITH VERO CELLS OR LIF PROTEIN ON POST-THAW SURVIVAL FOLLOWING CRYOPRESERVATION

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In a previous study, by transferring five embryos per recipient, we found that ovine embryos produced in modified SOFaaBSA [Thompson et al., *J Reprod Fertil* 2000;118:47–55] survived freezing less readily than those produced in sheep oviducts [Pugh et al., *Theriogenology* 2001;55:314]. At 25 days after transfer, 42% of recipients of the former and 90% of recipients of the latter were pregnant; 10 and 39% of recovered embryos were viable, respectively. Since use of sheep oviducts is an inappropriate culture system from an ethical view, we investigated whether Leukemia Inhibitory Factor (LIF) either as a pure protein or as a factor secreted during co-culture with Vero cells would improve survival of embryos after cryopreservation. Cumulus–oocyte complexes ( $n = 4687$ ) were aspirated from abattoir-derived ovaries and matured and then fertilized under standard conditions. Stripped putative zygotes were then randomly allocated to one of the following treatments and cultured at 39 °C in humidified (5% CO<sub>2</sub>, 7% O<sub>2</sub>, and 88% N<sub>2</sub>) for 7 days: (T1): modified SOFaaBSA; (T2): modified SOFaaBSA + 10 ng/ml rhLIF (Silenus Laboratories); (T3): co-culture on Vero cells in modified SOFaaBSA + 1% FCS. Grade 1 and 2 blastocysts were frozen in 1.5 M ethylene glycol + 0.1 M sucrose from 0 to –35 °C at 0.5 °C/min and then plunged into liquid nitrogen. After thawing and direct dilution, the embryos were grouped, within each culture treatment, as grade 1 or 2 and then non-surgically transferred to recipients (5 embryos/recipient). Only embryos cultured in rhLIF or on Vero cells were transferred. Recipients were slaughtered on Day 25, 26 or 27, the conceptuses recovered and graded for viability. The experiment was replicated five times. There was no effect of the culture system on the proportion of zygotes cleaving (92.0% versus 86.6% versus 83.7%) or developing into blastocysts (39.3% versus 38.6% versus 49.7%) for T1, T2 and T3, respectively. After freezing, approximately 100 blastocysts from each group were thawed and cultured for 48 h to assess survival. The proportion that subsequently hatched was not significantly different (36.8% versus 46.5% versus 50.6%; T1 versus T2 versus T3). Pregnancy rates and embryo survival are presented in the following table. There was no effect of the culture system on the pregnancy rate or on viable embryos recovered. However, more embryos that were classified as grade 1 post-thaw survived than those scored as grade 2 (41.0% versus 25.0%;  $\chi^2 = 6.18$ ,  $P < 0.05$ ). These data suggest that IVC in rhLIF or Vero cells may result in frozen embryo survival equivalent to that following culture in sheep oviducts. However, it will be necessary to determine whether birth weight and health of calves resulting from these culture systems are affected.

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Culture system	Embryo grade	Number of recipients (%)		Number of embryos (%)		
		Transferred	Pregnant	Transferred	Recovered	Viable <sup>a</sup>
Vero	1	8	7 (87.5)	40	18 (45.0)	18 (45.0)
	2	10	9 (90.0)	50	16 (32.0)	14 (28.0)
rhLIF	1	10	10 (100.0)	50	19 (38.0)	19 (38.0)
	2	10	7 (70.0)	50	13 (26.0)	11 (22.0)

<sup>a</sup> Of transferred.

## RE-VITRIFICATION OF MOUSE EMBRYOS DOES NOT AFFECT DEVELOPMENTAL COMPETENCE OR VIABILITY

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Vitrification is a useful technique for cryopreservation of mammalian embryos. Recently it has been demonstrated that ultrarapid vitrification procedures such as the cryoloop can vitrify mouse embryos without any loss in viability. The aim of this study was therefore to examine embryo development and viability following re-vitrification at successive stages of development (1-cell, 2-cell, 8-cell and blastocyst) using the cryoloop vitrification procedure. Zygotes were collected from F1 hybrid (C57BL/6 × CBA) females at 21 h post-hCG. Zygotes ( $n = 590$ ) were then divided between two treatments. Treatment I (control) embryos were cultured 48 h in medium G1 followed by 48 h of culture in medium G2 (total culture period 96 h) in 6% CO<sub>2</sub>, 5% O<sub>2</sub>, 89% N<sub>2</sub>. Treatment II embryos were vitrified using the cryoloop at the zygote stage immediately after collection. Zygotes were subsequently warmed and grown to the 2-cell stage in G1. Vitrification and warming were repeated at the 2-cell stage and again at the 8-cell stage on the same embryos. Eight-cell embryos were then cultured in G2 for a further 48 h (total culture period of 96 h) in 6% CO<sub>2</sub>, 5% O<sub>2</sub>, 89% N<sub>2</sub>. Development in culture for both treatments was assessed by rates of compaction after 48 h of culture and blastocyst development and hatching after 96 h of culture. Blastocysts were then either stained for total cell number, inner cell mass (ICM), and trophoctoderm (TE) cell numbers or transferred to recipient females. For embryo transfers, blastocysts in Treatment II were also vitrified at the blastocyst stage before transfer to pseudopregnant recipients. On Day 15 of pregnancy, implantation rates and fetal development rates were assessed. Differences between treatments were determined by generalized linear modeling or Student's *t*-test. Compaction rates were not different between treatments: 70.2% for control embryos and 67.1% for re-vitrified embryos. Similarly, blastocyst development (90.2 and 86.4%) and blastocyst hatching (69.9 and 66.1%) were not different between treatments I and II. Total cell numbers of blastocysts ( $86.1 \pm 1.6$  and  $81.5 \pm 2.4$ ), and cell numbers of ICM ( $18.2 \pm 0.5$  and  $17.3 \pm 0.7$ ) and of TE ( $67.9 \pm 1.3$  and  $64.2 \pm 2.0$ ), and the percentages of ICM development ( $21.1 \pm 0.5$  and  $21.3 \pm 0.6\%$ ) were also not different between treatments I and II. Twenty-four blastocysts were transferred from each treatment. There was no difference in implantation, fetal development or fetal weights between the control blastocysts (75.0%, 54.2% and  $188 \pm 11$  mg) and re-vitrified blastocysts (54.2%, 50.0% and  $199 \pm 11$  mg). Vitrifying embryos at successive stages of development using the cryoloop is not detrimental to embryo development or viability. The significance of these data lies with the ability to re-cryopreserve embryos that have been previously cryopreserved.

EFFECT OF GLYCEROL AND DIMETHYLSULPHOXIDE ON CRYOPRESERVATION OF RHESUS MONKEY (*MACACA MULATTA*) SPERMATOZOA

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Glycerol and dimethylsulphoxide (DMSO) have been widely used as penetrating cryoprotectants for cryopreservation of spermatozoa of many species, but various concentrations of the cryoprotectants are used for different species. Studying the cryoprotective properties of those cryoprotectants will be helpful to understand mechanisms of animal sperm cryopreservation. The present study examined the effects of penetrating cryoprotectant at different concentration (2, 5, 10, 15% glycerol and 5% DMSO) in an extender containing 10% lactose, 1% glucose and 20% egg yolk on rhesus monkey spermatozoa cryopreservation. The frozen-thawed sperm function was evaluated by sperm motility, plasma membrane integrity, acrosome integrity and in vitro fertilization. Membrane integrity was evaluated by Hoechst 33258 staining, and acrosomal status was monitored by means of FITC-labeled peanut agglutinin. Data were subjected to arcsin (square root) transformation and analyzed by ANOVA and LSD test. The results showed that the highest recovery of post-thaw sperm motility and rate of intact acrosomes occurred when spermatozoa were frozen with 5% glycerol ( $45.5 \pm 4.0$  and  $82.4 \pm 1.8\%$ ;  $P < 0.05$ ), but there was no significant difference among 2, 5 and 10% glycerol on plasma membrane integrity ( $53.3 \pm 5.8$ ,  $61.6 \pm 5.5$  and  $55.6 \pm 7.2\%$ , respectively;  $P > 0.05$ ). The post-thaw motility, plasma membrane and acrosome integrity of spermatozoa frozen with 5% glycerol were significantly higher than those frozen with 5% DMSO ( $45.5 \pm 4.0$ ,  $61.6 \pm 5.5$  and  $82.4 \pm 1.8\%$  versus  $25.6 \pm 4.4$ ,  $47.0 \pm 7.0$  and  $69.1 \pm 6.2\%$ , respectively;  $P < 0.05$ ). Spermatozoa cryopreserved with 15% glycerol showed the lowest post-thaw sperm motility and plasma membrane integrity among the five groups ( $16.1 \pm 4.6$  and  $19.5 \pm 2.1\%$ ;  $P < 0.05$ ). The lowest acrosome integrity rate was found in the spermatozoa frozen with 10 and 15% glycerol; there was no significant difference between the two groups ( $45.1 \pm 9.4$  and  $34.9 \pm 8.1\%$ , respectively;  $P > 0.05$ ). The fertility of sperm cryopreserved with 5% glycerol was assessed by in vitro fertilization of oocytes collected from non-stimulated adult rhesus monkeys. Of the inseminated oocytes,  $79.9 \pm 19.7\%$  were fertilized and  $49.7 \pm 23.5$  and  $7.5 \pm 7.2\%$  of the resulting zygotes developed into morulae and blastocysts, respectively. The results indicate that the concentration of penetrating cryoprotectant can greatly affect sperm functional integrity of rhesus monkey during freezing and thawing. DMSO was less effective than glycerol for cryopreservation of rhesus monkey spermatozoa.

## CRYOPRESERVATION REDUCES UPTAKE OF PYRUVATE BY GIANT PANDA SPERM BUT NOT GLUCOSE

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Sperm cryopreservation enhances the potential of artificial insemination (AI) and in vitro fertilization (IVF) by extending the storage period of gametes and by facilitating intercontinental transport. Previous studies in our laboratory have revealed that freeze–thawing does not alter sperm motility or the ability of the cell to undergo capacitation or decondensation. In general, there is little available knowledge in the literature on the impact of cryopreservation on sperm physiology. In our continued efforts to enhance AI and sperm cryopreservation in the giant panda, this study tested the hypothesis that freeze–thawing influences sperm metabolism. Semen from each of five adult giant pandas was collected during the breeding seasons (February through May) of 2001 and 2002. Sperm were assessed immediately for percent motility and forward progressive status (scale 0–5, 5 = best). An aliquot (100  $\mu$ l) of semen was diluted immediately in 1 ml modified HEPES buffered Ham's F10 medium (MHH) with 1 mM glucose and pyruvate and 2 mg/ml bovine serum albumin, centrifuged (8 min, 300  $\times$  g), the supernatant removed and the sperm pellet resuspended in MHH at a final concentration of 10  $\times$  10<sup>6</sup> sperm/ml (minimum volume = 2 ml). Another aliquot was centrifuged (8 min, 800  $\times$  g) to remove all sperm and the supernatant frozen. All remaining sperm were diluted in TEST freezing medium with 5% glycerol, cooled to 5  $^{\circ}$ C over 4 h and cryopreserved rapidly in 0.25 ml straws over liquid nitrogen vapor. Sperm were thawed and diluted in MHH at 37  $^{\circ}$ C, centrifuged and resuspended in MHH (as above). Fresh and thawed sperm were incubated for 6 h, and sub-aliquots were removed at 0, 2, 4 and 6 h for assessment of sperm motility traits. Additional 50  $\mu$ l samples at each time point were centrifuged (800  $\times$  g) to remove all sperm and the supernatant frozen. All frozen seminal and medium aliquots were later analyzed by microfluorescence to determine glucose, pyruvate and lactate concentrations. Sperm metabolism was defined as the rate of change in substrate concentration. Mean ( $\pm$ S.E.M.) fresh (73.0  $\pm$  5.7%) and post-thaw (59.3  $\pm$  4.2%) sperm motility was comparable ( $P > 0.05$ ). On average, lactate concentration within seminal plasma (2.6  $\pm$  0.5 mM) was higher than that of pyruvate (0.2  $\pm$  0.0 mM) or glucose (42.0  $\pm$  23.1 nM). Pyruvate uptake by fresh sperm (41.6  $\pm$  8.0 pmol/10<sup>6</sup> sperm/h) was greater ( $P < 0.05$ ) than that of glucose (17.7  $\pm$  4.2 pmol/10<sup>6</sup> sperm/h). Cryopreservation did not alter ( $P > 0.05$ ) either glucose uptake (thawed, 19.6  $\pm$  4.7 pmol/10<sup>6</sup> sperm/h) or lactate production (fresh, 55.3  $\pm$  12.6 pmol/10<sup>6</sup> sperm/h versus thawed, 63.6  $\pm$  17.5 pmol/10<sup>6</sup> sperm/h). Cryopreserved and thawed sperm took up pyruvate at a slower rate (16.2  $\pm$  4.1 pmol/10<sup>6</sup> sperm/h;  $P < 0.05$ ) than fresh counterparts. Sperm motility was not correlated ( $P > 0.05$ ) with any seminal plasma energy substrate concentration or sperm metabolic index. Rapid pyruvate uptake reveals that fresh giant panda sperm metabolism is highly oxidative in nature. The reduced pyruvate uptake after freeze–thawing does not affect lactate production, suggesting that the majority of pyruvate taken up was oxidized, whereas the majority of lactate was a product of glucose metabolism. The decrease after freeze–thawing of uptake of pyruvate, but not glucose, may indicate that cryopreserved sperm sustain mitochondrial damage or that oxidative metabolism is suppressed temporarily by the cryopreservation medium or process.

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## IMPROVED SURVIVAL OF CRYOPRESERVED PORCINE MORULAE

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To develop an efficient cryopreservation protocol for porcine morula-stage embryos, three vitrification methods were compared, i.e., embryos cryopreserved in a plastic straw (ST) [Ishimori et al., 1993], in a gel-loading tip (GLT) [Tominaga et al., 2002], or by the minimum volume cooling method (MVC) [Kuwayama et al., 2000]. In addition, to try to improve embryo survival even more, embryos in which the amount of cytoplasmic lipid had been reduced were also vitrified by the MVC method. Morula-stage embryos were collected from superovulated gilts and sows 5 days after hCG injection. Two experiments were performed. In Exp. 1, for the ST and GLT methods, a vitrification medium consisting of 20% ethylene glycol (EG), 20% DMSO, and 0.6 M sucrose was used, while for the MVC method, the concentration of the same cryoprotectants was reduced to 15%, 15%, and 0.5 M, respectively. Embryos were loaded into ST, GLT, and MVC plates (Cryotop, Kitazato Supply, Japan) with volumes of vitrification media of about 10, 0.6, and  $\leq 0.05$   $\mu$ l, respectively. The ST was kept in LN<sub>2</sub> vapor for 2 min before being plunged into LN<sub>2</sub>, whereas the devices for the other two methods were plunged directly into LN<sub>2</sub>. To liquify the vitrified solutions, the ST was immersed in a 37 °C water bath, whereas the GLT and MVC plates were placed directly into diluent containing 0.25 or 1 M sucrose, respectively. Cryoprotectants were removed by stepwise dilution. In Experiment 2, morulae were centrifuged with 7.5  $\mu$ g/ml cytochalasin B at 12,000  $\times g$  for 20 min to polarize the cytoplasmic lipid droplets. Polarized lipids were removed from some embryos by micro-manipulation (delipation). These lipid-reduced embryos and control non-manipulated embryos were vitrified by the MVC method. In both experiments, embryo survival was assessed by in vitro culture in NCSU23 medium + 10% FCS for 48 h. Differences were analyzed by the Chi-square test, and the Student's *t*-test was used to compare mean cell numbers of embryos. Developmental rates of vitrified embryos into blastocysts were 20% (6/30) for ST, 39% (18/46) for GLT, and 60% (26/43) for MVC, and the proportion of transferable quality blastocysts were 0, 22, and 37%, respectively. Embryo survival and transferable-quality embryos were further improved by vitrification after delipation (95%, 35/37; 95%, 35/37) compared with non-manipulated vitrified embryos or lipid-polarized ones, i.e., 24/42 (57%) or 29/38 (76%),  $P \leq 0.05$ ; 21/42 (50%) or 21/38 (71%),  $P \leq 0.01$ . The proportion of transferable blastocysts (95%) and cell numbers of blastocysts ( $92 \pm 25$ ) derived from delipated vitrified-embryos were comparable to those derived from non-vitrified control embryos, i.e., 85% (34/40) and  $103 \pm 31$ . These results demonstrate that survival of vitrified morulae can be significantly improved using the MVC method to cryopreserve delipated embryos.

DEVELOPMENT OF NUCLEAR-TRANSFER EMBRYOS USING  
VITRIFIED-WARMED IN VITRO-MATURED BOVINE OOCYTES

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Cryopreservation would be a useful way to provide a steady source of oocytes for nuclear transfer and in vitro embryo production. The present study was conducted to examine the developmental ability of nuclear transfer embryos reconstituted from bovine fetal fibroblasts transfected with green fluorescent protein (GFP) and vitrified mature oocytes. The vitrification solution (VS) consisted of 40% ethylene glycol (EG) + 1 M sucrose in H-TCM 199 medium + 20% FBS. Cumulus-free oocytes with one polar body were equilibrated in 3% EG and then exposed to VS for 30 s. The oocytes in VS were dropped directly into liquid nitrogen using a micropipette (microdrop method). To warm and liquify them, vitrified oocytes were placed into 0.3 M sucrose at 37 °C for 3 min. Vitrified oocytes were considered to have normal morphology by observation of dark, evenly granulated cytoplasm. Oocytes that had an irregular shape with dark yellow cytoplasm, cracks in the zona pellucida or with a damaged membrane were considered abnormal. Surviving oocytes were used for parthenogenetic activation and nuclear transfer with bovine fetal fibroblasts transfected with GFP. The survival rate of oocytes vitrified by the microdrop method was >90%. Rates of cleavage and blastocyst development of parthenogenetically-activated oocytes were 45.1 and 10.8%, respectively. Of vitrified-warmed oocytes, enucleation and fusion rates were not significantly different compared to fresh oocytes ( $P > 0.05$ ). However, development rates of fresh and vitrified-warmed oocytes were different at the 2-cell (Fresh; 68.6% versus Vit; 48.0%,  $P < 0.05$ ), and blastocyst stages (Fresh; 24.8% versus Vit; 9.0%,  $P < 0.05$ ). All embryos and blastocysts resulting from nuclear transfer using GFP-transfected fibroblasts expressed GFP under the fluorescence microscope.

In conclusion, the present study indicates that vitrified bovine oocytes can be reconstituted from GFP-transfected cells, and that reconstructed embryos can develop to the blastocyst stage in vitro.

Group	Number (%)	No. of oocytes (%)	No. of enucleated oocytes (%)	No. of development <sup>1</sup>	
				Two cell	Blastocyst
Fresh-PA	132	–	–	117 (88.6) <sup>a</sup>	42 (31.8) <sup>a</sup>
Fresh-NT	193	180 (93.3)	105 (58.3)	72 (68.6) <sup>a</sup>	26 (24.8) <sup>a</sup>
VT-PA	111/120 (92.50)	–	–	50 (45.1) <sup>b</sup>	12 (10.8) <sup>b</sup>
VT-NT	218/236 (92.37)	188 (86.2)	100 (53.2)	48 (48.0) <sup>b</sup>	9 (9.0) <sup>b</sup>

PA: parthenogenetic activation, NT: nuclear transfer, VT: vitrified-warmed. Values in a column with different superscripts are significantly different ( $P < 0.05$ ).

<sup>1</sup> Based on fused oocytes.

# Developmental Biology

## INHIBITION OF TRANSCRIPTION IN BOVINE FETAL FIBROBLASTS EXPOSED TO *XENOPUS LAEVIS* EGG EXTRACTS

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For successful development after nuclear transfer it has been suggested that the donor nucleus must first abolish transcription and then re-establish the embryonic pattern of gene expression [Campbell, Cloning 1999;1:3–14]. The aim of this study was to investigate the effect of *Xenopus laevis* egg cytoplasm on the regulation of transcriptional activity in mammalian somatic cells. Bovine fibroblasts derived from a 45–60-day-old fetus were cultured for a maximum of four passages in DMEM + 10% FCS supplemented with 2 mM glutamine, 0.1 mM  $\beta$ -mercaptoethanol, 2 mM non-essential amino acids and antibiotics at 39 °C in 5% CO<sub>2</sub>. Cells were synchronized at G1/S by 48 h serum starvation (0.1% FCS) followed by 18 h in DMEM + 10% FCS with aphidicolin (5  $\mu$ g/ml). After trypsinization, the plasma membrane was permeabilized with digitonin [Adam et al., M Enzymol 1992;219:97–110] and the cells were incubated in xenopus egg extracts (2000 cells/ $\mu$ l extract) supplemented with an energy regenerating system for a period of 2 h at 21 °C [Meier et al., J Cell Sci 1991;98:271–279]. Incorporation of 5-fluorouridine (FurD) at 0–1 and 1–2 h after permeabilization was used to investigate transcription by immunocytochemistry. Data are summarized in Table 1. A marked reduction (79%) followed by a complete inhibition of FurD incorporation was detected during the first 2 h of incubation in xenopus egg extracts compared to permeabilized controls. This result suggests that factors present in xenopus egg extracts inhibit transcriptional activity in somatic cells. Whether this effect is due to a specific factor or due to chromatin rearrangement is unclear. However, permeabilized cells underwent DNA synthesis in the extract over a period of 9 h, whereas control cells did not. This suggests that inhibition of transcription did not interfere with cell cycle progression. Further experiments will focus on the chromatin modifications of treated cells. This interspecies experimental approach may be useful for the identification of oocytic factors involved in nuclear reprogramming.

Table 1  
FurD incorporation of bovine fibroblasts incubated in xenopus extracts

Group	FurD (%)	
	Positive	Negative
Control non-permeabilized 0–1 h	96	4
Control non-permeabilized 1–2 h	44	56
Permeabilized 0–1 h in DMEM	43	57
Permeabilized 0–1 h in XT	9	91
Permeabilized 1–2 h in DMEM	32	68
Permeabilized 1–2 h in XT	0	100

XT: *Xenopus* egg extract.

## DOES AMINO ACID TURNOVER PREDICT THE CAPACITY OF TROPHECTODERM INTEGRITY IN CULTURED HUMAN EMBRYOS?

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During preimplantation development, the mammalian embryo undergoes at least two major morphological changes: compaction, when blastomeres polarize and cell adhesivity increases, and cavitation, when two distinct cell lineages, the undifferentiated inner cell mass and the epithelial trophoctoderm (TE) segregate. The integrity of the nascent TE is achieved by a sequential program of expression and membrane assembly of junctional gene products. The adherens junction (AJ) protein E-cadherin (E-cad) is essential for compaction, the tight junction (TJ) proteins (e.g. occludin, occ; ZO-1, expressed as two isoforms:  $-\alpha-$  and  $-\alpha+$ ) are then assembled, and finally the desmosomes (e.g. desmocollin 2, DSC2) stabilize the TE. In the mouse, the transmembrane occ and ZO-1 $\alpha+$  assemble together at the cell membrane just before cavitation and seal the TJ while ZO-1 $\alpha-$  is assembled earlier during compaction. Correct expression and assembly of these junctional proteins is essential for viable blastocyst development and implantation and can be disturbed in human embryos. Amino acid turnover in human embryos increases during both developmental steps and could be a better predictor of viability than morphology alone. The present study relates amino acid turnover during compaction and cavitation with future TE capacity of integrity by visualizing membrane assembly of key junctional components in human blastocysts of varying morphological grade. Depletion or appearance of 18 amino acids in the culture medium, brought about by single, spare human embryos was measured non-invasively. In vitro generation of human embryos, morphological grading (1–2.5) and reverse-phase HPLC analysis were performed as described [Houghton et al., Hum Reprod 2002;17:999–1005]. Culture medium used during compaction ( $n = 15$ ) or cavitation ( $n = 17$ ) of embryos which developed to blastocysts by Day 6 was examined. Results were expressed as amino acid depletion/appearance in pmol/embryo/h. Total amino acid turnover was defined as the sum of depletion/appearance of all 18 amino acids from the medium. Within the same human embryos, membrane assembly of the AJ protein E-cad, the TJ proteins occ and the two ZO-1 isoforms,  $-\alpha+$  and  $-\alpha-$ , and the desmosomal protein DSC2 was examined by immuno-confocal microscopy of blastocysts as described [Eckert et al., Theriogenology 2002;57:635]. Membrane assembly (1 = perinuclear, 2 = membrane assembly in few areas, 3 = membrane assembly in all areas) was assessed in 3D z-series reconstructions. The Spearman Rank Order Correlation (SigmaStat 2.03 software package, Jandel Scientific) was used to identify significant ( $P < 0.05$ ) relations between junctional membrane assembly, amino acid metabolism and morphological grade. Total amino acid turnover during compaction was significantly correlated ( $P = 0.003$ ,  $r = 0.69$ ) with grade at that stage and, at cavitation, with membrane assembly of the TJ proteins occ and ZO-1 $\alpha+$  ( $P = 0.03$ ,  $r = 0.77$ ) which appeared to assemble together. These correlations persisted during cavitation ( $P = 0.04$ ,  $r = 0.56$  and  $P = 0.01$ ,  $r = 0.92$ ) although the amino acids mainly contributing to this correlation differed at each stage. Membrane assembly of the other junctional components (E-cad, ZO-1 $\alpha-$ , DSC2) was not correlated to either morphological grade or amino acid turnover. Our data demonstrate that amino acid turnover could predict the capacity of integrity of the TE in human embryos, particularly of two TJ components responsible for sealing the TE. A failure to tighten this seal may result in viability loss.

CULTURE OF PREIMPLANTATION MOUSE EMBRYOS WITH FETAL CALF SERUM AFFECTS FETAL EXPRESSION OF IMPRINTED GENES AND POST-NATAL GROWTH AND BEHAVIOR

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Evidence suggests that *in vitro* culture of preimplantation embryos can be associated with aberrant growth and specific phenotypic abnormalities during fetal and post-natal development. Several culture systems have been associated with these syndromes, but most of them involve the use of serum [Thompson et al., *Biol Reprod* 1995;53:1385–1391]. Here, we determined if mouse embryos cultured in the presence of serum produce aberrant epigenetic modifications that were maintained somatically during fetal development or affected growth and behavior at later stages. Female B6CBAF1 mice were superovulated and oocytes were inseminated with spermatozoa of the same background as described previously [Sztejn et al., *Biol Reprod* 2000;63:1174–1180]. One-cell *in vitro* produced embryos were cultured for 4 days in KSOM in presence of 10% of fetal calf serum (Group A) or in presence of 1 g/l of BSA (Control). Embryos that reached the blastocyst stage were transferred into CD1 pseudopregnant females. Messenger RNA was isolated from 15 E14 fetuses and the expression of *Mest*, *IGFbp2* and *Meg1* imprinting genes was measured by quantitative Real Time PCR. Forty-three mice (24 males and 19 females) obtained from transfers of Group A embryos (cultured in presence of serum) and 35 control mice (13 males and 12 females) also obtained by IVF but cultured in serum-free medium were submitted to the following behavior tests: somatometric, preweaning developmental test (Fox battery), neuromotor developmental profile (neurological, sensory, and motor test), locomotors activity on week 6 and 52 (open field), and exploratory/anxiety behavior (elevated plus maze, and open field). Comparison between embryos cultured with or without serum was made using analysis of variance followed by least significant difference (LSD) post hoc test. Presence of serum significantly inhibited *in vitro* results (embryo development rates to the blastocysts stage were 35 and 93% in media with or without serum, respectively). Also expression of imprinting genes was significantly reduced in those fetuses obtained from transferred blastocysts after culture in the presence of serum. At 52 weeks, post-natal weight gain of male mice produced *in vitro* in presence of serum was similar to control male but female mice produced *in vitro* in presence of serum were significantly heavier than their control counterparts from 13 weeks on. Two of the eight measures of preweaning development and some specific measures in neuromotor development were delayed in Group A indicating a later general psychomotor development. No significant differences were observed between groups in the elevated plus maze test or in the open field test at 6 weeks of age, but in the open field test, motor activity of 52-week-old females from Group A was higher than in control females and males produced in the presence of serum. Together, these data suggest that culture of preimplantation mouse embryos in presence of serum influences the regulation of growth-related imprinted genes, it delays the appearance of a few developmental milestones, and it leads, at least in females, to aberrant growth and behavior.

EFFECTS OF CYSTEAMINE ON GLUTATHIONE SYNTHESIS OF BUFFALO  
(*BUBALUS BUBALIS*) OOCYTES DURING IN VITRO MATURATION

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An improved developmental competence of oocytes matured in the presence of cysteamine was recorded in buffalo species [Gasparrini et al., *Theriogenology* 2000;54:1537–1542]. The purpose of this work was to investigate (1) if cysteamine supplementation during in vitro maturation (IVM) of buffalo oocytes increases GSH synthesis and (2) if the inhibition of glutathione synthesis during IVM by buthionine sulfoximide (BSO), in the presence or absence of cysteamine, affects subsequent embryo development and GSH synthesis. Cumulus–oocyte complexes (COCs), recovered from slaughtered animals, were matured in vitro in TCM 199 + 10% FCS, 0.5 µg/ml FSH, 5 µg/ml LH and 1 µg/ml 17-β-estradiol in the absence or presence of cysteamine (50 µM), with or without 5 mM BSO at 38.5 °C under 5% CO<sub>2</sub> in humidified air for 24 h. In Experiment 1 a total number of 149 oocytes, over eight replicates, was used for GSH measurements. Glutathione content was measured by high-performance liquid chromatography and fluorimetric analysis in oocytes immediately after follicle aspiration (before maturation) or at the end of in vitro maturation (IVM) for the remaining oocytes, which were evenly distributed in the four IVM groups. Experiment 2 was carried out on a total of 405 COCs, over three replicates. COCs were randomly assigned to the four different experimental groups of maturation, in vitro fertilized and cultured for 7 days in order to assess development into blastocysts. In vitro fertilization was carried out at 38.5 °C under 5% CO<sub>2</sub> in humidified air in TALP medium supplemented with 0.2 mM penicillamine, 0.1 mM hypotaurine and 0.01 mM heparin. Frozen-thawed sperm from a tested bull was treated by the swim-up procedure and used at a final concentration of 20<sup>6</sup>/ml. After 20–22 h presumptive zygotes were cultured in SOF medium, supplemented with essential and non-essential amino acids and 8 mg/ml BSA, in a gas atmosphere of 5% CO<sub>2</sub>, 7% O<sub>2</sub>, and 88% N<sub>2</sub>. Differences among treatments in each experiment were analyzed by ANOVA1. It was demonstrated that buffalo oocytes synthesize glutathione during IVM and that cysteamine increases glutathione synthesis (Table 1). Furthermore, buthionine sulfoximide neutralized the promoting effects of cysteamine on GSH synthesis and embryo development (Table 1). These results indicate that cysteamine supplementation during in vitro maturation of buffalo oocytes improves embryo development by increasing GSH synthesis.

Table 1

Effects of cysteamine (50 µM) in the absence or presence of buthionine sulfoximide (BSO: 5 mM) during IVM on subsequent embryo development (three replicates) and GSH synthesis

Treatments	Cleavage (%) (Mean ± S.D.)	Day 7 blastocysts (%) (Mean ± S.D.)	GSH content pmol/oocytes (Mean ± S.D.)
Before IVM	–	–	1.4 ± 1.1 <sup>a</sup>
Control	67.0 ± 8.5 <sup>a</sup>	19.5 ± 4.7 <sup>a</sup>	4.6 ± 0.8 <sup>b</sup>
Control + BSO	46.6 ± 7.5 <sup>b</sup>	3.1 ± 0.6 <sup>c</sup>	0.9 ± 0.2 <sup>a</sup>
Cysteamine	66.7 ± 5.7 <sup>a</sup>	30.1 ± 2.8 <sup>b</sup>	6.4 ± 1.5 <sup>c</sup>
Cysteamine + BSO	51.3 ± 2.6 <sup>b</sup>	4.8 ± 1.7 <sup>c</sup>	1.3 ± 0.3 <sup>a</sup>

Values with different superscripts differ within columns ( $P < 0.01$ ).

## ACTIVE-CASPASE-3, TUNEL AND ULTRASTRUCTURE OF APOPTOTIC CELL DEATH IN BOVINE BLASTOCYSTS

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Apoptosis is a possible marker of developmental potential in preimplantation embryos, since it may indicate adequate regulation of both early differentiation events and response to stress. It is, however, important to identify both the different elements involved in regulation of cell death in embryos as well as their functional sequence, to adequately evaluate the developmental significance of this process. The aim of the current experiment was first, to investigate if caspase-3 (a central element in execution of the apoptotic program) was involved in cell death in bovine blastocysts, and to reveal any relation with observable changes in nuclear morphology and DNA fragmentation, and second, to observe if these biochemical elements of apoptosis coincided with classic ultrastructural features of apoptosis. Bovine blastocysts were harvested from a standard *in vitro* production system [Avery and Greve, *Mol Reprod Dev* 2000;55:438–445] at Day 7 post-insemination (p.i.). The blastocysts were either: (a) cultured in 10 mM staurosporine (a protein kinase C inhibitor known to block intercellular signaling and to induce apoptosis) for 24 h or (b) left for continued normal culture for a similar period. At Day 8 p.i. embryos from both treatments were fixed for either: (1) immunocytochemical (IMC) visualization of active-caspase-3 with Texas-Red, DAPI staining for assessment of nuclear condensation and fragmentation (a key feature of apoptosis) and detection of DNA fragmentation (a central element in apoptotic dismantling of cellular structures) by FITC-conjugated dUTP and terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) or (2) transmission electron microscopy to observe ultrastructural characteristics of apoptosis. Fluorescence signals were detected by use of Multi-Photon Confocal Laser Scanning Microscopy. In blastocysts without staurosporine induction, some cells contained nuclei displaying morphological changes compatible with apoptosis. A proportion of these cells displayed lack of or weak TUNEL staining of their nuclei and in these cases the cytoplasm displayed strong staining for active-caspase-3. On the other hand, in cells with apoptotic nuclei in combination with a strong TUNEL labeling, only a weak cytoplasmic staining for active-caspase-3 was seen. At the ultrastructural level classical features of apoptosis such as condensation of cytoplasm and organelles, extensive vacuolization, chromatin margination and condensation, nuclear and cellular fragmentation and phagocytosis could be observed. In staurosporine induced blastocysts, the blastocyst had disintegrated, and 70–90% of the nuclei displayed morphological changes of apoptosis, most of these with an intensive TUNEL labeling. A strong background staining for active-caspase-3 could be observed, but a specific cytoplasmic localization to single cells was not evident. Ultrastructurally, apoptotic cells at various stages of degeneration and secondary necrosis could be observed scattered among occasional cells with normal appearing nuclei and extensive amounts of debris. In conclusion, our findings indicate that caspase-3 is activated and may be involved in at least a part of the cell death observed in bovine blastocysts. This activation concurs with changes in nuclear morphology but precedes DNA degradation detectable by TUNEL, but a direct correlation is not yet defined.

## HEAT-INDUCED APOPTOSIS IN PREIMPLANTATION PORCINE EMBRYOS

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Heat stress conditions have long been recognized as having significant detrimental effects on efficiency of livestock animal embryo production both *in vitro* and *in vivo*. In lymphatic and neuronal cell culture systems, hyperthermia has been shown to potently induce cell death or apoptosis. Apoptosis is a genetically programmed form of cell death whereby unhealthy, damaged, or superfluous cells are eliminated from an organism. A minimal amount of apoptosis is normal—even necessary—for proper development, maintenance, and function of most tissues. And, although basal levels of cell death are thought to be important to the developmental process, apoptosis should generally be viewed as a process in constant conflict with successful embryo development. These experiments were designed to demonstrate the effects that a moderate heat shock has on the developmental competence and physiological status of preimplantation porcine embryos. *In vitro*-produced embryos were subjected to a 9 h heat shock of 42 °C at 15 h after the fertilization period (HS). Control embryos were cultured at 39 °C (NHS). Embryos were collected and fixed at 48, 72, and 96 h post-heat shock. The embryos were then processed to reveal their apoptotic status: TUNEL staining determined the presence or absence of internucleosomal DNA fragmentation; fluorescently labeled synthetic peptides allowed for the detection of activated caspases—the executioners of cell death. Developmental rates to blastocyst were analyzed on subsets of each group of embryos created (HS and NHS) that were allowed to culture 7 days undisturbed. A summary of collected results is contained in the table below. Statistical analysis was performed with the general linear model of analysis of variance using SAS.

	Heat shock		Non-heat shock	
	Number of embryos	%	Number of embryos	%
Blastocyst	175	0.5 ± 1.2 <sup>a</sup>	176	17.9 ± 8.2 <sup>a</sup>
TUNEL	40	45.6 ± 11.6 <sup>b</sup>	49	15.0 ± 6.0 <sup>b</sup>
Caspase	89	31.6 ± 3.3 <sup>c</sup>	79	17.3 ± 3.7 <sup>c</sup>

<sup>a</sup> Values differ at a significance level of  $P < 0.0005$ .

<sup>b</sup> Values differ at a significance level of  $P < 0.05$ .

<sup>c</sup> Values differ at a significance level of  $P < 0.01$ .

A 9 h heat shock radically reduced the developmental capacity of the HS embryos as compared to controls. TUNEL staining and caspase activity were also considerably more prevalent in the HS embryos as compared to the NHS embryos at all time points examined, indicating that an imposed heat stress of moderate duration and magnitude is sufficient to initiate apoptosis in blastomeres of early preimplantation porcine embryos.

ALTERATIONS OF MITOCHONDRIAL DYNAMICS AFFECT THE DEVELOPMENTAL COMPETENCE OF MOUSE PREIMPLANTATION EMBRYOS

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Mitochondria regulate the energy metabolism of the cells and have their own genetic materials. The mitochondrial inheritance in mammals is known to occur mainly through the oocyte. The relationship between mitochondrial function and early embryonic development is unclear. The objective of this study was to evaluate the effect of mitochondrial alterations on the developmental competence of the mouse preimplantation embryos. The ovulated oocytes and early embryos were collected from oviducts and uterus of super-ovulated ICR mice. We also collected the early embryos during in vitro culture of 2-cell stage embryos with modified T6 medium. The copy number of mitochondrial DNA was analyzed by competitive PCR of mitochondrial NADH dehydrogenase subunit 3 gene, and the morphological change of mitochondria was observed by transmission electron microscopy. The copy numbers of mitochondrial DNA were almost same and did not increase from the ovulated oocyte to the blastocyst stage. The mean of the copy number by the competitive PCR analysis was 157,000 copy/embryo, approximately. The ultrastructure of mitochondria changed by their developmental stages as follows. Mitochondria from the ovulated oocytes to the 4-cell stage embryos showed round and condensed appearance with concentric cristae and electrical dense matrix. They became enlarged and took on an orthodox configuration with more numerous and transversely arranged cristae during further development of embryos from the 8-cell to the blastocyst stage. Interestingly, the mitochondria in the polar body were less differentiated comparing to those in the blastomeres at the morula and blastocyst stage. We think that both mitochondrial and embryonic genes are needed for the normal differentiation of mitochondria. The degenerated embryos after in vitro culture showed abnormal ultrastructural changes of mitochondria, such as vacuolization and poor differentiation of cristae. In the in vitro culture from 2-cell to blastocyst stage, the slow developing embryos had significantly lower numbers of mitochondrial DNA than those in the fast developing embryos. These data suggest that the in vitro culture system can induce the abnormal morphological changes in mitochondria, and the absolute number of mitochondria may correlated with embryo developmental competence. Therefore, we propose that the mitochondrial dynamics should be considered in the regulation of early embryonic development and the optimization of in vitro culture system for preimplantation embryos.

APOPTOSIS IN BOVINE EMBRYOS CULTURED UNTIL DAY 7  
POST-OVULATION AFTER COLLECTION AT DIFFERENT  
TIMES OF IN VIVO DEVELOPMENT

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Apoptosis has been demonstrated in in vitro cultured and in vivo-developed bovine preimplantation embryos. There are indications that suboptimal culture conditions increase the incidence of apoptosis. In this study we investigated whether the duration of in vitro culture influences the level of apoptosis. Therefore, embryos were collected ex vivo from 59 cows, superovulated with FSH, at three time points post-ovulation (p.o.): 45 h p.o. (45 h group), when most embryos are still in the oviduct, at 100 h p.o. (100-h group), when embryos enter the uterus and at Day 7 p.o. (in vivo group) when embryos are in the uterus. The embryos collected at 45 and 100 h p.o. were further cultured in synthetic oviductal fluid (SOF) medium with 4 g/l BSA fr. V until Day 7 p.o. A control group of morulae and blastocysts was obtained from abattoir oocytes after IVM/IVF and IVC in SOF medium (in vitro group). The developmental stage of the embryos was determined with a stereo microscopy. The embryos were fixed with paraformaldehyde in PBS and apoptotic nuclei were detected using fluorescein-conjugated dUTP and terminal nucleotidyl transferase-mediated nick end labeling (TUNEL, In Situ Cell Death Detection kit; Roche, Hvidovre, Denmark), all nuclei were counterstained with propidium iodide. For positive controls embryos were incubated in DNase before TUNEL reaction, and for negative controls the terminal transferase was omitted from the reaction. Embryos were scored for total cell number as well as apoptotic nuclei within the inner cell mass (ICM), i.e. the regular ICM and the overlying polar trophoctoderm, and the trophoctoderm (TE), i.e. the mural trophoctoderm, using confocal laser scanning microscopy. Nuclei and fragments thereof were only counted as apoptotic if they contained condensed and/or fragmented DNA in combination with TUNEL staining. Apoptotic index was calculated as number of apoptotic nuclei relative to total cell number. The apoptotic index was lower in blastocysts developed after temporal or completely in vivo development compared to blastocysts cultured completely in vitro, for the 45-h group this difference was significant ( $P < 0.05$ ). These differences were related to a significantly lower apoptotic index in the ICM of the 45-h and in vivo group compared to the in vitro group. The apoptotic index in the ICM was in all groups significantly higher compared to the TE. In conclusion, in vitro production does influence the apoptotic index in bovine embryos, but the duration of in vitro culture appears not to affect the level of apoptosis. The relatively small differences between the apoptotic indices for the total embryo indicate that SOF medium provides an environment in which apoptosis in bovine embryos occurs fairly similar to that in vivo (as shown in Table). Apoptotic index  $\pm$  S.E.M. (number of embryos) in morulae (m)/early blastocysts (eb) and blastocysts (b), expanded (xb) and hatched blastocysts (hb) and incidence in ICM and TE after different times of in vivo development.

Group	m + eb	b + xb + hb	ICM	TE
In vitro	9.3 $\pm$ 2.5 <sup>a</sup> (6)	9.4 $\pm$ 1.4 <sup>a</sup> (28)	12.4 $\pm$ 1.6 <sup>aA</sup> (24)	6.3 $\pm$ 1.7 <sup>aB</sup> (24)
45 h	8.6 $\pm$ 4.2 <sup>a</sup> (9)	5.9 $\pm$ 0.8 <sup>b</sup> (42)	6.3 $\pm$ 0.7 <sup>bA</sup> (36)	4.1 $\pm$ 0.8 <sup>aB</sup> (36)
100 h	not pr	7.8 $\pm$ 0.7 <sup>ab</sup> (48)	9.1 $\pm$ 0.9 <sup>abA</sup> (43)	5.5 $\pm$ 0.7 <sup>aB</sup> (43)
In vivo	7.96 $\pm$ 0.2 <sup>a</sup> (28)	6.4 $\pm$ 0.6 <sup>ab</sup> (33)	7.1 $\pm$ 0.9 <sup>bA</sup> (26)	2.9 $\pm$ 0.5 <sup>aB</sup> (26)

(a, b) Within columns, values with different superscripts are significantly different ( $P < 0.05$ ) analyzed by ANOVA and Bonferroni test; (A, B) within rows, values with different superscripts are significantly different ( $P < 0.05$ ) analyzed by *t*-test; not pr: stage not present for analysis. The total cell number was 1.3–1.7 times higher for b + xb + hb than for m + eb stage embryos.

## EFFECT OF CADMIUM ON THE DEVELOPMENT AND DNA SYNTHESIS OF MOUSE EMBRYOS CULTURED IN VITRO

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Cadmium (Cd) is a widely distributed environmental pollutant that has been shown to exert deleterious effects on reproductive processes. The effect of cadmium exposure during mouse preimplantation development and outgrowth in vitro was examined. Preimplantation mouse embryos were collected from stimulated CD-1 mice (48 days old) at 3 (Experiment 1) and 4 (Experiment 2) days after eCG administration. In the Experiment 1, 8–16 cell stage embryos ( $n = 175$ ) were randomly allocated in three different treatments and cultured at 37 °C and 5% CO<sub>2</sub> in CZB medium + 0.1% BSA supplemented with 0 (control), 2 and 20 µM CdCl<sub>2</sub>. After 48 h embryos from each treatment were randomly divided into two groups, one of which was stained with Hoechst 33342 and propidium iodide to evaluate cell viability, and the other one was incubated overnight with 5 µM BrDU to detect DNA neo-synthesis. BrDU labeled nuclei were evidenced by immunocytochemistry after incubation with an anti-BrDU primary antibody and a FITC labeled secondary antibody. In the Experiment 2, collected mouse blastocysts ( $n = 64$ ) were randomly divided in three groups (control:  $n = 20$ ; 2 µM:  $n = 23$ ; 20 µM:  $n = 21$ ) and cultured in TCM 199 + 10% FCS for hatching and outgrowth to the bottom of the petri dishes until the inner cell mass (ICM) was well distinguishable from the trophoblastic cells. At this time, Cd was added and after 24 h the ICM and trophoblastic cells were processed for BrDU incorporation as described above for preimplantation embryos. The results show that at low (2 µM) and high (20 µM) doses, Cd adversely affects subsequent embryonic development and all embryos were decompacted; 20 µM Cd significantly affects cell viability ( $P < 0.01$ ) and 30% of cells showed membrane degeneration as evidenced by the red staining of PI, compared to 5% in 2 µM Cd and 0% in the control. BrDU incorporation analysis showed that Cd affects the DNA synthesis. In 2 µM treatment only 32% of embryonic nuclei were positive to BrDU incorporation compared to 86% of the control. Positive fluorescence seems attributed to the nuclei of the ICM. In the 20 µM Cd treatment BrDU incorporation was completely inhibited. These results were confirmed by the Experiment 2, where it has been observed that DNA synthesis was significantly reduced ( $P < 0.01$ ) in the 2 µM treatment (31%) compared to the control (92%), and it was practically absent in the trophoblastic compartment. Higher doses of Cd (20 µM) determined degenerative effects in cultured cells with only 7% of BrDU positive nuclei. However, these toxic effects were less evident and fast than those observed in the preimplantation stages. In conclusion, the presence of Cd in the culture media clearly shows a toxic effect in mouse embryonic cells altering cell junctions and DNA synthesis. The trophoblastic cells show a high sensitivity to Cd presence while the inner cell mass portion is able to continue the DNA synthesis even in presence of a significant amount of this heavy metal. Supported by IZS of Sardinia.

## DISTINGUISHABLE FATE OF BLASTOMERES OF THE TWO-CELL PIG EMBRYO: LINEAGE TRACING STUDIES

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Asymmetric divisions just before compaction lead to the formation of two phenotypically different cell types, which give rise to the first two cell lineages of the future blastocyst, trophoblast and the inner cell mass. However, little if any determinants that could specify cell fate at earlier preimplantation stages are known. To address whether the organization of pig preimplantation blastocyst is reflected by the first cleavage division plan, we conducted lineage tracing studies where distribution of the 2-cell stage blastomere progeny was traced over the preimplantation period using expression of a microinjected plasmid-borne GFP gene. Oocytes obtained from abattoir ovaries were matured followed by both in vitro fertilization (IVF) and parthenogenetic activation (PGA). In vitro culture conditions were previously described [Kikuchi et al., *Biol Reprod* 2002;66:1033–1041]. Plasmid-encoded GFP gene was microinjected into cytoplasm of one blastomere of a 2-cell stage embryo. GFP expression in the embryos was assayed using the Nikon Diaphot 200 inverted fluorescent microscope equipped with a mercury arc lamp (100 W) and a DM505 fluorescein filter set (consisting of a 450–490 nm excitation filter and a 520 nm emission filter). Following injection of the transgene into one blastomere of a 2-cell stage embryo, we modulated mosaic GFP expression over the preimplantation period. In the most normally developing embryos with GFP expression, the number of GFP fluorescing cells was near half of the total cell number. This allowed us to directly monitor the organization of cleavage-stage embryos that appeared to consist of two spatially separated cell populations, both descendants of the 2-cell stage blastomeres. Therefore, we were able to determine which cells occupied the embryonic or abembryonic portion of the blastocyst. Specifically, the vast majority, around 70%, of in vitro produced embryos showed asymmetrical first cleavage division that resulted in distinguishable size differences between blastomeres of the newly formed 2-cell embryos. However, size of the blastomere did not reflect in vitro developmental capacity, allowing the vast majority, 69.4% (179/258), of 2-cell stage embryos in the four consecutive experiments to develop to full blastocyst. More important is that the larger sized blastomere at the 2-cell stage divided first and preferentially contributed its progeny to the embryonic part of the blastocyst (including inner cell mass). Thus, the first cleavage division plan appears indicative of the fate of 2-cell stage blastomeres. Some angular displacement of the boarder line between GFP-fluorescing and non-fluorescing cell populations from the embryonic–abembryonic axis was observed in IVF produced blastocysts but not in the blastocysts obtained from PGA oocytes, suggesting the intrinsic role of sperm in spatial patterning of the early porcine embryo.

DEVELOPMENTAL ABILITY OF BLASTOMERES SEPARATED FROM  
TWO-, FOUR-, AND EIGHT-CELL IN VITRO-PRODUCED BOVINE EMBRYOS

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Recently, a few reports have described the production of monozygotic calves by separation of blastomeres returned to the zona pellucida of in vitro produced bovine embryos. If it is possible for blastomeres without the zona pellucida to develop to blastocysts, it will be possible to produce identical calves simply. The objective of the present study was to evaluate the developmental ability, in a small amount of culture medium (8–10  $\mu$ l), of blastomeres without the zona pellucida from 2-, 4-, and 8-cell in vitro-matured and -fertilized bovine embryos. The separation of blastomeres from 2-, 4-, and 8-cell embryos was performed at 28, 40, and 48 h post-insemination, respectively. As a control, intact 2-, 4-, and 8-cell embryos were cultured. The zona pellucida were removed from embryos by digestion for 1–2 min in 0.5% pronase solution, and the blastomeres were separated by mechanical pipetting in PBS(–) supplemented with 3 mg/ml BSA. The single blastomeres were isolated from each 2-cell embryo (1/2), 4-cell embryo (1/4) and two blastomeres from each 8-cell embryo (2/8). Blastomeres were cultured in micro-wells filled with 8–10  $\mu$ l. Blastomeres and intact embryos were cultured in CR1aa supplemented with 5% calf serum for 9 days (fertilization = Day 0). Total cell numbers in blastocysts were counted by means of the air-drying method. The development rates to blastocysts were analyzed using the chi-square method, and total cell numbers were analyzed using ANOVA. The results are presented in Table 1. There were no differences in the development rate of blastocysts between 1/2-embryos and the control, but that of 1/4- and 2/8-embryos was significantly lower than that of the control ( $P < 0.05$ ). The blastocyst rate of 1/2-embryos was higher than that of 1/4- and 2/8-embryos. The twin embryo rate of 1/2-embryos was 43.8%, the twin or more embryo rate of 1/4-embryos was 46.6%. However, twin embryos alone were obtained from 2/8-embryos, while quadruplet embryos were not obtained. Total cell number (mean  $\pm$  S.E.M.) in the blastocysts of separated embryos (1/2:  $51.7 \pm 3.7$ , 1/4:  $29.2 \pm 2.0$ , 2/8:  $36.2 \pm 3.9$ ) was significantly lower than that of the control (2-cell:  $124.2 \pm 11.8$ , 4-cell:  $111.2 \pm 6.8$ , 8-cell:  $122.6 \pm 7.5$ ) ( $P < 0.05$ ). The total cell number of blastocysts of 1/2 was significantly higher than those of 1/4 and 2/8 ( $P < 0.05$ ). These results indicate that blastomeres without the zona pellucida separated from 2- and 4-cell bovine embryos cultured in a small amount of culture medium have the possibility of development to blastocysts.

Table 1  
Developmental ability of blastomeres separated from 2-, 4-, and 8-cell in vitro-produced bovine embryos

Treatment	No. of embryos	Blastomeres	No. of blastocysts	No. of twin embryos (%)	No. of triple embryos (%)	No. of quadruplets embryos (%)
1/2-embryo	32	64	38 (59.4) <sup>a</sup>	14 (43.8)	–	–
Intact 2-cell	35	–	22 (62.9)	–	–	–
1/4-embryo	58	232	78 (33.6) <sup>b,c</sup>	14 (24.1)	10 (17.2)	3 (5.2)
Intact 4-cell	73	–	41 (56.2) <sup>d</sup>	–	–	–
2/8-embryo	22	88	22 (25.0) <sup>b,c</sup>	5 (22.7)	0 (0.0)	0 (0.0)
Intact 8-cell	60	–	35 (58.3) <sup>d</sup>	–	–	–

<sup>a,b</sup>Values within a column with different superscripts differ between separated-group ( $P < 0.05$ ).

<sup>c,d</sup>Values within a column with different superscripts differ between separated-group and control ( $P < 0.05$ ).

## VIABILITY OF EMBRYOS AFTER EXPOSING TO HIGH HYDROSTATIC PRESSURE

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It has been demonstrated that temperature, pH, osmotic pressure, light and hydrostatic pressure affect the survival of embryos in vitro. The aim of our present study was to build a protocol through which embryos kept in high hydrostatic pressure environment for long periods of time could survive. One-cell stage mouse embryos were collected and cultured at 37 °C with 5% CO<sub>2</sub> and maximal humidity in air in G 1.2 and G 2.2 media (Vitrolife, Göteborg) to the expanded blastocyst stage. Blastocysts were loaded into 0.08-ml straws (7–9 embryos/straw) with M2 (Sigma, St. Louis, MO). Straws were placed into the chamber, filled with M2, of a special lab-made device that is capable of generating and precisely detecting hydrostatic pressure up to 1500 atmospheric pressure (atm). Embryos were exposed to 900 bar pressure for 30, 60 and 120 min, then the pressure was gradually released in nine steps within 30, 60, 90, 120 and 150 min. Finally, the embryos were either cultured in vitro in G 2.2 or transferred to Day 3 pseudopregnant recipients. In vitro survival was judged on morphological characteristics and hatching rate of blastocysts. In vivo survival was based on number of offspring born. Eighteen to 23 embryos were used in each treatment group; each trial was repeated three times. Data were analyzed by chi-square analysis. The survival of embryos exposed to 900 bar pressure showed positive correlation with the duration of pressure release. All embryos exposed to 900 bar for 30 min and released for 30 or 60 min died. However, embryos released for 90 min showed 100% survival and 90% hatching rate ( $n = 54$ ). Sixty minutes of exposure with 60, 90 and 150 min release time resulted in 0% survival. But in the group of embryos released for 120 min 83% survival rate with 50% hatching rate was found ( $n = 66$ ). None of the embryos survived after treatment with 900 bar for 120 min paired with 60 and 90 min release time. Twenty two percent survival rate was found in the group of embryos ( $n = 54$ ) released for 150 min. However, when the duration of release was 120 min 60% of the embryos ( $n = 60$ ) survived and showed 50% hatching rate. Thirteen, 9, 11 and 11 embryos exposed to 900 bar for 30, 60, 120 and 60 min and released for 90, 120, 120 and 60 min were implanted into recipients and 10 (76.9%), 8 (88.8%), 10 (90.9%) and 0 offspring were born. The results provide additional information to the basic features of preimplantation embryos. Our study indicates that embryos can survive long lasting high hydrostatic pressure if they are retrieved gradually. Each impact with different parameters had an optimal retrieval protocol. The retrieval time shortens proportionally as the duration of the impact increases. Embryos die if the pressure is released promptly after the impact (10 min at 900 bar is lethal for 50% of the embryos).

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RESETTING THE “HISTONE CODE” IN THE MAMMALIAN OVUM:  
A ‘PROOF-OF-PRINCIPLE’ STUDY

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Cellular differentiation is believed to be accompanied by an ever increasing amount and complexity of epigenetic modifications. Post-translational histone N-terminal tail modifications, including acetylation, phosphorylation, and methylation, represent one type of epigenetic modification or “mark” [Wolffe and Matzke, *Science* 1999;286:481–486]. It has long been proposed that either some or all of these marks must be cleared from the chromatin during passage through the germline in order to reset the chromatin from the terminally differentiated state of the ovulated ovum to the totipotent state of the early embryo. To begin to investigate which (if any) histone marks are erased in the ovum or early embryo, we studied changes in the amount and distribution of five histone tail modifications by indirect immuno fluorescence during late oogenesis and throughout preimplantation mouse development. Three independent developmental trials consisting of: germinal vesicle, metaphase II, fertilized, 2-, 4-, 8-cell, morula, and blastocyst staged embryos were observed for each modification using confocal microscopy. The modifications studied were of three categories: (1) arginine or lysine methylation, which is often, but not always associated with long-term gene silencing, (2) lysine acetylation, which is generally associated with dynamic, transcriptional activation, and (3) serine phosphorylation which, in the case of histone H3, correlates closely with mitotic chromosome condensation as well as gene activation [Cheung et al., *Cell* 2000;103:263–267]. Observed staining was localized to the chromatin by co-staining with Sytox nucleic acid stain. Results showed that histone tail modification H3 lysine 4 methylation and H4 serine 1 phosphorylation were present throughout all stages tested with no apparent net changes in quantity or localization (Group A). Histone modifications H3 arginine 17 methylation, H4 arginine 3 methylation, and hyperacetylated H3 and H4, were present in immature oocytes, absent (or significantly diminished) in mature and fertilized ovum, and then became evident again from the pronuclear to blastocyst stage (Group B). These results suggest that clearance of the histone modifications found in Group B may be a necessary prerequisite for gene expression reprogramming during the maternal to embryonic transition.

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## IMPROVING THE VIABILITY OF WEAK PREMATURE CALVES WITH ACTH

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During the last week of gestation increased fetal cortisol secretion stimulates maturation of the organs. If calves are born before this glucocorticoid increase, their neonatal adaptation is impaired because of immature organ development. Synthetic glucocorticoids administered to cows to induce delivery pass the placenta and initiate the maturation of organs (imitating the fetal endogen cortisol increase) until labor occurs within 2–3 days. In order to develop a model for treatment of calves with impaired viability, for instance cloned calves, we created such weak calves by delivering them prematurely and then investigated whether treatment with ACTH improved the beneficial effects of glucocorticoid treatment of the pregnant mother. In 22 dairy cows, the calves were delivered by at  $255 \pm 1$  days of gestation (90% of term). For 13 of those, the cows were treated with 25 mg dexamethasone (DEX) 40 h before, and the remaining nine served as untreated controls (NOT). Seven calves delivered by Caesarean section close to term ( $275 \pm 1$  days) served as mature controls (TERM). Blood gas values and body temperature were recorded at 0, 0.5, 1, 1.5, 2.5, 3, 4, 5, 6, 10, 21, 22, 23 until 24 h of age, when the calves were euthanised and internal organs weighed. Two times daily, the calves were fed with 40 ml colostrum/kg bodyweight and treated with antibiotic (1 ml IV Baytril<sup>®</sup>, Bayer, Germany). Six of the DEX-calves were at 0, 10 and 21 h treated with ACTH (Synachthen<sup>®</sup>, Ciba-Geigy, 50 mg IV) while the other 23 did not get further treatment. Four of the nine NOT-calves died during the first hour post-partum. Blood gas and glucose values for the ACTH-calves were similar to the TERM-calves and different from the values in DEX-calves and in particular those in NOT-calves (Table 1). The body temperature in ACTH-calves was intermediate between the values in DEX-calves and TERM-calves and in the NOT-calves, the thermoregulation was compromised, since their body temperature was the lowest of all groups. The relative weight of thymus, which is reduced by progressing maturation, was lowered in the ACTH-calves towards those in TERM-calves, whereas the weight was higher in the NOT-calves than in any of the other groups. This experiment confirms that increased fetal cortisol level is essential to induce maturation of the organs and in premature calves DEX treatment of the mother initiates that maturation. The results suggest that post-natal ACTH treatment enhances the incipient maturation of the lungs, glucose homeostasis and thermoregulation. This treatment regimen may improve the survival rate of calves with impaired post-natal viability for instance weak calves born after transfer of nuclear transfer embryos.

Table 1

Values (means  $\pm$  S.E.M.) at 24 h after birth in ACTH-calves compared to DEX-calves (\*) and to NOT-calves (+)  $P < 0.05$

Treatment	DEX ( $n = 7$ )	ACTH ( $n = 6$ )	NOT ( $n = 5$ )	TERM ( $n = 7$ )
Acidity (pH)	$7.22 \pm 0.02$	$7.36 \pm 0.01^{*+}$	$7.18 \pm 0.05$	$7.32 \pm 0.01$
O <sub>2</sub> (ml/dl)	$3.1 \pm 0.8$	$8.9 \pm 0.3^{*+}$	$5.2 \pm 0.7$	$7.2 \pm 1.2$
CO <sub>2</sub> (mmHg)	$65.1 \pm 4.8$	$55.6 \pm 1.6^{+}$	$67.2 \pm 4.1$	$54.6 \pm 3.5$
Glucose (mM)	$1.7 \pm 0.3$	$3.7 \pm 0.3^{*+}$	$2.1 \pm 0.1$	$4.2 \pm 0.4$
Body temperature (°C)	$37.7 \pm 0.1$	$38.1 \pm 0.1^{*+}$	$36.4 \pm 0.5$	$38.6 \pm 0.2$
Thymus (g/kg)	$3.31 \pm 0.42$	$2.45 \pm 0.34^{+}$	$5.98 \pm 0.49$	$2.27 \pm 0.33$

EMBRYONIC DEVELOPMENT IN VIVO RELATIVE TO THE PREEVULATORY  
LH SURGE AFTER SUPEROVULATION WITH A CONTROLLED  
LH SURGE IN HEIFERS

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Data on preimplantation embryo development in vivo in relation to time are essential to improve in vitro embryo techniques. Therefore, it was investigated whether superovulation (SO) with a controlled LH surge could be used to obtain embryos at precisely defined times after ovulation. Before SO, all follicles  $\geq 5$  mm were ablated by puncture at Day 8 (estrus = Day 0) in synchronized, cyclic HF heifers, ( $n = 134$ ). To control the endogenous LH surge a Crestar ear implant (3 mg norgestomet; Intervet International B, Boxmeer, The Netherlands) was inserted at Day 9 without accompanying injection, and SO was started at Day 10 by oFSH i.m. (Ovagen ICP, Auckland, New Zealand) in decreasing doses twice daily during 4 days (total dose of 10 ml). Prostaglandin (22.5 mg PG; Prosolvin, Intervet) was administered i.m. concomitant with the fifth dose of FSH. Ear implants were removed 48–54 h after PG. GnRH (1.0 mg Fertagyl or 0.021 mg Receptal; Intervet) was administered i.m. immediately after removal of the implant. All heifers were inseminated 12–14 h after GnRH administration with two straws of semen from a bull of known high fertility. Embryonic structures were collected upon slaughter at defined times and qualified within 2 h by stereomicroscopy (Table 1). All heifers showed a similar, normal LH profile as analyzed by RIA of plasma samples taken every hour for 8 h after GnRH. The response to SO was  $16.4 \pm 11.4$  (S.D.) corpora lutea per animal counted after slaughter. In total  $11.1 \pm 7.2$  embryonic structures per animal were collected (recovery rate  $69.8 \pm 26.2\%$ ). The various developmental stages showed a maximum number at successive times during embryonic development: 1-cell at 40 h, 2–4 cell at 62 h, 5–8 cell at 72 h, 9–16 cell at 97 h, 9–16 cell at 108 h, 9–16 cell at 126 h, morula at 155 h and blastocyst at 191 h. It can be concluded that the SO procedure with a controlled LH surge can be effectively used to collect embryos at specific developmental stages. However, at distinct times a variable proportion (on average 40%) of the collected embryonic structures did not belong to one specific stage.

Table 1

Occurrence of different embryonic stages (number per heifer) at fixed times after a defined LH surge

h after LH	# heifers	# embryos	Deg	1 cell	2–4 cell	5–8 cell	9–16 cell	17–32 cell	Mor	Blast
40	1	16	0.0	16.0	0.0	0.0	0.0	0.0	0.0	0.0
62	13	149	0.0	7.3	4.2	0.0	0.0	0.0	0.0	0.0
72	34	359	2.6	ND	3.1	4.3	0.6	0.0	0.0	0.0
97	11	109	1.7	ND	0.7	2.0	5.5	0.0	0.0	0.0
108	13	167	6.5	ND	0.2	1.7	4.4	0.0	0.0	0.0
126	26	298	3.7	ND	0.6	1.2	2.8	2.4	0.8	0.0
155	10	135	2.6	ND	0.1	1.3	0.3	0.0	8.6	0.6
191	26	255	3.7	ND	0.1	0.0	0.0	0.0	2.7	3.2

# embryos: total number collected; deg: degenerated; mor: morula; blast: blastocyst; ND: not determined.

## Early Pregnancy/Pregnancy Recognition

### EFFECTS OF A SINGLE INTRAUTERINE INFUSION OF LIPOSOMAL RECOMBINANT BOVINE INTERFERON-TAU ON INTERESTROUS INTERVALS IN HEIFERS

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Interferon-tau (IFN- $\tau$ ) is produced in high amount by trophoblastic cells in conceptus during their elongation period and is mostly known for its antiluteolytic functions in ruminants. And daily intrauterine infusion of recombinant bovine IFN- $\tau$  (rboIFN- $\tau$ ) derived from baculovirus expression system can extend interestrous intervals in cyclic heifers [Geshi et al., *Theriogenology* 2001;55:325 (abstract)]. Liposomes have been proposed to improve the delivery of proteins because of their potential to function as sustained release systems. These sustained release systems may allow for reduced infusion frequency. The purpose of this study was to investigate the effect of a single intrauterine infusion of liposomal rboIFN- $\tau$  in heifers on their interestrous intervals.

Recombinant boIFN- $\tau$  derived from baculovirus expression system were prepared by the same methods previously described [Geshi et al., *Theriogenology* 2001;55:325 (abstract)]. Purity of rboIFN- $\tau$  was 87%, and its biological activity measured using a standard plaque reduction assay was  $5.0 \times 10^5$  units/mg. For a control sample, wild type viruses were used instead of recombinant viruses. COATSOME EL-A-01 (anionic liposomes with particle size of 100–300 nm, NOF Corporation, Tokyo, Japan) were used for preparation of liposomal rboIFN- $\tau$  and liposomal control. The total number of heifers in the study was three, and each received both treatments at an interval of 6 months. Their signs of estrus were monitored twice a day (dawn and dusk). Ovaries were examined using a real-time ultrasonic scanner on Day 6, 12, 15 and every third day thereafter until standing estrus (day of estrus = Day 0). The liposomal control or liposomal rboIFN- $\tau$  (2 mg) was introduced into the uterine horn ipsilateral to the corpus luteum by the cervical route using a sterile stainless steel embryo transfer gun on Day 13. All recipients received epidural anesthesia 5–10 min before intrauterine transfer. Data were analyzed by ANOVA using Statview software.

Heifers receiving liposomal rboIFN- $\tau$  in utero had estrous cycles of 27.5, 30.0 and 30.0 days. In contrast, the same heifers treated with control had estrous cycles of 20.5, 20.5 and 23.0 days, respectively. The mean interestrous interval was significantly prolonged ( $P < 0.05$ ) in the liposomal rboIFN- $\tau$  treated group compared with that in the control group ( $29.17 \pm 0.83$  days versus  $21.50 \pm 0.61$  days). These results indicate that a single intrauterine infusion of liposomal rboIFN- $\tau$  derived from baculovirus expression system in heifers can extend their interestrous intervals (corpus luteum lifespan).

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## CO-EXISTENCE OF FIBROBLAST GROWTH FACTOR-2 AND HEPARAN SULFATE PROTEOGLYCAN DURING THE FORMATION OF BOVINE PLACENTA

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It is well known that co-expression of fibroblast growth factor-2 (FGF-2) and extracellular matrix heparan sulfate proteoglycan (HSPG) plays an important role in the angiogenetic and morphogenetic processes [1]. To obtain insight into correlation of these two molecules for the formation of bovine placenta, we investigated the localization and the expression of FGF-2 and HSPG in the early bovine placenta. Furthermore, the expression of FGF-2 was analyzed regarding cultured trophoblastic (TB) cells in vitro. Bovine uterine samples were collected from Holstein cows on Day 60–80 of gestation ( $n = 6$ ). To study expression of FGF-2 mRNA, the placentae were separated into fetal cotyledonary and intercotyledonary, as well as maternal caruncular and intercaruncular tissues and their total RNAs were isolated. These RNAs were subjected to reverse transcription followed by polymerase chain reaction (RT-PCR). For immunohistochemical analysis, placental samples were divided into placentome and interplacentome, then frozen-sectioned. Anti-bovine FGF-2 and anti-mouse HSPG were used as primary antibodies, respectively. To investigate whether FGF-2 is trapped in HSPG, the sections were treated previously with or without Heparitinase I before the immunoreaction. Additionally, the expression of FGF-2 was investigated in an in vitro model. Bovine TB cells were obtained from blastocysts produced in vitro [2]. After 30–35th passage, TB cells were cultured on Matrigel. In the bovine placenta, heparanase expression has been detected in the maternal caruncle, but no significant expression in the intercaruncle [3]. Therefore, to recreate an environment similar to the placenta in vivo, Matrigel was treated previously with or without Heparitinase I that is an analogue of heparanase. Treated Matrigel was thought to be a placentome model, while non-treated Matrigel as an interplacentome model in this study. After 6 days of culture, the cells were collected and mRNA was isolated. Semi-quantitative PCR was performed to compare the amounts of expressed FGF-2 mRNA by using a LightCycler Instrument (Roche Diagnostics, Mannheim, Germany). Statistical analysis was carried out using Student's *t*-test. In RT-PCR, FGF-2 mRNAs were detected in all tissues examined. It suggests that FGF-2 may be produced in each tissue. In fact the immunohistochemical localization of FGF-2 was detected in the cells of fetal villi, uterine epithelium, stroma and the basement membrane (BM). HSPG also was distributed in the BM and the stroma. These two molecules were co-localized along with the BM and in the stroma restrictedly. Furthermore, the positive staining of FGF-2 in those areas was weak or abolished in sections previously treated by Heparitinase I. Thus, FGF-2 may be trapped by HSPG in the bovine placenta. Relative expression of FGF-2 mRNA in TB cells cultured on Heparitinase I-treated Matrigel (i.e. placentome model) was higher compared to non-treated Matrigel (i.e. interplacentome model) ( $0.338 \pm 0.075$  versus  $0.147 \pm 0.041$ ,  $P < 0.05$ ). These observations suggest that bovine TB cells located on placentome possibly produce more FGF-2 depending on the state of HSPG in the basement membrane and might participate in the formation of the placentome.

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## DETRIMENTAL EFFECTS OF PROSTAGLANDIN F<sub>2α</sub> ON PREIMPLANTATION DEVELOPMENT OF BOVINE EMBRYOS

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Elevated uterine concentrations of prostaglandin F<sub>2α</sub> (PGF) decreased embryonic development, quality, and pregnancy rates in cattle [Schrick et al., *Biol Reprod* 1993;49:617–621; Hockett ME et al., *J Anim Sci* 1998;76(Suppl 1):241 (abstract); Seals et al., *Prostaglandins* 1998;56:377–389]. Moreover, addition of PGF to culture media inhibited development of rabbit [Maurer and Beier, *J Reprod Fertil* 1976;48:33–41] and rat embryos [Breuel et al., *Biol Reprod* 1993;48(Suppl 1):173 (abstract)] and decreased hatching rates of in vivo-derived bovine morula embryos in culture [Scenna et al., *Theriogenology* 2002;53:512 (abstract)]. Furthermore, administration of flunixin meglumine (an inhibitor of prostaglandin synthesis) at the time of embryo transfer improved pregnancy rates in cattle [Schrick et al., *Theriogenology* 2001;55:370 (abstract)].

The objective of the present study was to determine effects of PGF addition to culture media on continued embryonic development of pre-compacted (in vitro-produced) bovine embryos. Pre-compacted (16- to 32-cell) embryos were washed four times, evaluated for quality, and randomly assigned during seven replicates to receive either: (1) control (KSOM media supplemented with 0.3% polyvinyl alcohol (KSOM-PVA);  $n = 168$ ); (2) PGF-1 (1 ng/ml PGF; Cayman Chemical Company; Ann Arbor, MI) in KSOM-PVA;  $n = 143$ ); (3) PGF-10 (10 ng/ml PGF in KSOM-PVA;  $n = 168$ ); (4) PGF-100 (100 ng/ml PGF in KSOM-PVA;  $n = 136$ ); or (5) PGE-5 (5 ng/ml PGE-2; Cayman Chemical Company; Ann Arbor, MI) in KSOM-PVA;  $n = 62$ ). Addition of PGE<sub>2</sub> to culture media served as a positive prostaglandin control [Gurevich et al., *Reprod Fertil Dev* 1993;5:281–283] and was performed in the final three replicates. Embryos were cultured in 5.5% CO<sub>2</sub>, 7% O<sub>2</sub>, and 87.5% N<sub>2</sub> at 38.5 °C until assessment of development 4 days later by experienced technicians uninformed of treatments. To validate concentrations of PGF during culture, media samples were collected at time of assessment of embryonic development and analyzed by RIA (control, 0.4 ng/ml; PGF-1, 1.3 ng/ml; PGF-10, 11 ng/ml; PGF-100, 103.5 ng/ml; and PGE-5, 0.3 ng/ml). An incomplete block design was used to determine effects of PGF on continued embryo development. ANOVA was performed using mixed models and contrasts were tested to identify differences across levels of PGF. Analyses were then validated with chi-square analysis.

Continued development of 16- to 32-cell bovine embryos to blastocyst was compromised by addition of PGF in culture media (control, 51.8%; PGF-1, 30.4%; PGF-10, 41.4%; PGF-100, 33.3%;  $P = 0.002$ ). In addition, culture of embryos in media containing 1 ng/ml of PGF resulted in reduced continued development compared to the addition of 10 ng/ml of PGF ( $P < 0.05$ ), with PGF-100 not differing from either PGF-1 or 10. These results would suggest that effects of PGF on continued development of embryos are not receptor-mediated events. Furthermore, development to blastocyst did not differ between control and PGE<sub>2</sub> treatments ( $P > 0.10$ ). In conclusion, this study indicates a direct negative effect of PGF on continued embryonic development of pre-compacted IVP bovine embryos.

## CHARACTERIZATION OF PROGESTERONE AND TGF- $\beta_1$ LEVELS OF NUCLEAR TRANSFER RECIPIENT IN KOREAN NATIVE CATTLE

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Nuclear transfer still has limited efficiency in terms of live calves born due to high fetal loss after embryo transfer. Progesterone and TGF- $\beta_1$  are known to be required in mammalian species for maintenance of pregnancy, and the functional withdrawal of progesterone in association with labor may account for reduced TGF- $\beta_1$  at parturition [Power et al., J Reprod Immunol 2002;56:19–28]. The objective of this study was to investigate the interaction between progesterone and TGF- $\beta_1$  in NT recipients during pregnancy in Korean Native cattle.

Tissue and blood samples were collected from eight NT recipients and five control (AI) of Korean Native cattle. Blood plasma was obtained intravenously at Day 50, 70, 110 and 150 of pregnancy and pre-parturition in both NT recipients and controls. All NT recipient and control animals were induced by cesarean section 1 day before anticipated parturition ( $283 \pm 2$  days) and the placentas were obtained. Cytoplasm of homogenized placenta tissues was collected following ultracentrifugation at  $105,000 \times g$  for 1.5 h. Plasma progesterone and TGF- $\beta_1$  concentrations were analyzed by Progesterone RIA kit (DPC, USA) and TGF- $\beta_1$  ELISA kit (Promega G7591, USA), respectively. Differences between both groups were analyzed by the Welch's *t*-test using Statview 4.0.

In control, progesterone levels in plasma increased gradually during gestation and decreased rapidly at parturition (mean  $\pm$  S.E.M.;  $0.10 \pm 0.01$  ng/ml). However, progesterone levels in plasma in NT recipients were not decreased at parturition ( $6.82 \pm 0.56$  ng/ml) and were similar to those at mid-pregnancy. Thus, the progesterone levels in NT recipients at parturition were higher than those in control ( $P < 0.01$ ). Cortisol levels after parturition were high in both cloned and control calves and decreased dramatically until 30 days (data not shown). Thus, the delivery signal sequence was not functional in NT recipients. TGF- $\beta_1$  levels in plasma and placenta tissue in NT recipients also were higher significantly ( $P < 0.01$ ) than those in the control group ( $107.52 \pm 10.73$  ng/ml versus  $72.22 \pm 7.79$  ng/ml in plasma;  $21.72 \pm 1.06$  ng/g versus  $15.76 \pm 1.14$  ng/g wet placenta tissue).

In conclusion, the delivery signal of the cloned fetus may not be transferred to the placenta of the recipient and may alter immune responses regulated by TGF- $\beta_1$ , etc. Further studies are warranted to examine the immunoregulatory mechanisms during the pre- and post-parturition periods of NT recipients.

## Embryo Culture

### DEVELOPMENT OF MAMMALIAN EMBRYOS AND STEM CELLS AT MICRO-GRAVITY CONDITIONS AT THE INTERNATIONAL SPACE STATION

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During the launch of the Soyuz rocket and Space Capsule No. 33 by Energia, the Russian Rocket and Space Corporation, from Baikonur, Kazakhstan, we sent mammalian embryos and stem cells for a period of 10 days in a portable incubator (Biotherm, Australia) with Mark Shuttleworth (the first African in space) to the International Space Station (ISS). One-cell stage embryos were collected from multi-ovulated local Tegeres sheep ewes, and the 1-cell mice embryos ( $n = 510$ ) were collected from F1-hybrid mice bred in Moscow, Russia. The sheep and mice embryos were collected at Baikonur, close to the Soyuz launching site. The embryonic stem cells were grown, using a standard procedure, at our laboratory at the University of Stellenbosch, South Africa. The embryos and stem cells were cultured at 38.5 °C at the ISS. During the flight the culture medium in the test tubes containing the embryos and the stem cells was changed every second to third day by injecting the medium from reservoir tubes into the specially adapted culture test tubes in the portable Biotherm incubator. The culture medium used was TCM-199 with 20% FBS, 1% antibiotics, and 0.5% of both essential and non-essential amino acids. A filter in the culture test tubes prevented the suspended experimental embryos and the stem cells from being flushed out of the culture tubes. The spent medium was collected into a plastic bag by means of a fixed tube. Control embryos and stem cell samples were cultured under similar conditions on earth for the duration of the space flight. After their return from outer space, the embryo and cell samples and the corresponding control samples were frozen immediately in liquid nitrogen, using ethylene glycol as a cryo-preservative. The control and space samples were then transported to the laboratory at the University of Stellenbosch for evaluation and further analysis. It was shown that the sheep and mouse embryos grown under micro-gravity conditions for the 10-day interval at the ISS developed past the hatching stage of the embryo, and an enlargement and outgrowth of the inner cell mass were observed. Of the sheep embryos cultured at the ISS, 37.5% reached the hatched blastocyst stage, while 25% of the control sheep embryos hatched. Stem cells from outer space developed more than one layer of cells, forming a multi-layered pattern.

EFFECT OF CITRATE AND TAURINE ON BOVINE EMBRYO DEVELOPMENT  
IN DIFFERENT CULTURE MEDIUM UNDER 5% CO<sub>2</sub> IN HUMIDIFIED AIRL.S.A. Camargo<sup>1</sup>, W.F. Sa<sup>1</sup>, J.H.M. Viana<sup>1</sup>, A.M. Ferreira<sup>1</sup>, A.A. Ramos<sup>2</sup>,  
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Sodium citrate has a metabolic role and is also a metal chelator when used at 0.5 mM in embryo culture under defined conditions in 5% O<sub>2</sub>. Taurine is an amino acid that may act as an antioxidant, improving embryo development in defined culture medium. However, in contrast to sodium citrate, better results have been observed under 20% O<sub>2</sub> or humidified air. Nevertheless, both can have a protective action against free radicals. The aim of this work was to verify if there is any synergistic effect between sodium citrate and taurine on in vitro embryo culture in defined, semi-defined and undefined culture medium under humidified 5% CO<sub>2</sub> in air. COCs, aspirated from bovine ovaries collected at slaughterhouse, were matured and fertilized in vitro and presumptive zygotes were randomly allocated into treatments according to the experimental design. The culture was performed under humidified 5% CO<sub>2</sub> in air at 38.8 °C. Data was analyzed for cleavage and blastocyst rate and total blastocyst cell number. The first experiment ( $n = 510$  presumptive zygotes, four replicates) evaluated the effect of 0.5 mM of sodium citrate and 3.0 mM taurine (C + T) in defined culture medium (CR2aa medium with 3 mg/ml of polyvinyl alcohol) and in semi-defined culture medium (CR2aa medium with 3 mg/ml of BSA). Controls were the same culture medium without citrate and taurine and also an undefined culture medium (CR2aa added with 10% of fetal calf serum). In second experiment, the presumptive zygotes ( $n = 580$ , six replicates) were cultured in undefined culture medium with citrate and taurine. Cleavage and blastocyst rates were compared by  $\chi^2$  analysis and total blastocyst cell number by Student's  $t$ -test. There was no effect ( $P > 0.05$ ) of citrate and taurine in defined or semi-defined medium on cleavage, blastocyst and total cell number (77.0%, 12.7% and  $57.49 \pm 7.7$ ; 80.1%, 16.6% and  $64.8 \pm 6.2$ ; 80.6%; 9.7% and  $49.7 \pm 7.2$ ; 85.7%, 14.3% and  $67.4 \pm 6.8$  for defined control group; defined C + T; semi-defined control group and semi-defined C + T, respectively). However, the undefined control group had a higher ( $P < 0.05$ ) blastocyst rate and total cell number (31.9% and  $104.9 \pm 5.1$ , respectively). In the second experiment, no effect was observed ( $P > 0.05$ ) for citrate and taurine on cleavage, blastocyst and total cell number (65.5%, 23.5% and  $108.5 \pm 7.5$ ; 66.9%, 24.0% and  $111.1 \pm 6.3$  for undefined control group and undefined C + T, respectively). Despite previous reports of an improvement in embryo development when citrate and taurine were used alone in defined medium, in this study no synergistic effect was observed when both were used together in defined, semi-defined and undefined medium. It may mean that citrate and taurine together are not fully able to protect against the detrimental effects of atmospheric O<sub>2</sub> concentration during embryo culture in defined and semi-defined medium. Supported by CNPq.

## EFFECT OF PYRROLINE-5-CARBOXYLATE AND GLUCOSE ON POST-COMPACTION DEVELOPMENT OF IN VITRO PRODUCED BOVINE EMBRYOS

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Under routine in vitro conditions, bovine embryos accumulate excessive lipids. One possible cause is inability of embryos to shunt sufficient glucose to the pentose phosphate pathway (ppp), so too much of this substrate is processed via glycolysis to lactate and other inappropriate metabolic end products. The aim of this experiment was to test the capability of pyrroline-5-carboxylate (p-5-c), an electron acceptor for NADPH, to improve the post-compaction development of bovine embryos, by shunting glucose from glycolysis to ppp.

Oocytes were retrieved from slaughterhouse ovaries and matured in TCM-199 plus 10% FCS and hormones and fertilized in F-CDM (chemically defined medium with nonessential amino acids, 0.5% FAF BSA and heparin) in 0.5 ml in wells at 38.5 °C in 5% CO<sub>2</sub> in air. After 18–21 h of co-culture, sperm and cumulus cells were removed, and presumptive zygotes placed in culture in CDM-1 (with nonessential amino acids and EDTA) for 2 days, and then CDM-2 (CDM-1 plus essential amino acids, but without EDTA) for 2 more days, all in 5% CO<sub>2</sub>/5% O<sub>2</sub>/90% N<sub>2</sub>. Compact morulae then were selected ( $n = 1440$ ) and randomly allocated (~10 per subclass) to 16 treatments (0, 9, 27 and 81 µM p-5-c × 0, 0.5, 2 and 8 mM glucose) for an additional 72 h in CDM-2; the experiment was replicated 4 and 5 times with semen from each of 2 bulls. At 36 and 72 h, embryos were evaluated for percentage blastocysts, stage of development, morphological quality (scale of 1–4), degree of lightness as a presumptive measure of lipid accumulation (subjective scale of 1–5), and quality of the inner cell mass (scale of 1–4). Embryos then were fixed and stained to count cells. Wells were coded to make evaluations blind. Data were analyzed by factorial ANOVA, with factors p-5-c (4), glucose (4) and bulls (2).

One of the bulls produced more embryos that also were more advanced at the end of the treatments than the other ( $P < 0.01$ ); otherwise, sperm from both bulls behaved similarly. No main effects were observed for the different levels of p-5-c and glucose evaluated ( $P > 0.05$ ), but a tendency was observed for the p-5-c × glucose interaction for embryo quality at 36 h ( $P = 0.07$ ). Embryos cultured in 8 mM glucose tended to be of better quality at all levels of p-5-c compared with no p-5-c. Although no beneficial effects in post-compaction embryo development can be attributed to p-5-c in culture medium under the conditions of this experiment, a possible protective effect of p-5-c could be inferred when embryos were challenged with high glucose levels (8 mM) in the culture medium.

## EVALUATION OF DIFFERENT METHODS FOR IN VITRO CULTURE OF A SINGLE BOVINE EMBRYO

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It has been shown that in vitro development of bovine embryos is improved when they are cultured in groups. However, with widespread use of the OPU and in vitro production of embryos for commercial purpose, it is common to retrieve only one oocyte from a donor, which requires an individual embryo culture system. In the present study, we evaluate the embryo development when they were culture individually in the well on well system (WOW) [Vajta et al., Mol Reprod Develop 2000;55:256–264] or in a microdrop of medium. In the first experiment, 345 bovine oocytes were matured and fertilized in vitro, and presumptive zygotes were randomly distributed into two treatments (4 replicates): culture of 25–30 zygotes in a 400 µl drop of medium from Days 1–8 (T1); culture of 1 zygote in each well of the 25 made on a 4-well dish (WOW), covered with 400 µl of medium (T2). The blastocyst rate was evaluated on D7 and the hatching rate on D8. In the second experiment, after IVM and IVF, presumptive zygotes were randomly distributed into 3 treatments. Twenty-five zygotes were cultured in 400 µl of medium from Day 1 to Day 8 (T1-control); 25 zygotes were cultured individually in 25 WOW-wells, each covered with a 20-µl drop of medium (T2); 25 zygotes were cultured individually in drops of 20 µl of medium (T3). The embryos were evaluated on D6, D7 and D8. In each replicate a sample of embryos at the blastocyst stage was removed from all treatments, fixed and stained with orcein, for cell number determination. For all groups, cumulus cells that remained following IVF were maintained during embryo culture. The culture medium was SOFaa in an atmosphere of 5% CO<sub>2</sub> in air. Data were analyzed by  $\chi^2$  test. In the first experiment no differences were observed ( $P > 0.05$ ) in cleavage (72.5 and 71.2%), blastocyst at Day 7 (38.0 and 39.3%) and hatching at Day 8 (46.1 and 52.6%), for the control (T1) and for the WOW system (T2), respectively. The results of embryo development for the second experiment are presented in Table 1. Although no statistical differences were observed among treatments, the zygotes that were culture individually in a 20-µl drop showed lower blastocyst at Day 7 and lower hatching rates than the other groups. The percentages of embryos that showed less than 64 cells, from 64 to 120 cells and more than 120 cells were, respectively, 0, 60 and 40% for T1; 18.2%, 45.4%, 36.4% for T2 and 50, 20 and 30% for T3. The results suggest that individual zygotes can be cultured in the WOW system in the presence of cumulus cells and high O<sub>2</sub> tension with no detrimental effect in embryo development. The culture of individual zygotes in a small drop showed a tendency to produce less embryos on Day 7, lower hatching rate and higher frequency of embryos with low number of cells, than the other methods. However, additional studies are needed to evaluate the pregnancy rate of the embryos produced in those systems.

Table 1

Development of bovine embryos cultured in group of 25 (T1) or individually in WOW system (T2) and in a small drop (T3) of SOFaa medium from Days 1–8

Treatment	Number of oocyte	Cleavage (%)	Blastocyst (%, D6)	Blastocyst (%, D7)	Blastocyst (%, D8)	*Hatched/blastocyst (%)
T1-400 µl	85	69 (81.2)	25 (29.4)	34 (40.0)	39 (45.9)	13/24 (54.2)
T2-WOW + 20 µl	85	70 (82.4)	20 (23.5)	32 (37.6)	33 (38.8)	7/22 (31.8)
T3-20 µl drop	85	67 (78.8)	19 (22.4)	28 (32.9)	32 (37.6)	6/22 (27.3)

Differences in the same column were not significant ( $P > 0.05$ ) by  $\chi^2$  test. \*The hatching rate was calculated using the number of the hatched blastocyst over the number of total blastocysts that remained in culture after the samples were taken out for cell number evaluation.

**BOVINE TROPHECTODERM CELL DIFFERENTIATION AFTER CULTURE  
IN SIMPLE MEDIUM WITH REDUCED OSMOLARITY**

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Intracellular osmolality plays an important role during in vitro embryo development [Thompson, *Theriogenology* 1996;45:27–40]. Culture media (CM) for mammalian embryos routinely use NaCl for adjusting osmolality and maintaining ionic balance [Lim et al., *Theriogenology* 1994;42:421–432]. Reduced NaCl concentration in completely defined medium is beneficial for bovine embryo development [Roh et al., *Theriogenology* 1999;51:252]. The present work studies the effect of osmotic pressure in a defined or semi-defined CM on bovine embryo development and trophectoderm cell differentiation. Cumulus–oocyte complexes were matured for 24 h in TCM199-HNaCO<sub>3</sub> with 10% FCS, FSH, LH, E<sub>2</sub> and cysteamine, fertilized with swim-up separated sperm and cultured in Synthetic Oviduct Fluid (SOF) [Holm et al., *Theriogenology* 1999;52:683–700] under mineral oil. Two NaCl stocks (107.6 and 88 mM) were used to obtain SOF with high (275–285 mOsm) and low (240–250 mOsm) osmolality. Macromolecules (MMs) used to supplement media were BSA (3 mg/ml) or PVA (4 mg/ml). Zygotes were cultured up to Day 9 at 39 °C, 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. As a criterion of embryo quality, nuclei from trophectoderm (TE) and inner cell mass (ICM) were counted in Day 8 expanded and hatched blastocysts [an Soom et al., *Mol Reprod Dev* 1996;45:171–182]. Data were analyzed by ANOVA and Duncan's multiple range test and presented as means ± S.E.M. Low osmolality improved development in presence of both BSA and PVA. A reduction in total cell counts ( $P < 0.08$ ), which can be accounted for lower TE cell numbers, was noted in BSA with high osmolality. As compared to BSA, PVA under low osmolality increased ICM numbers and ICM/total cell rate, although TE and total cell counts decreased ( $133.1 \pm 14.4$  for PVA and  $109.6 \pm 9.5$  for BSA;  $P < 0.08$ ) (data not shown in table). No one of these effects could be observed under high osmolality. Low osmolality is beneficial to development, and combinations of MM and osmolality influence TE differentiation. More research is needed to determine the role of CM on the cellular differentiation process. Supported by AGL2001–0379.

Embryo development and cell number in SOF with high (275–285 mOsm) or low (240–250 mOsm) osmolality and supplemented with BSA or PVA.

Culture medium

Osmotic pressure	MM	N	% Blastocysts		N	Cell counts		
			Day 7	Day 8		ICM	TE	ICM/total
High	BSA	208	16.32 ± 3.3 <sup>bg</sup>	21.13 ± 3.9 <sup>b</sup>	25	35.3 ± 3.4	73.8 ± 6.6 <sup>x</sup>	33.4 ± 3.4 <sup>a</sup>
High	PVA	205	6.36 ± 3.7 <sup>cyh</sup>	11.99 ± 5.2 <sup>by</sup>	17	26.3 ± 3.2 <sup>d</sup>	81.4 ± 6.3 <sup>a</sup>	24.0 ± 3.2
Low	BSA	206	27.47 ± 3.2 <sup>axf</sup>	32.35 ± 4.2 <sup>ax</sup>	14	24.9 ± 3.1 <sup>a</sup>	110.0 ± 14.8 <sup>by</sup>	20.1 ± 3.6 <sup>b</sup>
Low	PVA	205	14.24 ± 3.1 <sup>yg</sup>	18.09 ± 3.1 <sup>b</sup>	9	37.3 ± 5.9 <sup>bc</sup>	72.3 ± 9.5 <sup>x</sup>	34.4 ± 4.2 <sup>a</sup>

Replicates: 10 and 6 for embryo development and cell numbers respectively. Different superscripts within columns differ significantly: <sup>a,b,c</sup> ( $P < 0.05$ ); <sup>x,y</sup> ( $P < 0.01$ ); <sup>d,e</sup> ( $P < 0.08$ ); <sup>f,g,h</sup> ( $P < 0.09$ ).

IMPROVED DEVELOPMENT AFTER TREATMENT OF DONOR SOMATIC CELLS AND CLONE EMBRYOS WITH HEMOGLOBIN AND/OR  $\beta$ -MERCAPTOETHANOLW.S. Hwang<sup>1,2</sup>, E.S. Park<sup>1</sup>, G. Jang<sup>2</sup>, S.K. Kang<sup>2</sup>, B.C. Lee<sup>2</sup>, and J.M. Lim<sup>1</sup><sup>1</sup>School of Agricultural Biotechnology, <sup>2</sup>College of Veterinary Medicine, Seoul National University, Suwon, South Korea

We previously suggested that apoptosis is one of the major causes for developmental retardation found in somatic cell nuclear transfer (SCNT) embryos. Based on this hypothesis, we further hypothesized that development of cloned embryos can be improved by treatment with apoptosis inhibitors. This experiment was subsequently conducted to assess whether treatment of donor somatic cells or reconstructed embryos with putative apoptosis inhibitors could promote preimplantation development of clone embryos. IVM and IVF of oocytes, SCNT and activation, and IVC of embryos were performed by our standard procedure [Cho et al., *Theriogenology* 2002;57:1819–1828] and ear fibroblasts subcultured 3–5 times were employed as a donor somatic cell of SCNT. Hemoglobin (Hb, 1  $\mu$ g/ml), a nitric oxide scavenger, and  $\beta$ -mercaptoethanol (Me, 10  $\mu$ M), an antioxidant, were used as apoptosis inhibitors. Morphogenetic evaluation monitoring preimplantation development, apoptosis detection by a terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling assay, counting inner cell mass (ICM) cells and trophectodermal (TE) cells in blastocysts by immunostaining were employed as experimental parameters. A generalized linear model consisting of ANOVA and the least squares method in the SAS program was used for statistical analysis. In Experiment 1, thawed ear fibroblasts of a donor somatic cell were cultured and serum-starved in DMEM supplemented with Hb and/or Me. Apoptosis index of SCNT embryos showing the ratio of apoptotic to normal blastomere number was significantly ( $P < 0.05$ ) decreased by ME treatment ( $0.058 \pm 0.005$  versus  $0.083 \pm 0.006$ ). In this treatment, significantly improved blastocyst formation (41 versus 34%) with increasing blastomere ( $131 \pm 1.3$  versus  $123 \pm 1.3$  cells/blastocyst) and ICM cell ( $31.9 \pm 0.9$  versus  $27.6 \pm 0.9$  cells/blastocyst) numbers and ICM to TE cell ratio ( $0.24 \pm 0.01$  versus  $0.22 \pm 0.01$ ) was achieved compared with no treatment. In Experiment 2, embryos reconstructed with ME-treated ear fibroblasts were further cultured in mSOF medium, to which Hb and/or ME were added. Culture of SCNT embryos in ME + Hb-containing medium significantly reduced apoptotic index ( $0.085 \pm 0.005$  to  $0.069 \pm 0.004$ ) and increased all parameters of cell number ( $123 \pm 1.3$ – $155 \pm 1.4$ ,  $27 \pm 0.5$  to  $41 \pm 0.6$ ,  $98 \pm 1.0$  to  $114 \pm 1.0$  and  $0.22 \pm 0.01$  to  $0.26 \pm 0.01$  in total blastomere, ICM, TE and ICM to TE ratio, respectively). In conclusion, we confirmed our hypothesis that apoptosis occurs in developing SCNT embryos and causes developmental retardation. Furthermore, it was demonstrated that preimplantation development could be improved by the treatment of donor somatic cell and SCNT embryos with ME and ME + Hb, respectively, which significantly reduced blastomere apoptosis.

## EFFECTS OF INTERLEUKIN-11 (RHIL-11) ON THE DEVELOPMENT, SEX RATIO AND CELL NUMBER OF HEAT STRESSED BOVINE EMBRYOS

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Interleukin-11 (IL-11) is a pleiotropic cytokine that has been thought to play a role in the maintenance of haematopoietic system, in the induction of acute phase proteins and to have a protective effect on the gastrointestinal mucosa. Expression of IL-11 has been demonstrated in murine adult & embryonic tissues in early post-implantation development of rats [Davidson et al., Stem Cells 1997;15:119–124]. The aim of the present study was to investigate the role of recombinant human IL-11 (rhIL-11) added to bovine embryo culture system to determine whether this cytokine protects bovine embryos from heat stress-related cellular damage in vitro. Cumulus–oocyte complexes (COCs) obtained after follicular aspiration of ovaries were matured in TCM-199 for 24 h and expanded oocytes were inseminated with frozen thawed sperm. After 18 h of co-incubation the presumptive zygotes were cultured in S1 media and sub-grouped as following: (1) Control: embryos cultured in S1/S2 medium for a period of 8 days, (2) Heat stressed: Presumptive zygotes cultured in S1 medium for 72 h, heat stressed at 42 °C for 3 h followed by culture in S2 medium for 8 days, (3) IL-11 group (C + IL): Embryos were cultured in S1/S2 medium supplemented with 1500 IU of rhIL-11 for 8 days without heat stress, (4) IL-11 (1500 IU) + heat stress (H + IL-1500): embryos were cultured for 72 h in S1 supplemented with 1500 IU rhIL-11, heat stressed at 42 °C for 3 h followed by transfer into S2 media + 1500 IU of rhIL-11. The blastocysts harvested at the end of the culture period (8 days) from each subgroup were differentially stained to assess total cell number (TCN) and inner cell mass count (ICM) before sexing them by PCR amplification. Results of the study (Table 1) showed a substantial reduction in blastocyst yield (about half) and skewing of the sex ratio in favor of females (0.43) in the heat stressed group compared to the control group. While there was no increase in blastocyst yield in IL-11 supplemented group (C + IL), the IL-11 supplemented and heat stressed group (H + IL-1500) yielded blastocyst rate comparable to that of control group. The TCN and ICM assessment revealed a significant reduction in cell allocation to both lineages of heat stressed group compared to the control. Although the sex ratio of the embryos of heat stressed and IL-11 supplemented groups remained close to 1:1, the cell numbers were significantly reduced in these groups compared to control groups and the viability of heat stressed male embryos was significantly improved in IL-11 supplemented group. Taken together the results suggest that IL-11 may have an embryo protective effect under heat stress conditions. The reversal of the skewed sex ratio in heat stressed embryos in the presence of IL-11 indicates that IL-11 offers protection to male bovine embryos that are more vulnerable to environmental insults as imposed by elevated temperature in vitro.

Table 1

Impact of heat stress with and without IL-11 supplementation, on blastocyst yield (Blast), total cell number (TCN), cells allocated to inner cell mass (ICM) and male female ratio (M:F) in blastocysts

Group	Total oocytes	Cleaved (%)	% Blasts	M:F ratio	TCN-male	ICM-male	TCN-female	ICM-female
Control	598	430 (71.9)	22.1	1.0	148.5 ± 11.3 <sup>a</sup>	40.5 ± 4.9 <sup>a</sup>	139.2 ± 11.6 <sup>a</sup>	37.6 ± 5.6 <sup>a</sup>
Heat stress	576	420 (72.9)	10.9	0.43	116.7 ± 11.4 <sup>b</sup>	28.1 ± 3.3 <sup>b</sup>	111.5 ± 10.6 <sup>b</sup>	28.6 ± 2.6 <sup>b</sup>
Control + IL	481	342 (71.1)	21.9	1.0	146.8 ± 10.6 <sup>a</sup>	38.5 ± 3.6 <sup>a</sup>	130.4 ± 11.7 <sup>a</sup>	34.4 ± 5.7 <sup>a</sup>
H + IL 1500 IU	690	489 (70.8)	20.1	0.91	114.8 ± 13.9 <sup>b</sup>	28.6 ± 3.7 <sup>b</sup>	115.8 ± 9.5 <sup>b</sup>	28.8 ± 2.7 <sup>b</sup>

<sup>a,b</sup>Different superscripts in same column differ significantly ( $P < 0.05$ ).

## ASPARTATE AND LACTATE NEGATE THE REQUIREMENT FOR PYRUVATE FOR THE FIRST CLEAVAGE DIVISION IN THE MOUSE

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Mouse zygotes appear to have a requirement for pyruvate (P) to complete the first cleavage division. In contrast, 2-cell embryos can use lactate (L) as an energy source. Regulation of this switch in substrate utilization in embryos is proposed to be by the redox equivalent mitochondrial shuttle, the malate-aspartate shuttle (MAS). Initial experiments have identified that the MAS is active in 2-cell mouse embryos enabling lactate to be used as a substrate but has very low or no activity in 1-cell embryos. The MAS is composed of four enzymes which transfer the reducing equivalent NADH across the mitochondrial membrane, cytoplasmic aspartate aminotransferase (cAAT), mitochondrial aspartate aminotransferase (mAAT), cytoplasmic malate dehydrogenase (cMDH) and mitochondrial malate dehydrogenase (mMDH). The aim of this study was to investigate how MAS is regulated in 1-cell and 2-cell mouse embryo by examining the presence and activity of these four enzymes.

Mouse zygotes and 2-cell embryos were collected from F1 hybrid (C57BL/6 × CBA) mice. For genetic analysis reverse transcription-PCR methods were used to establish the presence of the transcripts of the four enzymes. Amplified cDNA was analyzed on a 1.6% agarose gel and products sequenced to confirm the product identity. For analysis of enzyme activity embryos were separated into mitochondrial and cytoplasmic compartments using a digitonin method. LDH and  $\alpha$ -ketoglutarate dehydrogenase were used to confirm the purity of the compartments. Kinetics of enzyme activity were analyzed using ultramicrofluorescence. For embryo culture, zygotes were collected in substrate-free MOPS-buffered medium and cultured in a simple medium lacking amino acids and EDTA with the appropriate carbohydrates. Development to the blastocyst stage and cell numbers were assessed after 96 h of culture. Differences between treatments were analyzed using generalized linear modeling or one-way ANOVA and Bonferroni's post-test for multiple comparisons.

Transcripts for all four MAS enzymes were present in both 1-cell and 2-cell embryos. Therefore, control of MAS activity was not through gene expression. Analysis of enzyme activity revealed that activity of all 4 enzymes could be detected in both 1-cell and 2-cell embryos. The  $K_m$  of cAAT for aspartate was 3.14 mM. This concentration is above the levels of aspartate in embryo culture media (0.1 mM). To examine if this  $K_m$  was a controlling mechanism of shuttle activity, zygotes were cultured in media containing either lactate as the sole substrate (negative control), L and P (positive control), L with 10 mM aspartate or L with 10 mM aspartate and 0.2 mM aminooxyacetate (AOA, an inhibitor of the shuttle). In the medium with L as the sole substrate 4.0% of zygotes developed to the blastocyst stage with cell numbers of  $11.5 \pm 2.0$ , compared to 97.2% ( $P < 0.01$ ) blastocyst development in the presence of L and P and cell numbers of  $78.3 \pm 5.6$  ( $P < 0.01$ ). Addition of aspartate to the medium containing L resulted in 78.2% of zygotes developing to the blastocyst stage (cell numbers  $70.1 \pm 3.8$ ), which reduced to 21.6% ( $P < 0.01$ ) and blastocyst cell numbers of  $55.9 \pm 6.1$  ( $P < 0.05$ ) in the presence of AOA.

The malate-aspartate shuttle appears regulated by the cytoplasmic concentration of aspartate due to the high  $K_m$  of the enzyme cAAT. The inability of mouse zygotes to use L can be overcome by increasing the concentration of aspartate in the culture medium.

## MICROSENSOR OXYGEN MEASUREMENTS AROUND IN-VITRO DEVELOPING CATTLE EMBRYOS: PRELIMINARY OBSERVATIONS

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The actual gas concentration around an in-vitro developing embryo is not well defined during culture and during handling outside the incubator. Only few studies have addressed this problem, primarily due to the lack of suitable technology. The aim of the present study was to test a non-invasive and rapid oxygen microsensor system on in-vitro produced day 7–8 cattle blastocysts [Holm et al., *Theriogenology* 1999;52:683–700] in SOF-medium covered by paraffin oil and at the bottom of 4-well dishes or in WOW-microwells [Vajta et al., *Mol Reprod Dev* 2000;55:256–264]. They were placed in a thermally-controlled microscope stage box with constant flow of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> at 38 °C and 100% humidity. Oxygen recordings were made with a Unisense oxygen microsensor (<http://www.unisense.com>) with the tip (approximately 5 µm in diameter) placed against the embryo's zona pellucida. The sensor was moved by a motorized, PC controlled micro-manipulator using Unisense Profix software, resulting in point measurements and oxygen concentration profiles. Exp. 1: a dish with SOF-medium under oil was first exposed to 100% N<sub>2</sub> for 3 days to ensure complete anoxia, then in atmospheric air (21% O<sub>2</sub>) to investigate equilibration with the overlying atmosphere. Repeated oxygen measurements over 900 min showed that 90% equilibrium was first reached after approx. 9 h. An embryo's exposure to oxygen can therefore be quite variable if the controlled atmosphere around the dish is disturbed during the in-vitro culture period. Exp. 2: to show the effect of grouping, the oxygen partial pressure was recorded at different points in a group of 30 embryos. The partial pressure in this group's middle was 6.1% atmospheric saturation (1.2% absolute O<sub>2</sub>), but increased to about 12% at the group's periphery and to 25–27% away from the group (approx. 5% absolute O<sub>2</sub>). The effect of this difference for the resulting embryo quality is uncertain. Exp. 3: the oxygen partial pressure was measured around two single embryos placed in a WOW or in a normal well. In the WOW the partial pressure of 11.9% atmospheric saturation below (2.5% absolute saturation) and 16% above the embryo was considerably lower compared to the embryo on the surface (14.5 and 18%, respectively). Therefore, WOWs influence the diffusion space around the embryo, being important for its exposure to oxygen. Exp. 4: the oxygen partial pressure was measured at the surface of two single embryos with good or poor morphology. The partial pressure was 15% of atmospheric saturation (3% absolute oxygen) for the good quality embryo, while it was clearly higher (20.5%) for the poor quality embryo, perhaps due to its reduced oxygen consumption rate. The results demonstrate that (1) the microenvironment around an in-vitro developing embryo may vary considerably according to its respiration and surrounding diffusion space even though the oxygen partial pressure in the overlying gas atmosphere is kept constant; (2) embryo culture conditions can be further optimized; (3) the oxygen microsensor system could be a method for quality assessment of single embryos.

A COMBINED MORPHOLOGICAL EVALUATION OF IN VITRO PRODUCED  
BOVINE EMBRYOS AT DAYS 6 AND 7 OF CULTURE, ENHANCES  
DISCRIMINATION IN EMBRYO QUALITY WITH  
REGARD TO PREGNANCY RATE

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The need to judge the quality of embryos with regard to the establishment of pregnancies following embryo transfer (ET) is obvious. The simplest but also the most subjective way to judge an embryo prior to transfer is to evaluate its morphology. The bovine in vitro embryo production procedure gives the opportunity to use additional embryonic developmental information to come to a final embryo classification. The aim of this study was to investigate the effect of a combined morphological embryo evaluation at Days 6 and 7 (Day 6–7) on the discrimination in embryo quality with regard to the establishment of pregnancies. Immature cumulus–oocyte complexes (COCs) were obtained twice weekly by ultrasonic guided transvaginal oocyte collection. COCs were matured in TCM199/FCS/LH/FSH, fertilized with frozen–thawed Percoll-separated semen and subsequently cultured for 7 days in SOFaaBSA. At Day 6, embryos were evaluated with respect to the chance of becoming a transferable embryo at Day 7. Embryos were classified as A (sure; morula 1 (IETS standard) and early blastocyst 1) or B (doubtful; early morulae 1, 2 and morulae/early blastocysts 2). At Day 7, morulae up to expanded blastocysts, classified as 1 or 2, were transferred either fresh or frozen/thawed. The total experiment covered one year. Results were analyzed by logistic regression. From 295 out of 1427 embryos produced, the relation between embryo quality at Day 6 and Day 7 could be assured, i.e. only one class of embryo was present at Day 6. In a retrospective view, the level of discrimination of a Day 7 and Day 6 evaluation was similar for both fresh and frozen embryos. However, when the information of Day 6 and Day 7 is combined, resulting in four new quality grades (A-1, A-2, B-1 and B-2), level of discrimination increases (see Table 1).

These results show that additional in vitro embryonic developmental information by morphological examination at Day 6, can be used to enhance discrimination in embryo quality evaluation at the day of transfer. It must be mentioned that the discrimination level at Day 6 is based on only those embryos transferred at Day 7. In general, all class A embryos at Day 6 become transferable at Day 7. However, only half of the class B embryos present at Day 6 become transferable at day 7. So if all class B embryos present at Day 6 would have been transferred at Day 7, the pregnancy rate of this group would probably have been much lower. To make a Day 6–7 evaluation possible for each embryo produced, class A embryos should be put in separate wells/drops at Day 6 upon evaluation.

Table 1  
Pregnancy rate of fresh IVP embryos

Day 7			Day 6			Day 6–7		
Quality	<i>n</i>	Pregnancy (%)	Quality	<i>n</i>	Pregnancy (%)	Quality	<i>n</i>	Pregnancy (%)
1	135	51.9	A	11	53.2	A-1	85	56.4 <sup>a</sup>
2	55	39.9	B	79	40.4	A-2	26	43.1
						B-1	50	44.0
						B-2		30.9 <sup>b</sup>

Effect of embryo morphology evaluation at Day 6, Day 7 or Days 6–7 on discrimination of embryo quality.

<sup>a,b</sup>Superscripts in column differ significantly ( $P < 0.03$ ).

## EFFECT OF VITAMIN B12 ON THE IN VITRO MATURATION AND DEVELOPMENT OF OVINE OOCYTES

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Vitamin B12 is a co-factor in the enzyme methionine synthase which plays a key role in the cellular metabolism of methionine and folate. Deficiency of vitamin B12 may reduce DNA synthesis via alterations in the availability of thymidine, and may also impact on DNA methylation. Culture media such as Medium 199 (M199) and Synthetic Oviduct Fluid (SOF) do not contain vitamin B12 but this vitamin may be present as a contaminant when bovine serum albumin (BSA) is used as a protein source. The aim of the present study was to determine the effect of presence or absence of vitamin B12 on the in vitro maturation of ovine oocytes, and on development to the blastocyst stage. Ovine oocytes were aspirated from abattoir-derived ovaries and those with evenly granulated cytoplasm and >3 layers of cumulus cells were matured, fertilized and cultured at 38.5 °C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub>. Oocytes were matured for 24 h in supplemented M199 prior to co-incubation for 18 h with frozen-thawed spermatozoa from a single ejaculate (1 × 10<sup>6</sup> live sperm ml<sup>-1</sup>). Presumptive zygotes were cultured for 7 days in supplemented SOF and blastocysts produced were fixed and stained (Hoechst 33258) to count cell numbers. Maturation and culture media contained 0.4% w/v standard Fraction V BSA (vBSA) (Sigma A3311) or 0.4% w/v vitamin B12-deficient BSA (dBSA) (Sigma A3902) or the same albumin plus 600 pg ml<sup>-1</sup> vitamin B12 (dBSA + B12). Treatments were replicated seven times. Data were tested by ANOVA and binomial analysis (Generalized Linear Model) in Genstat. There was no effect of treatment on the percentage of oocytes that cleaved, or on the mean (±S.E.M.) diameters or cell numbers of the blastocysts produced (Table 1). Almost twice as many blastocysts were produced in media containing Fraction BSA rather than vitamin B12-deficient BSA (*P* < 0.001). Addition of vitamin B12 to media containing the vitamin B12-deficient BSA did not result in a significant improvement in blastocyst production. Results of this preliminary study therefore suggest that (i) the processing of BSA to remove contaminating vitamin B12 also removed additional factors that are beneficial to zygote development, and (ii) the early cleavage stage ovine zygotes contained adequate endogenous vitamin B12 and (or) were unable to take up this vitamin from the culture environment.

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Table 1  
In vitro development of ovine oocytes and zygotes in media with contrasting BSA and vitamin B12 content

	vBSA	dBSA	dBSA + B12
No. of oocytes matured/fertilized	330	330	330
No. of oocytes cleaved (% of matured)	309 (94%)	283 (86%)	285 (86%)
No. of blastocysts Days 6 + 7 (% of cleaved)	87 (28%) <sup>A</sup>	47 (17%) <sup>B</sup>	60 (21%) <sup>b</sup>
Mean ± S.E.M. blastocyst diameter (μ)	194 ± 2.0	194 ± 2.9	195 ± 2.6
Mean ± S.E.M. blastocyst cell number	83 ± 3.6	80 ± 4.4	80 ± 3.8

Means with different superscripts are significantly different: (AB), *P* < 0.001; (Ab), *P* < 0.05.

## BOVINE BLASTOCYST PRODUCTION BY AN INDIVIDUAL OOCYTE CULTURE DURING IVM/IVF/IVC USING A CHEMICALLY DEFINED MEDIUM

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An individual-IVP system in which the oocyte or embryo is cultured individually may be helpful to clarify the relationship between follicular environment and developmental competence. There are several studies reporting individual-IVP systems, but the addition of serum or BSA, or a co-culture system was used. The present study was performed to establish an individual bovine oocyte culture throughout IVP using a chemically defined simple medium (mSOFaa containing 0.1% polyvinyl alcohol: PVA). Effects of epidermal growth factor (EGF) during oocyte maturation on IVM/IVF/IVC were also examined. Oocytes were collected by aspirating follicles of 2–8 mm in diameter and matured for 24 h at 39 °C in 5% CO<sub>2</sub>, in mSOFaa containing 0.1% PVA (control medium) or supplemented with 1, 10, 50 ng/ml of EGF, and two further groups (TCM199 and mSOFaa supplemented with 10% FCS) were also included. All maturation media were added 0.02 IU/ml of porcine FSH (Antrin<sup>TM</sup>, Denka Chemical Co., Ltd., Kawasaki, Japan) and 1 µg/ml estradiol-17β. After IVM, oocytes were inseminated with frozen-thawed spermatozoa for 22 h in the control medium containing 20 µM penicillamine, 10 µM hypotaurine and 2 µM epinephrine and 10 IU/ml heparin. After insemination, oocytes were denuded from cumulus cells and were cultured in modified SOFaa (with inositol and citrate) supplemented with 0.1% PVA. In the individual-IVP system, IVM/IVF/IVC were performed in a drop of 10 µl, and drops of 50/50/25 µl were used in the group-IVP system, respectively. Meiotic status of bovine oocytes after IVM, the incidence of fertilization after IVF and embryonic development to the blastocyst stage 8 days after IVF were examined, as were cell number of each blastocyst developed in group- and individual-IVP systems. In the group-IVP system, the proportion of matured oocytes in the control medium (62.7 ± 5.0%) was significantly ( $P < 0.05$ ) lower than other treatments (73.4–81.4%), and in the individual-IVP system, the maturation rate in the control medium supplemented with 1 ng/ml EGF (76.2 ± 5.4%) was significantly ( $P < 0.05$ ) higher than that in the control medium without EGF (57.1 ± 14.4%). The addition of EGF did not affect the proportions of penetrated and normally fertilized oocytes in either individual- or group-IVP system. In the group-IVP system, no significant difference was found in the rate of blastocyst development among all treatments, whereas in the individual-IVP system, the control medium supplemented with 10 ng/ml EGF resulted in a significantly ( $P < 0.05$ ) higher blastocyst rate (20.0 ± 5.2%) than that in the control medium without EGF (6.2 ± 3.5%) (Table 1).

These results indicate that bovine oocytes can develop to blastocysts in an individual-IVP system using a chemically defined medium. The effect of EGF during oocyte maturation differed depending on whether embryos were cultured individually or in a group.

Table 1  
Effect of adding EGF to IVM media on development to the blastocyst stage of bovine oocytes cultured by an individual-IVP system

IVM treatment medium	Protein EGF (ng/ml)		<i>N</i>	Blastocyst (%)	<i>N</i> <sup>a</sup>	No. of cells per blastocyst
TCM199	FCS	–	77	19.5 ± 6.2 <sup>ab</sup>	10	104 ± 14
mSOFaa		–	75	14.7 ± 5.9 <sup>abc</sup>	7	98 ± 26
mSOFaa	PVA	0 (Control)	81	6.2 ± 3.5 <sup>c</sup>	4	95 ± 24
		1	81	14.8 ± 7.4 <sup>abc</sup>	8	108 ± 31
		10	85	20.0 ± 5.2 <sup>a</sup>	13	111 ± 19
		50	70	11.4 ± 7.2 <sup>bc</sup>	5	109 ± 22

Values are mean ± S.E.M. <sup>a-c</sup>Different superscripts in the same column are significantly different ( $P < 0.05$ ).

<sup>a</sup> Number of blastocysts examined for cell number.

## IN VIVO RATES OF MOUSE EMBRYO DEVELOPMENT CAN BE ATTAINED IN VITRO

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Mouse embryos cultured in conventional culture media, lacking amino acids and being based on a single formulation, exhibit delayed development in vitro compared to their in vivo counterparts. Newer culture systems use a sequential media approach that reflects both the changes that occur in the female reproductive tract and the changing requirements of the embryo. Therefore, this study was designed to compare the development of embryos cultured in either a single medium formulation, or in sequential media, to embryos developed in vivo.

Zygotes were collected from F1 (C57BL/6 × CBA) females at 21 h post-hCG. Zygotes were cultured in either medium HTF, KSOMAA, P1/blastocyst, or G1/G2 media at 37 °C in 6% CO<sub>2</sub>, 5% O<sub>2</sub>, 89% N<sub>2</sub> (10 embryos in 20 µl of medium supplemented with 5 mg/ml HSA under paraffin oil). After 48 h of culture embryos in media P1 and G1 were transferred to blastocyst medium and G2, respectively. The mean cleavage time from the 4-cell to the 8-cell stage, percent compaction after 48 h of culture and blastocyst development after 72 h of culture were all assessed. In vivo developed embryos were flushed and scored at equivalent time points. Blastocyst cell number, inner cell mass (ICM) development, and trophoctoderm (TE) cell number were subsequently determined. Differences between treatments were assessed using either generalized linear modeling or Kruskal–Wallis with a Dunn post comparison test.

Mean cleavage time to the eight-cell stage of in vivo developed embryos was 64.3 ± 0.2 h post-hCG. The mean cleavage time of embryos cultured in G1 (64.4 ± 0.2 h post-hCG) and KSOMAA (65.0 ± 0.2 h post-hCG) was not different to in vivo. In contrast, the mean cleavage time was significantly slower for embryos cultured in P1 (65.9 ± 0.2 h post-hCG) or HTF (70.5 ± 0.2 h post-hCG). Compaction of cultured embryos was significantly higher in G1 embryos (74.4%) compared to all other treatments KSOMAA (60.2%;  $P < 0.01$ ), P1 (66.0%;  $P < 0.01$ ) and HTF (26.4%;  $P < 0.01$ ). At 93 h post-hCG 82.2% of all embryos flushed from the reproductive tract were at the blastocyst stage, similar to that obtained in G1/G2 (89.4%). In contrast, blastocyst development in KSOMAA and P1/blastocyst was significantly reduced ( $P < 0.05$ ), 64.5 and 61.4%, respectively. Blastocyst development in HTF was significantly less than in any other media system (18.0%,  $P < 0.01$ ). Total blastocyst cell numbers were not different between in vivo (45.6 ± 0.8), G1/G2 (47.4 ± 2.2) and KSOMAA (43.9 ± 1.5). However, culture in P1/blastocyst (37.0 ± 2.6;  $P < 0.05$ ) and HTF (28.5 ± 1.8;  $P < 0.01$ ) resulted in significantly fewer cells than the in vivo controls. ICM was not different between in vivo (17.8 ± 0.4) and G1/G2 (16.6 ± 0.8). In contrast, all other media resulted in a significant decrease in ICM development (KSOMAA, 12.5 ± 0.9,  $P < 0.01$ ; P1/blastocyst, 11.7 ± 1.1,  $P < 0.01$ ; HTF, 6.9 ± 0.6,  $P < 0.01$ ).

These data show that by using physiologically based sequential media, such as G1/G2, it is possible to obtain development in vitro at an equivalent rate and quality to that in vivo.

## EFFECT OF SERUM DURING BOVINE EMBRYO CULTURE ON BLASTOCYST DEVELOPMENT, CRYOTOLERANCE, AND MESSENGER RNA EXPRESSION

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The post-fertilization culture environment is crucial in determining blastocyst quality [Rizos et al., *Mol Reprod Dev* 2002;61:234–248]. Therefore, any improvement in the quality of blastocysts produced *in vitro* is likely to derive from modification of the post-fertilization culture conditions. The objective of this study was to examine the effect of serum during this period on cleavage rate, developmental kinetics, blastocyst yield, and blastocyst quality. Bovine blastocysts were produced following *in vitro* culture in synthetic oviduct fluid (SOF) +3 mg/ml BSA with or without 10% fetal calf serum (FCS). Significantly, more blastocysts were present by Day 6 in the presence of FCS (20.0% versus 4.6%,  $P < 0.001$ ). By Day 7, however, this difference had disappeared and there was no difference in overall blastocyst yield at Day 9 between the groups (42.1% versus 39.1%, respectively). The presence of serum significantly decreased the survival of blastocysts after vitrification (24 h: 34.0% versus 72.9%; 72 h: 22.6% versus 54.2%, respectively,  $P < 0.01$ ). The quantification of all gene transcripts was carried out by real-time quantitative RT-PCR. Differences in relative mRNA abundance among the two groups of blastocysts analyzed were found for genes related to apoptosis (Bax), oxidative stress (MnSOD, CuZnSOD and SOX), communication through gap junctions (Cx31 and Cx43), maternal recognition of pregnancy (IFN- $\tau$ ) and differentiation and implantation (LIF and LR- $\beta$ ). The presence of serum during the culture period resulted in a significant increase ( $P < 0.05$ ) in the level of expression of MnSOD, SOX, Bax, LIF and LR- $\beta$ . The level of expression of Cx31 and Cu/ZnSOD also tended to be increased although the difference was not significant. In contrast, the level of expression of Cx43 and IFN- $\tau$  was decreased in the presence of serum. In conclusion, using a combination of measures of developmental competence and blastocyst quality to give a more complete picture of the consequences of modifying medium composition on the embryo, we have shown that conditions of post-fertilization culture can affect the speed of embryo development and the quality of the resulting blastocysts.

THE PRESENCE OF NON-POLAR ESSENTIAL AMINO ACIDS IN A MEDIUM BEFORE THE 4-CELL STAGE DISRUPTS ACTIN FILAMENT DISTRIBUTION IN PORCINE PARTHENOGENETIC DIPLOIDS

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Composition of the culture medium plays an important role in the development of embryo. Nuclear migration and embryo cleavage are dependent on normal actin filament distribution and protein phosphorylation during early development. This study was designed to examine actin filament distribution and tyrosine-phosphorylated proteins in porcine parthenogenetic diploids that were cultured in the presence of non-polar essential amino acids (Np-EAA) in a protein-free medium during the first and second cell divisions. Porcine parthenogenetic diploids were produced by a single electro-stimulation (El-St; 100  $\mu$ s at 1500 V/cm) and 5.0  $\mu$ g/ml cytochalasin B (CB) treatment of in vitro matured oocytes. The culture medium for development was based on Whitten's medium (WM), containing 0.5 mg/ml polyvinyl alcohol (PVA) and 5.0 mg/ml hyaluronic acid (PVA-WM). A two-step culture system involving changes in osmolarity was used, in which 290 mOsmol was used before the 4-cell stage (72 h after El-St, PVA-WM 290) and subsequently 256 mOsmol (PVA-WM<sub>256</sub>) up to 168 h after El-St. In Experiment 1, the diploids were cultured in PVA-WM<sub>290</sub> supplemented with Np-EAA at the same concentration in Eagle's basal medium (BME), 19 amino acids (without glutamine), or without amino acids for the first 72 h, and then subsequently cultured in PVA-WM<sub>256</sub> including 19 amino acids without glutamine until 168 h after El-St. In Experiment 2, actin filament distribution in the 1-, 2- and 4-cell stages that were cultured in PVA-WM<sub>290</sub> + Np-EAA, PVA-WM<sub>290</sub> + 19AA or PVA-WM<sub>290</sub> was examined by confocal microscopy after staining with 1  $\mu$ g/ml FITC-conjugated phalloidin. In Experiment 3, tyrosine-phosphorylated proteins in 2- and 4-cell diploids that were cultured in the media as described in Experiment 2 were detected by Western blotting. A control medium for all experiments was WM supplemented with 4 mg/ml BSA instead of PVA (BSA-WM). The frequencies at which diploids developed to each stage in Experiment 1 were subjected to an arcsine transformation for each replication. The transformed values were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. The results of these experiments showed that the presence of Np-EAA before the 4-cell stage caused a severe delay of the first and second division of diploids and inhibition of development beyond the 4-cell stage. Very few actin filaments were distributed in the cortex at the region of attachment of blastomeres and especially in the perinuclear area of diploids cultured with Np-EAA. The presence of Np-EAA caused a decrease of tyrosine-phosphorylated proteins compared with diploids cultured in the absence of Np-AA and controls for the first 72 h after El-St. In conclusion, the presence of Np-EAA before the 4-cell stage affects the normal distribution of actin filament, and decreases tyrosine phosphorylated proteins, resulting in a 4-cell block of porcine parthenogenetic diploids.

## FRAGMENTATION AND APOPTOSIS IN BOVINE EMBRYOS

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Fragmentation is frequently present in bovine embryos which have been cultured in vitro to the morula-blastocyst stage. It can also occur in embryos collected from the uterus of superovulated cows, indicating that it is not an in vitro artifact. Both cytoplasmic fragmentation and apoptosis or programmed cell death are mechanisms which can be used by the embryo to eliminate cells with undesired properties. It was the purpose of this study to examine whether the amount of cytoplasmic fragmentation of the embryo at the morula stage was related to the incidence of apoptotic nuclei as assessed by TUNEL staining. Bovine embryos were obtained using routine methods for IVM, IVF and IVC. Zygotes were cultured in groups of 25 in 50  $\mu$ l droplets of SOF + 5% FCS at 39 °C in 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. The medium was not replenished during IVC. Embryos at the morula stage with varying degrees of fragmentation were selected at Day 6. Amount of fragmentation was estimated by placing embryos in 0.3 M sucrose. Cytoplasmic fragments show no osmotic reaction to hypertonic solution and can therefore easily be distinguished. Morulae were assigned a grade according to the amount of fragmentation: (1) no fragmentation, (2) <25% fragmentation, (3) 25–50% fragmentation and (4) >50% fragmentation. The embryos were fixed in paraformaldehyde 4% for 30 min at RT. After washing in PBS, they were permeabilized in Triton X-100 0.5% in PBS for 1 h, washed and incubated in fluorescein-dUTP and terminal deoxynucleotide transferase (In Situ Cell Death Detection kit, Boehringer, Mannheim, Germany) for 1 h at 37 °C in the dark. Positive controls were incubated in DNA-ase 1 (0.05 mg/ml) for 1 h and washed before TUNEL. Negative controls were incubated in nucleotide mixture only, in the absence of transferase. Samples were incubated in RNase A (50  $\mu$ g/ml) for 1 h at RT and nuclei were counterstained with propidium iodide (0.5  $\mu$ g/ml). Embryos were evaluated by means of fluorescence and confocal laser scanning microscopy. A total of 898 zygotes (three replicates) were cultured and 400 reached the morula stage (45%). The number of morulae assigned to grade 1 was 115 (29%), to grade 2, 102 (26%), to grade 3, 56 (14%) and to grade 4, 127 (32%). One hundred 76 morulae (two replicates) were evaluated for apoptotic cell index (no. of TUNEL positive nuclei/total cell no.  $\times$  100) (Table). Data were analyzed by means of ANOVA and logistic regression. From the results it appears that apoptosis and fragmentation are two independent processes which confirm data obtained earlier with human embryos [Antzack and Van Blerkom, Hum Reprod 1999;14:429]. While fragmentation was clearly correlated with total cell no., ACI did not differ between embryos showing up to 50% fragmentation. The negative impact of fragmentation upon embryo development must therefore be attributed to other factors. Whether fragmentation of bovine embryos may influence the polarized distribution of important regulatory proteins, as occurs in human embryos, remains to be determined.

Morula grade	No. of morulae	Total cell number (X $\pm$ S.E.M.)	Apoptotic cell index (ACI) (X $\pm$ S.E.M.)	Percent of morulae with ACI < 10%
1	63	59 $\pm$ 3 <sup>a</sup>	8 $\pm$ 2 <sup>a</sup>	79 <sup>a</sup>
2	38	51 $\pm$ 3 <sup>a</sup>	8 $\pm$ 2 <sup>a</sup>	76 <sup>a</sup>
3	43	36 $\pm$ 2 <sup>b</sup>	8 $\pm$ 1 <sup>a</sup>	67 <sup>a</sup>
4	32	23 $\pm$ 2 <sup>c</sup>	16 $\pm$ 2 <sup>b</sup>	38 <sup>b</sup>

<sup>a-c</sup>Values with different superscripts within the same column differ significantly ( $P < 0.05$ ).

## PRODUCTION OF LIVE PIGLETS FOLLOWING IN VITRO EMBRYO CULTURE IN A MICROFLUIDIC ENVIRONMENT

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In vitro embryo culture is a critical step of in vitro production of mammalian embryos as well as many assisted reproductive technologies. However, the efficiency of embryo culture and pregnancy rates following assisted reproductive technologies is relatively low. With the development of the micro fluidic environments, it is believed that the efficiency of embryo culture and pregnancy rates may be increased. The objectives were to determine if embryos cultured in the microchannels would produce live piglets, and to determine reproductive fecundity of these animals. Prepubertal gilts were synchronized with an injection of PG600<sup>®</sup> at approximately 180 days of age. Gilts in standing estrus were mated to produce crossbred embryos. Four-cell embryos were surgically collected in HEPES-buffered Beltsville Embryo Culture Medium-3 supplemented with 1% BSA from the donor gilts. Donor embryos were divided equally between microchannels and controls. Embryos were cultured in North Carolina State University -23 medium supplemented with 4 mg/ml BSA at 39 °C in a humidified 5% CO<sub>2</sub> in air atmosphere. Control embryos were cultured in a 4-well Nunc dish in 1 ml of medium with 3 ml of medium in the inner well with no oil overlay. Development was observed every 24 h. Data was analyzed by a Generalized Linear Model procedure in SAS. There was no significant difference ( $P > 0.05$ ) in blastocyst formation in the microchannels (79%) compared to controls (84%). Blastocysts ( $n = 16$ ) cultured in the microchannels were surgically transferred to the uterus of an asynchronous recipient. Pregnancy was diagnosed on Day 25 by ultrasonography using a 3.5 mHz transducer attached to an Aloka ultrasound. Recipient CET 2 farrowed three females and two males. At birth, body conditions of the piglets were considered normal. All five animals had an average weaning weight of 7.34 kg and were reared to reproductive age. At puberty (6 months), the two boars were mated to Yorkshire females and the three females were mated to a Duroc male. The females were allowed to gestate to term (average 116 days). The two Yorkshire females mated to the boars have farrowed with an average litter size of 11.5 piglets with an average of 10 live piglets born and an average birth weight of 1.45 kg. Currently, one female, mated to a Duroc male, has farrowed with a litter of five piglets. In conclusion, we have demonstrated that microchannels supported blastocyst formation of 4-cell in vivo produced and produced live offspring following embryo transfer to a suitable recipient. Although there was no difference in blastocyst formation and live piglets were born, optimization of embryo culture in the microchannels was not part of the present study. Further parameters will be investigated to maximize blastocyst formation and live piglets born after embryo transfer.

EFFECTS OF ELAND AND BOVINE SERUM ON THE DEVELOPMENT OF IN VITRO-DERIVED BOVINE EMBRYOS

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Bovine serum and/or serum albumin is used to culture embryos from a diverse group of species. However, murine and human embryo culture studies indicate the use of heterologous serum may be detrimental [Prescott, Aust J Exp Biol Med Sci 1983;61:47; Moneret-Vautrin et al., Allergy 1991;46:228]. Moreover, the effect of heterologous serum on the development of interspecies nuclear transfer couplets is unknown. In a preliminary trial, after interspecies nuclear transfer of eland somatic cells into enucleated bovine oocytes, some embryos developed to blastocysts during culture in medium supplemented with eland serum. Therefore, the primary purpose of the present study was to evaluate the effect of serum type (eland versus bovine) on in vitro development of IVM/IVF bovine embryos. In addition, we evaluated in vitro development of bovine embryos cultured in medium (without serum) supplemented with insulin-transferrin-sodium selenite (ITS; 5 and 5 µg/ml and 5 ng/ml, respectively) and non-supplemented control medium. Oocytes were matured during an overnight shipment using TCM-199 supplemented with FBS, LH and estradiol, and gassed with 5% CO<sub>2</sub> at 39 °C. IVF was done in TALP with heparin, penicillamine-hypotaurine-epinephrine and non-essential amino acids. At 18 h post-insemination, presumptive zygotes were vortexed, washed and placed in IVC. The IVC medium was modified BM-3 [McKiernan et al., Mol Reprod Dev 1995;42:188–199] containing 0.2 mM glucose, 1 mM glutamine, and 1× MEM nonessential and 0.5× BME amino acids. The four treatments were modified BM-3 + (1) 5% eland serum; (2) 5% FBS; (3) ITS; and (4) Control (Ctrl). Cleavage frequency was evaluated on Day 2 post-insemination. Embryos were placed in fresh medium and % serum in treatments 1 and 2 was increased to 10% on Day 4 post-insemination. On day 8, blastocyst development and the diameter (µm) of expanded and hatched blastocysts were recorded. Eland serum was harvested from blood collected from a cycling female. Serum was extracted after centrifugation at 1500 × *g* for 6 min, heat inactivated and stored at –80 °C until use. Data were analyzed with ANOVA using GraphPad Instant<sup>®</sup> 3.0 program and Tukey's test Values were assumed statistically different when *P* < 0.05 Cleavage and blastocyst yield indicate that eland serum was not detrimental to bovine embryos. Embryos produced in bovine serum were larger than those produced in the control medium. In conclusion, eland serum could be used as an alternative source of external protein/nutrients for bovine IVF embryos.

Treatment	Oocytes inseminated ( <i>n</i> )	Day 2 cleaved (% ± S.E.M.)	Day 8 blastocyst (% ± S.E.M.)	Diameter (mean ± S.D.) ( <i>n</i> )
1 (+ES)	143	72.4 ± 2.0	41.0 ± 2.4	240 ± 62 <sup>ab</sup> (37)
2 (+FBS)	134	68.2 ± 4.9	33.7 ± 1.5	249 ± 75 <sup>b</sup> (23)
3 (+ITS)	136	79.4 ± 2.2	34.1 ± 4.9	222 ± 46 <sup>ab</sup> (23)
4 (Ctrl)	130	80.6 ± 6.5	31.4 ± 6.4	196 ± 42 <sup>a</sup> (16)

<sup>a,b</sup>Values with different superscripts are different (*P* < 0.05).

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# Embryo Manipulation

## EFFECT OF ELECTROFUSION METHOD AND TIME OF CLEAVAGE ON BOVINE EMBRYO DEVELOPMENT AFTER BLASTOMERE FUSION AT THE 2-CELL STAGE

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Electrofusion of 2-cell blastomeres has been used in an attempt to produce tetraploid embryos in several mammalian species. The aim of this experiment was to assess the fusion rates and subsequent development of bovine embryos using two fusion protocols. The effect of time of first cleavage on cleavage and blastocyst development post-treatment was also evaluated. Oocytes were obtained from a commercial source (BoMed, Madison, WI) and were fertilized with frozen semen using standard in vitro fertilization procedures. Oocytes were observed for cleavage at 30, 32, 34 and 36 h post-insemination and 2-cell embryos were then selected for electrofusion using a BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA). Fusion buffer consisted of 0.3 M mannitol, 0.1 M MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05 mM CaCl<sub>2</sub>, 0.5 mM HEPES and 0.4% BSA. Embryos were aligned using a 5 s, 7.5 V ac pulse. Two fusion protocols were used: Method A consisted of a single 1.4 kV/cm 100 µs dc pulse while Method B consisted of two 1.4 kV/cm 50 µs dc pulses. Treated embryos were observed after 1 h for fusion of cell membranes between the two blastomeres. Cleavage was assessed at 60 h post-fusion. Blastocyst rate was assessed on days 6 and 7 post-fusion. Data for fusion rates, cleavage rates, and blastocyst rates were analyzed using ANOVA. Fusion method had no effect on the percentage of embryos that fused, cleaved or developed to the blastocyst stage following treatment. Overall, 518 of 709 embryos treated had fused at 1 h post-treatment. Only 4% of the embryos treated lysed after fusion. In total, 62% of the fused embryos cleaved following treatment and 14% of those cleaved embryos developed to the blastocyst stage. Time of first cleavage post-insemination had no effect on the percentage of embryos that fused but did affect the percentage of fused embryos that cleaved and had formed blastocysts following fusion ( $P < 0.05$ ). Embryos that cleaved and were fused at 30 h post-insemination were more likely to cleave and form blastocysts after fusion than those that cleaved at 34 and 36 h post-insemination (Table 1).

Table 1  
Effect of timing of first cleavage on fusion and following cleavage rates of fused embryos

Time (h) <sup>†</sup>	<i>n</i> <sup>‡</sup>	No. of fused	Percent fused	No. of cleaved *	Percent cleaved**
30	255	188	74	140	75 <sup>a</sup>
32	195	144	74	93	65 <sup>ab</sup>
34	149	99	66	55	56 <sup>bc</sup>
36	110	87	79	34	39 <sup>c</sup>
Total	709	518	73	322	62

<sup>†</sup>Post-insemination; <sup>‡</sup>number of 2-cell embryos exposed to electrical pulse; \*assessed at 60 h post-fusion, based on the number of fused oocytes; \*\*based on the number of fused oocytes; <sup>abc</sup>numbers in columns with different superscripts differ ( $P < 0.05$ ).

## IN VITRO PRODUCTION OF BOVINE ANDROGENETIC EMBRYOS

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Androgenetic embryos are important in understanding the mechanism of imprinting. The objective of this study was to investigate the developmental capacity of bovine androgenetic embryos. Bovine oocytes were collected via slicing from slaughterhouse ovaries, matured in TCM 199 and fertilized with frozen-thawed semen of proven fertility. Androgenetic embryos (Andro) were created by fertilization of enucleated matured (19–20 h) oocytes. Their in vitro development was compared with that of sham-treated controls (denuded and zona slit; Co–) and nontreated IVF controls (Co+). For enucleation the denuded oocytes were incubated with cytochalasin B (5 µg/ml) and Hoechst 33342 (5 µg/ml) for three minutes. Andro and Co– were inseminated with either 50,000 (1) or 500,000 (2) sperms/ml. Co+ were inseminated with 1 million sperms/ml. After 18 h of coincubation all groups of embryos were cultured in SOFaa with BSA in 5% CO<sub>2</sub> and 5% O<sub>2</sub>. Cleavage and developmental rates were recorded at days 3, 5 (only Andro-groups) and 7/8 of culture. These data were compared by Tukey's test. Androgenetic embryos inseminated with 50,000 sperms/ml resulted in significantly ( $P < 0.003$ ) lower cleavage (Andro1: 37/119, 31.1% versus Andro2: 74/108, 68.5%) and developmental rates at day 5 (Andro1: 9/119, 7.5% versus Andro2: 40/108, 37%) compared to 500,000 sperms/ml. In both groups most of the embryos arrested in development at the 8–16-cell stage. Only 2/119 (1.7%) and 4/108 (3.7%) developed in Andro1 and 2 to morula and blastocyst stages, respectively. No significant differences were found in the CO-groups (cleavage; Co-1: 15/40, 37.5%; Co-2: 29/54, 53.7%; blastocysts at day 7/8; Co-1: 5/40, 12.5%; Co-2: 12/54, 22.2%). However, the data differ significantly ( $P < 0.02$ ) from the rates of Co+ (cleavage; 210/252, 83.3%; blastocysts at day 7/8; 101/252, 40.7%). Further analysis of androgenetic embryos ( $n = 96$ ) by lacmoid-staining showed that 37.5% of the embryos had one pronucleus, while 9.4% of the embryos developed two or more (4.2%) pronuclei. Since previous studies had shown that diploid androgenetic embryos have a higher developmental capacity, experiments were initiated to diploidize the genetic content by 6-DMAP or cytochalasin B treatment. Both treatments did not enhance the development of androgenetic embryos over the blocking stage. Currently, experiments are underway to produce diploid androgenotes by pronuclear transfer in haploid androgenetic embryos. The results of this study indicate that the sperm dose at fertilization can influence cleavage and day 5 developmental rates of androgenetic embryos. However, their developmental capacity to morulae and blastocysts is restricted.

## TROPHOBLASTIC VESICLES AS PUTATIVE CARRIERS OF MOUSE EMBRYONIC STEM CELLS

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It is known that mice fully derived from embryonic stem (ES) cells can be produced either by aggregation of ES cells with tetraploid carrier embryos [Nagy et al., *Development* 1990;110:815–821] or by injection of ES cells into blastocysts from which the ICMs have been previously microsurgically removed [Modlinski et al., *Anim Reprod Sci* 1996;42:437–446] or in which ICM cell divisions have been selectively blocked by short, acute heat shock [Amano et al., *Theriogenology* 2000;53:1449–1458]. Here, we describe the production of ICM-free mouse blastocysts (trophoblastic vesicles; TVs)—which could be used as putative carriers of ES cells—by prolonged exposure of early cleaving embryos to mild heat shock (MHS). Two-cell, early and late 4-cell and 8-cell embryos were cultured at 40 °C in modified M16 medium until the blastocyst stage. Both trophoblastic vesicles and normal blastocysts were obtained after chronic mild heat treatment. The rate of T formation increased with the age of the embryos treated. Only 2.7% of TVs was obtained from 2-cell embryos, whereas early and late 4- and 8-cell embryos produced 6.2, 18.2 and 35.6% of TVs, respectively. The number of normal blastocysts was also higher when later stage embryos were exposed to MHS (8.3% from 2-cell embryos versus 41.3 and 39.4% from early 4- and 8-cell embryos) with the unexpected exception of late 4-cell stage (2.3% of blastocysts). In control groups (culture at 37 °C) TVs were not formed and rate of blastocyst formation reached 93.7%. The mean cell number ( $\pm$ S.D.) of blastocysts developed at 40 °C from 2-, 4-, and 8-cell embryos was 27.5,  $39.6 \pm 4.2$  and  $47.5 \pm 3.5$ , respectively. Cell numbers in TVs were similar to those of blastocysts. To check the quality of the obtained blastocysts and TVs, they were transferred to 3.5 day pseudopregnant recipients. Out of 12 blastocysts from 4-cell embryos, 9 young were born and out of 18 blastocysts developed from 8-cell embryos, 13 young were born. The transferred TVs induced a strong decidual reaction. Fifty-two TVs were injected with D3 ES cells (derived from pigmented 129/SvJ strain), each with about 13–15 cells. TVs originated from albino strains (Swiss albino, BALB/cByJ). After 24 h of culture 28 vesicles, with the compacted group of ES cells adhering to the inner surface of the trophoblast, were selected for transfer to 3.5-day pseudo-pregnant recipients. So far, two 13-day fetuses (one of them alive and morphologically normal and one dead) and one normal live birth were obtained. That pup was male; its sex, pigmented eyes and darkish skin indicated its D3 origin. These results show that prolonged mild heat treatment of early embryos does not preclude their viability and also suggest that trophoblastic vesicles obtained after MHS can be successfully used as carriers for production of fully ES cell-derived mice.

# Embryo Transfer

## THE USE OF CIDR IN THE SUPEROVULATORY PROTOCOL OF NELORE DONORS AT DIFFERENT STAGES OF ESTRUS CYCLE

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Elaborate superovulatory protocols which allow the use of a larger number of donors per program, such as the synchronization of follicular wave emergence using steroid hormones, have minimized the routine problem of the estrus cycle control in large herds in the northern region of Brazil. This type of protocol allows the veterinarian, even when far from the property, to determine the most suitable period for the performance of the embryo transfer program [Andrade et al., Anim Reprod Sci 2002;69:9–14]. However, as the administration of steroid hormones has been performed without control of the estrus cycle stage, it remains the necessity to be determined if the effectiveness of this treatment is the same in all stages of the estrus cycle. Thus, the objective of this study was to evaluate the superovulatory response and ova/embryo recovery from Nelore donors following the administration of CIDR (controlled internal drug releasing device) program in all stages of estrus cycle. The control group (TI;  $n = 30$ ) was submitted to a standard protocol of superovulation and was formed by females between days 9 and 12 of their cycle (estrus = day 0). The donors that received CIDR (intravaginal device of 1.9 g progesterone + intramuscular injection of 2 mg estradiol benzoate) were at the day 0 (TII;  $n = 30$ ), between days 2 and 6 (TIII;  $n = 30$ ), 7 and 12 (TIV;  $n = 30$ ), 13 and 16 (TV;  $n = 30$ ) and 17 and 20 (TVI;  $n = 30$ ) of the estrus cycle. Superovulation was induced with p-FSH, divided into eight decreasing doses at intervals of 12 h (80 Bid; 60 Bid; 40 Bid; 20 Bid). The donors received PGF $2\alpha$  48 h after the beginning of the treatment and intravaginal devices were removed 12 h later. Artificial inseminations (AI) were done at 12 and 22 h after the initiation of estrus, and the embryo collections were done 7 days after AI. In the donors which displayed behavioral estrus, the mean ( $\pm$ S.E.M.) of total ova, viable (transferable) and degenerated embryos were, respectively,  $14.2 \pm 11.3$ ,  $7.4 \pm 6.9$  and  $3.2 \pm 3.5$  (TI),  $13.3 \pm 10.4$ ,  $7.1 \pm 6.2$  and  $3.3 \pm 6.2$  (TII),  $13.5 \pm 7.0$ ,  $8.1 \pm 6.7$  and  $2.3 \pm 3.0$  (TIII),  $17.4 \pm 9.9$ ,  $9.4 \pm 6.9$  and  $4.0 \pm 4.4$  (TIV),  $16.9 \pm 8.8$ ,  $9.8 \pm 8.1$  and  $2.7 \pm 2.5$  (TV) and  $13.0 \pm 7.8$ ,  $7.2 \pm 6.9$  and  $2.3 \pm 2.5$  (TVI). In those animals which did not display behavioral estrus, the mean values of total ova, viable (transferable) and degenerated embryos were, respectively,  $6.5 \pm 7.2$ ,  $3.5 \pm 3.7$  and  $1.1 \pm 1.2$  (TI;  $n = 7$ ),  $11.7 \pm 10.8$ ,  $7.2 \pm 7.4$  and  $2.3 \pm 1.8$  (TII;  $n = 3$ ),  $11.9 \pm 8.2$ ,  $9.7 \pm 8.3$  and  $1.8 \pm 1.5$  (TIII;  $n = 3$ ),  $15.1 \pm 12.2$ ,  $10.1 \pm 8.1$  and  $3.4 \pm 3.8$  (TIV;  $n = 2$ ) and  $10.3 \pm 9.3$ ,  $6.4 \pm 5.6$  and  $2.1 \pm 2.4$  (TVI;  $n = 2$ ). No significant differences ( $P \geq 0.05$ ) were observed among groups. Pregnancy rates of 67.0% (TI;  $n = 86/128$ ), 60.8% (TII;  $n = 59/97$ ), 63.5% (TIII;  $n = 73/115$ ), 64.1% (TIV;  $n = 84/131$ ), 72.3% (TV;  $n = 81/112$ ) and 60.6% (TVI;  $n = 63/104$ ) were obtained with embryos transferred from these collections. Pregnancy rates were not different ( $P \geq 0.05$ ) among groups. Results of this study allow us to conclude that a combination of steroid hormones could be used before superovulation in Nelore donors, at any stage of the estrus cycle, without decreasing the efficiency of embryo transfer. These treatment protocols minimize costs of embryo transfer by permitting the use of a large number of donors in a short period of time, making it a valuable tool for animal production programs that use this technology.

PREGNANCY RATES IN RECIPIENT HEIFERS AFTER ADMINISTRATION OF HUMAN CHORIONIC GONADOTROPIN OR A GONADOTROPIN-RELEASING HORMONE AGONIST ON DAY FIVE OF THE ESTROUS CYCLE

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Administration of hCG or a GnRH-agonist on Day 5 of the estrous cycle caused ovulation of the first wave dominant follicle with formation of an induced corpus luteum (CL). Interestingly, when the accessory CL was induced by such treatments, plasma concentrations of progesterone (*P*) in heifers were greatly increased. Indeed, *P* supplementation during the luteal phase of the estrous cycle could increase pregnancy rates in cattle. Thus, we aimed to determine whether an injection of hCG or GnRH-agonist on Day 5 after estrous (=Day 0) can improve conception rates following embryo transfer programs. Holstein heifers were randomly assigned to an hCG group (1, 500 IU; *n* = 69) or a GnRH-agonist group (Buserelin, 8 µg; *n* = 71) both treated on Day 5, or a control group (*n* = 89). Blood was collected on Days 5 and 15 by jugular venipuncture (hCG: *n* = 32, GnRH-agonist: *n* = 36, and control: *n* = 33), and plasma was immediately separated and stored at -20 °C until assayed for P. Plasma P concentrations were determined by the enzyme immunoassay as described earlier by Miyamoto et al. [J Endocrinol 1992;135:103-114]. The P levels on Day 5 or Day 15 were compared among the hCG, GnRH-agonist and control groups. The heifers were classified into pregnancy or non-pregnancy on Day 30. Bovine embryo transplantation rates (no. of transferred/injected), pregnancy rates (no. of pregnancy/transferred) and conception rates on Days 30 and 60 (no. of pregnancy/injected) were compared among the groups. Means of plasma P concentrations were analyzed by ANOVA followed by Student's *t*-test. Chi-square test was used to compare differences in pregnancy or conception rates among groups. Plasma P concentrations on Day 15 were higher (*P* < 0.05) in hCG and GnRH-agonist ( $5.34 \pm 1.29$  and  $4.91 \pm 1.61$  ng/ml, respectively) than those in control group ( $3.54 \pm 1.16$  ng/ml). Similarly, the increases in P concentrations (values on Day 15 - values on Day 5) were higher (*P* < 0.05) in the groups of hCG and GnRH-agonist ( $4.12 \pm 1.33$  and  $3.77 \pm 1.65$  ng/ml, respectively) than those in control group ( $2.52 \pm 1.25$  ng/ml). However, plasma P concentrations did not differ between the group of pregnancy and non-pregnancy on Day 30. Also, there was no significant difference in transplantation rate (hCG: 91.3; GnRH-agonist: 78.8 and control: 84.3%), pregnancy rate on Day 30 (hCG: 77.8; GnRH-agonist: 82.1 and control: 73.3%) and Day 60 (hCG: 69.8; GnRH-agonist: 75.0 and control: 66.6%), and conception rate on Day 30 (hCG: 71.0; GnRH-agonist: 64.8 and control: 61.8%) and Day 60 (hCG: 63.8; GnRH-agonist: 59.2 and control: 56.2%). Unexpectedly, the treatment with GnRH-agonist decreased conception rates because transplantation rates became lower than those in hCG and control groups. In conclusion, the present results showed that a treatment with hCG or GnRH-agonist on Day 5 after estrus increased plasma P concentrations on Day 15, which may potentially increase pregnancy rates because of a slightly higher pregnancy and conception rates obtained in the present study. However, these results indicate that further investigations are needed to increase pregnancy rates using hCG or GnRH-agonist.

## OVARIAN RESPONSE AND TRANSFERABLE EMBRYOS FROM SUPEROVULATED ANGUS COWS USING TRIUB AND ESTRADIOL BENZOATE

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The variable and unpredictable ovarian response of donors to superovulatory treatments is one of the most limiting factors in an embryo transfer program. The day of treatment initiation in the estrous cycle is one of the more important extrinsic factors that affect ovarian superovulatory response. A study was designed to compare the efficacy of a protocol using an intravaginal progesterone device (Triub) in combination with estradiol benzoate versus conventional initiation of super-ovulation treatment on day 10 of the estrous cycle. This research was done in the fall of 2002 in Marcos Paz, Buenos Aires, Argentina, using Angus cows. Normal cyclic cows ( $n = 44$ ) were randomly allocated into two groups. Twenty-four cows were synchronized receiving an intravaginal progesterone device (Triub, Elastecnicas, Argentina) and 2 mg of estradiol benzoate (EB) i.m. on the same day; the remaining 20 cows served as controls. On day 4 after Triub insertion or on day 10 or 11 after estrus, superstimulatory treatment was initiated with a decreasing dose of FSH (Pluset, Callier, Spain; i.m.) administered every 12 h for 4 days. Prostaglandin f2alpha (2 ml, i.m.) was administered to all animals, and Triub devices were removed on the same day. At heat detection, cows were artificially inseminated and received a dose of GnRH by i.m. injection. Cows were again inseminated at 12 and 24 h after heat. Seven days after estrus and before embryo collection, the ovarian response was evaluated by determining the number of CLs and anovulatory follicles imaged with transrectal ultrasound using a 7.5 MHz transducer (Pie Medical, The Netherlands). Recovered ova/embryos were evaluated as described (IETS manual). Student's *t*-test was used to compare variables between groups. For Triub versus Control Groups, respectively, the mean number of CL  $\pm$  S.D. ( $16.6 \pm 6.4$  and  $15.6 \pm 5.2$ ), anovulatory follicles ( $4.5 \pm 2.1$  and  $3.9 \pm 2.0$ ), and transferable embryos ( $5.0 \pm 3.9$  versus  $5.6 \pm 5.2$ ) were not different. The number UFO and degenerate embryos that were recovered were not different, although there was a numerical increase in the number UFOs in the Triub Group. Total number of ova/embryos was not different between groups (15.6 versus 12.4), with a collection efficiency of 93 and 87% respectively. All transferable embryos were frozen for transfer in the spring. In summary, the number of CL, ova/embryos, and transferable embryos were not different between groups. These results indicate that using an intravaginal progesterone device, such as Triub, in combination with EB is as effective as starting superovulatory treatment on Day 10 of the estrous cycle. Use of Triubs allowed the initiation of superovulation on any day of the estrous cycle.

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## NON-SURGICAL EMBRYO TRANSFER IN SWINE: PRELIMINARY RESULTS

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Recently, a new device for non-surgical embryo transfer (NET) in pigs has been developed [Martinez et al., Proceedings of the Sixth International Conference on Pig Reproduction, 2001, p. 133]. Initial results using this procedure were encouraging [Martinez et al., Theriogenology 2002;57:549]. However, further research is needed to realize the full potential of this procedure. The objective of this study was to assess the effect of the day of embryo collection on pregnancy outcome after NET. Crossbred gilts ( $n = 41$ ) were used as embryo donors and crossbred gilts ( $n = 10$ ) and sows ( $n = 10$ ) were used as embryo recipients. Heat detection was performed once a day and donor animals were inseminated at 12 and 24 h after heat detection. Embryos were collected surgically on days 5–7 after onset of estrus by flushing each uterine horn with 30 ml TALP-HEPES (transfer media). Embryos were assessed, selected, washed three times and transferred within 1–6 h of collection. Embryo transfers were performed to recipients that were detected in heat the same day or up to 48 h after the onset of estrus in the donors, and an average of 24 embryos was used for each transfer. The embryo transfer procedure was carried out in a gestational crate (sows) or in a portable chute (gilts), without sedation of the recipient females. A commercial artificial insemination spirette containing a specially designed flexible catheter (FC) (1.5 m length, 4 mm outer diameter) was introduced through the vagina and inserted into the cervix from where the FC was moved forward toward the uterine body. Once the cervix was cleared, the FC was propelled deep inside one uterine horn. Embryos were transferred in 0.1 ml of transfer media and the catheter was flushed with 0.6 ml to clear the embryos from the catheter. Pregnancy diagnosis was performed 25–30 days after onset of estrus by ultrasound. A total of 471 embryos was transferred. Day 5 embryos were classified as morula (69.9%) or blastocyst (27.6%), while day 6 embryos were classified as morula (27.0%), blastocyst (55.2%) and hatched blastocyst (17.8%). Day 7 embryos were mostly hatched blastocyst (90.1%). A total of 20 transfers was performed and 12 pregnancies were obtained (60%). Data were analyzed using Chi-square. Data sorted by day of embryo collection are presented in Table 1. Day of embryo collection did not ( $P > 0.43$ ) affect pregnancy rate. In addition, pregnancy rate did not differ ( $P > 0.36$ ) between gilts and sows. Although more research is needed in this area, these preliminary data suggest that this new procedure has the potential to be applied commercially and could provide a means of reducing the risk of disease transmission while transporting valuable genetic material

Table 1

Pregnancy results after non-surgical embryo transfer (NET) of embryos collected on days 5–7 after onset of estrus

Day of embryo collection	No. of NETs	No. of pregnant recipients (%)
Day 5	5	4 (80)
Day 6	10	6 (60)
Day 7	5	2 (40)

PREGNANCIES OBTAINED AFTER COLLECTION AND TRANSFER OF  
OOCYTES FROM OVARIES OF EUTHANIZED MARES

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Euthanasia or death of valuable mares results in the loss of future genetic potential. Development of new reproductive techniques allows the clinician to offer clients a method to obtain offspring after death of a mare. Here, we report results of collections and transfers of oocytes from five client mares that were euthanized. Donor mares were 23, 14, 9, 10 and 16 years. Reasons for euthanasia were neoplasm (Donor 1), musculoskeletal (Donors 2, 4 and 5) and gastrointestinal (Donor 3) conditions with poor prognoses. Ovaries of Donors 1–3 were transported <30 min to Colorado State University, whereas ovaries from Donors 4 and 5 were transported in an Equitainer [Hamilton-Thorne S, Hamilton, MA] at ambient temperature (Donor 5) or 5. C (Donor 4). Interval between ovarian removals and oocyte collections for transported ovaries was 8–10 h. Oocytes were collected and cultured in SOF-based or TCM-199 media. Donors 1 and 2 received hCG on the day before euthanasia; oocytes were collected from their preovulatory follicles ( $n = 2$ ) and cultured for 6.5–8 h before transfer. Between 8 and 15 oocytes (mean  $\pm$  S.D.,  $11 \pm 3$ ) were collected from the small follicles of Donors 1, 3, 4 and 5 and cultured for 24–30 h. Oocytes were transferred into oviducts through flank laparotomies [JAVMA 2001;218:87–91]. Only the preovulatory oocyte was collected and transferred for Donor 2; an average of  $9 \pm 4$  oocytes was transferred per remaining donors. Ten recipients were used (1–3 per donor) and were inseminated with cooled, transported ( $n = 9$ ) or frozen ( $n = 1$ ) semen before and/or after transfer. Sixteen days after transfer, 4 of 10 recipients were diagnosed pregnant with at least one embryonic vesicle. From 36 transferred oocytes, 6 embryonic vesicles were imaged by ultrasound (17%). The single embryonic vesicles imaged for Donors 1, 3 and 4 underwent embryonic death by Days 45, 62 and 38, respectively. The single recipient for Donor 5 received 5 oocytes, and 3 embryonic vesicles were imaged on Day 13. The two smallest embryonic vesicles were manually crushed on Days 13 and 14, and the remaining vesicle resulted in a live foal. The low pregnancy and high embryo loss rates indicated that optimal conditions for shipment of equine ovaries has not been determined. However, apparently this is the first report of a live foal being produced from transported ovaries after the death of a mare.

VAGINAL CYTOLOGY FOR DETECTION OF OVULATION IN  
*MACACA FASCICULARIS*

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*Macaca fascicularis* (*Mf*) does not exhibit overt external signs of ovulation that are characteristic in other macaque species (e.g. *M. nemestrina*). Therefore, the use of non-invasive methods for detecting ovulation in *Mf* would be useful in situations where other more invasive methods are not possible. Here we have undertaken an analysis of vaginal cell distributions and their use in detecting an “ovulation window” by comparing known and novel cytological maturation algorithms to a very elegant algorithm for detection of ovulation (day of ovulation/cycle length ratio, DO/CL =  $0.48 \pm 0.08$ ) as described previously for *Mf* [Dukelow et al., J Med Primatol 1979;8:39–47]. The precision of the different algorithms for prediction of the mid-point of the expected ovulatory window in recipient females receiving IVF derived embryos was also compared. Parabasal (Pb), cornified (Cn), pyknotic (Py) and anucleate (An) cell types were counted in vaginal swabs collected from cycling female *Mf* ( $n = 35$ , 3–4 regular cycles) during the follicular and early luteal phases of the menstrual cycle (range: Days 2–21). Cell counts were used to develop maturation indices for vaginal cytological patterns according to previously published algorithms and novel algorithms based on cell distributions during the cycle. Square root transformed ( $X' = \sqrt{X} + \sqrt{X + 1}$ ) Pb, Py and An cell counts were normally distributed throughout the observation period and exhibited significant positive or negative correlation with the day of the menstrual cycle. In contrast, transformed Cn cell counts were not normally distributed and were not correlated with the day of the cycle (Table 1). The female *Mf* examined had an average cycle length of  $31.9 \pm 0.58$  days (137 cycles) representing an ovulation window of  $11.1 \pm 0.20$  days to  $19.5 \pm 2.1$  days with a mid-point of  $15.3 \pm 0.30$  days as determined using the mean DO/CL  $\pm 1$  S.D. Of three previously published maturation indices that use Cn cell counts only one (karyopyknotic index (KPI) correlated with the DO/CL ( $r = 0.42$ ,  $t = 2.68$ ,  $P < 0.02$ ). However, the KPI underestimated the mid-point of the expected ovulatory window described by the DO/CL by  $-3.6 \pm 0.60$  days. A logarithmic ratio of maximal Py/Pb cell counts correlated with the DO/CL ( $r = 0.61$ ,  $t = 4.44$ ,  $P < 0.001$ ) and provided a better estimate of the mid-point of the expected ovulatory window compared to the KPI ( $-1.29 \pm 0.32$  days,  $P = 0.001$  versus KPI). Log Py/Pb derived estimates of the mid-point of the expected ovulatory window in pregnant ( $-1.72 \pm 0.48$  days,  $n = 11$ ) and non-pregnant ( $-2.12 \pm 0.59$  days,  $n = 11$ ) recipients of IVF derived embryos were not significantly different ( $P = 0.60$ ). This study demonstrates that vaginal cytology may be useful for detection of ovulation in *Mf*. The use of specific cell types in the calculation of maturation indices will impact on the precision of the estimate. The benefits of using Pb and Py cell counts in preference to Cn cell counts are discussed.

Table 1  
Vaginal cell distributions in *Mf*

	Cell		Type	
	Parabasal	Cornified	Pyknotic	Anucleate
Mean	6.93	8.57	13.06	11.27
Standard deviation	4.89	3.04	3.08	3.37
Kurtosis	0.80	-0.45	0.48	-0.52
Skew	1.22	0.002	-0.68	-0.36
Normal	Yes	No	Yes	Yes
Correlation vs. cycle day	-0.53	0.04	0.46	0.19
<i>P</i> for correlation	<0.001	>0.05	<0.001	<0.001
<i>N</i>	356	356	356	356

REDUCED DIETARY PROTEIN IMPROVES PREGNANCY RATES FOLLOWING  
TRANSFER OF IN VITRO PRODUCED BOVINE EMBRYOS

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Evidence exists that high levels of degradable intake protein may lead to elevated blood urea nitrogen concentrations which in turn may influence uterine pH and decrease embryo survival following transfer. Here we propose that embryos derived from artificial insemination (AI), embryo transfer (ET), and in vitro production (IVP) possess varying levels of sensitivity to changes in uterine environment. Embryos maintained in vivo following AI may adjust to their environment while embryos deposited into recipient uteri on Day 7 or 8 of the estrous cycle lack this opportunity of adjustment and may be more immediately susceptible to damage. To test this hypothesis, a 2 × 3 factorial experiment was performed over a 3-year period with two levels of dietary protein and three breeding methods. Crossbred heifers and cows were fed either a high protein diet (100% alfalfa) or a diet with reduced protein (40% alfalfa and 60% cup-plant, a non-legume, high fiber feedstuff). The high protein diet was approximately 18% protein while the reduced protein diet was approximately 14% protein upon feedstuff analysis. These diets were fed for a period lasting 2 weeks prior to and 4–6 weeks after transfer or insemination. Thereafter, all recipients were pooled and fed grass and legume species. Animals were bred using the same proven Angus semen with one of three methods: AI, in vivo/ET, or IP/ET. In vivo produced embryos had been previously frozen with ethylene glycol as cryoprotectant, and were transferred via direct transfer. IVP embryos were transferred fresh. Culture medium for IVP embryos was either modified KSOM or SOF with amino acids; there was no effect of medium on pregnancy rates. All embryos for ET or IVP were quality Grade 1. Pregnancy was diagnosed via ultrasonography by at least 28 days of gestation. Pregnancy rates were compared within breeding method using a chi-squared test ( $P < 0.05$ ). No difference was observed between dietary protein levels for AI or in vivo transfers (Table 1). There was no donor effect on pregnancy rates. For recipients receiving IVP embryos, pregnancy rates were higher for animals fed reduced protein diets as compared to high protein diets (47.7% versus 28.0%, respectively). Further data will be collected for the last year of this study, including pregnancy diagnoses later in gestation as well as information about parturition and calving. Blood urea nitrogen (BUN) levels sampled at the time of transfer will also be analyzed for correlation with pregnancy rates. These preliminary data suggest that levels of dietary protein influence pregnancy rates of IVP embryos. Observations from this study also support ongoing efforts to improve the viability of IVP embryos.

Table 1

Pregnancy rates ( $\geq 28$  days) for cattle receiving a high protein or reduced protein diet and bred by AI, in vivo frozen/ET, or IVP fresh/ET

	High protein		Reduced protein	
	Total animals	Pregnant (%) (n)	Total animals	Pregnant (%) (n)
AI	23	65.2 <sup>a</sup> (15)	31	67.7 <sup>a</sup> (21)
In vivo	15	46.7 <sup>a</sup> (7)	12	41.7 <sup>a</sup> (5)
IVP	50	28.0 <sup>a</sup> (14)	44	47.7 <sup>b</sup> (21)

Values with different superscripts (a, b) within a row are significantly different ( $P < 0.05$ ).

RELAXIN ADMINISTRATION DURING IVF/ET IN *MACACA FASCICULARIS*E.S. Hayes<sup>1,2</sup>, E.C. Curnow<sup>1,2</sup>, A.O. Trounson<sup>1</sup>, and E.N. Unemori<sup>3</sup><sup>1</sup>Monash Institute of Reproduction and Development, Clayton, Vic., Australia,<sup>2</sup>University of Washington-NPRC, Seattle, WA, USA, <sup>3</sup>Connetics Corporation

*Macaca fascicularis* (*Mf*) rarely (<0.1% of conceptions) carries multiple pregnancies [Resuello, Vet Rec 1987;121:155] and therefore represents a useful model for the study of mechanisms that affect primate uterine receptivity. Here we have studied the effect of relaxin, a known inducer of implantation-associated growth factors [Unemori et al., Hum Reprod 1999;14:800–806], on implantation and early pregnancy following in vitro fertilization (IVF) and embryo transfer (ET) in *Mf*. Mature oocytes collected from cycling *Mf* stimulated with recombinant human FSH were fertilized in vitro, cultured to the 4–8-cell stage, slow cooled in 1,2-propanediol and stored in liquid nitrogen prior to thawing and ET. The effect of relaxin ( $n = 11$ ) or vehicle ( $n = 11$ ) on the endometrium in early pregnancy was examined during continuous infusion (pump implantation = Day 0) for 21 days during the peri-implantation period. Two embryos were transferred to each oviduct of each recipient (Day 7). Endometrial thickness and the number and sizes of gestational sacs and fetuses were examined by ultrasound on Days 21, 28 and 67. At Day 0 endometrial thickness (transverse plane) was  $0.22 \pm 0.02$  and  $0.22 \pm 0.01$  cm for vehicle and relaxin-treated animals, respectively. On Day 7 endometrial thickness was  $0.25 \pm 0.02$  and  $0.33 \pm 0.02$  cm for vehicle and relaxin-treated animals, respectively ( $P < 0.005$ ) but this effect was transient such that endometrial thickness at Days 21, 28 and 67 was comparable in both groups. Implantation rates (gestational sacs or fetuses/embryo transferred) on Day 21 were 29.5% (13/44) and 40.9% (18/44) for vehicle and relaxin-treated recipients, respectively. Relaxin-treatment-associated implantation rates were not significantly different compared to vehicle at Days 28 (36.4% versus 27.2%) and Day 67 (22.7% versus 13.6%). Pregnancy rates (pregnant recipients/total) on Day 21 were high in both groups (vehicle, 91% versus relaxin, 82%). Sustained pregnancy rate on Day 67 was 54.5% (6/11) and 45.4% (5/11) for vehicle and relaxin-treated recipients, respectively. Multiple pregnancy rates (multiple gestations/pregnant recipient) at Day 67 were 0% (0/6) and 60% (3/5, two twin and one triplet) in vehicle and relaxin-treated animals, respectively ( $P = 0.06$ ). At Day 67, ultrasound assessment of placental size (surface area, circumference) indicated that placental sizes were comparable in relaxin-treated pregnancies ( $0.87 \pm 0.18$  cm<sup>2</sup>,  $3.37 \pm 0.33$  cm) and vehicle-treated pregnancies ( $0.59 \pm 0.17$  cm<sup>2</sup>,  $2.73 \pm 0.68$  cm). Fetal greatest length measurements were  $7.73 \pm 0.48$  and  $6.22 \pm 0.35$  cm in vehicle and relaxin-treated recipients, respectively ( $P = 0.04$ ). Smaller fetal sizes in relaxin-treated recipients were very likely the result of increased multiple gestations in those animals. This study demonstrates that excellent IVF/ET success rates can be achieved in *Mf* using cryostored embryos and, for the first time, a triplet pregnancy using assisted reproductive technologies in *Mf*, a species that rarely carries multiple gestations. Administration of human relaxin to regularly cycling *Mf* during the peri-implantation period resulted in a transient increase in endometrial thickness and increased placental size despite smaller fetal sizes. These results are consistent with a role of relaxin in modulating endometrial physiology during the implantation period and suggest that further work on relaxin's effect on the primate endometrium is warranted.

SERUM INSULIN AND IGF-I IN RECIPIENT HEIFERS FED WITH  
PROPYLENE-GLYCOL PRIOR TO EMBRYO TRANSFER

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Pregnancy rates (PR) at Day 60, corpus luteum (CL) quality and Day 7 serum progesterone (P4) increase in recipients fed with oral propylene-glycol (PG) before embryo transfer (ET) [Hidalgo et al., AETE Proc 2002, in press]. Plasma insulin increases after PG administration [Grummer et al., J Dairy Sci 1994;77:3618–3623], bringing about increased IGF-I [O’Callaghan et al., Anim Sci 1999;68:299]. Insulin and IGF-I are known to affect ovarian function and early embryonic development. In the present work we wanted to investigate non-acute serum insulin and IGF-I in the above cited heifers treated with PG and subsequently receiving embryos. Cyclic Holstein heifers ( $n = 64$ ), aged 13–16 months, from 11 farms were oestrus synchronized with two doses of PGF<sub>2α</sub>, and distributed as follows: (1) heifers fed daily with 250 ml PG for 20 days until Day 6 in cycle; and (2) untreated heifers. Blood samples were taken early at oestrus (Day 0), at Day 6 at noon (post-prandial) and at Day 7 (time of ET) early in the morning (pre-prandial). As a preliminary study, insulin and IGF-I (RIA) were analyzed besides glucose, urea and triglyceride (TG) (Hitachi Autoanalyzer), at the end of the treatment period, on a prandial basis (see table). Data were analyzed by one-way ANOVA and Duncan’s multiple range test and presented as LSmeans  $\pm$  S.E. Insulin values tended to differ between Day 7 (PG+) and Day 0 (PG–) ( $14.3 \pm 3.52$  and  $24.9 \pm 2.1$ , respectively;  $P < 0.08$ ), and between PG+ and PG– in pre-prandial conditions (Table 1;  $P < 0.08$ ). IGF-I values showed differences according to cycle day ( $P < 0.01$ ), but not to PG treatment ( $316 \pm 18$ , Day 0 (PG+);  $310 \pm 19$ , Day 0 (PG–);  $268 \pm 29$ , Day 7 (PG+);  $265 \pm 31$ , Day 7 (PG–)). Relevant correlations were noted between PR (data in press) and urea ( $0.41$ ;  $P = 0.01$ ), PR and TG ( $0.41$ ;  $P = 0.001$ ), IGF-I and urea ( $-0.30$ ;  $P = 0.008$ ), IGF-I and insulin ( $0.28$ ;  $P = 0.01$ ), and glucose and TG ( $0.40$ ;  $P = 0.0004$ ). No significant correlations were obtained for P4 and CL quality (data in press) as related to the variables analyzed. Although plasma acute modifications on TG, insulin, IGF-I and glucose levels are expected in response to PG, the studied reproductive parameters are likely to be more dependent on sustained effects. Thus, heifers receiving PG showed increased pre-prandial insulin, nondependent on glucose. Supported by AGL2001-379. In collaboration with ASCOL.

Table 1

Serum levels (LSmeans  $\pm$  S.E.) of insulin, IGF-I, glucose, urea and triglyceride (TG)

Serum samples	PG	N	IGF-I (ng/ml)	Insulin ( $\mu$ UI/ml)	Glucose (mg/dl)	Urea (mmol/l)	TG (mg/dl)
Pre-prandial	+	13	$261 \pm 23^a$	$21.9 \pm 3.3^d$	$41.5 \pm 4.7^x$	$4.2 \pm 0.2^x$	$30.9 \pm 1.9$
	–	12	$264 \pm 24^{a,d}$	$14.1 \pm 3.9^e$	$43.8 \pm 4.8^x$	$3.4 \pm 0.2^y$	$30.3 \pm 2.0^a$
Post-prandial	+	29	$325 \pm 14^b$	$21.0 \pm 3.2$	$65.5 \pm 2.8^y$	$4.0 \pm 0.1^a$	$23.9 \pm 1.7^b$
	–	29	$293 \pm 15^c$	$19.3 \pm 2.1$	$65.6 \pm 3.0^y$	$3.9 \pm 0.1^{b,z}$	$24.2 \pm 1.7$

Different superscripts within columns differ significantly: (x, y, z) ( $P < 0.01$ ); (a, b, c) ( $P < 0.05$ ); (d, e) ( $P < 0.08$ ).

## COMPARISON OF THE PROPERTIES OF CERVICAL MUCUS IN PREGNANT AND NON-PREGNANT HOLSTEIN HEIFERS AND COWS

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Most embryo transfer technicians evaluate the quality of the corpus luteum (CL) of recipient animals by transrectal palpation to determine whether the animal is acceptable as an embryo recipient. However, evaluation of the CL by rectal palpation is a subjective judgment. Therefore, a more objective way of determining the condition of the recipient is necessary. The objective of this study was to investigate the relationship between properties of cervical mucus and pregnancy rates after artificial insemination and embryo transfer in dairy cows. Samples of cervical mucus were collected from heifers and cows 7 days after artificial insemination or just before embryo transfer. Cervical mucus was collected using a swab from the ostium uteri externum, and measured for pH, type of staining figure (Diff-Quik Staining), and proportion of epithelial cells having collapsed nuclei. The type of staining figure was classified [Higaki et al., Bull Natl Inst Agric Sci 1953;G7:51–59] into one of five groups such as filiceous (Pattern 1), taenia (Pattern 2), claustral (Pattern 3), nubecula (Pattern 4), and aqueous (Pattern 5). In Experiment 1, 70 Holstein cows were used. Fourteen of 70 cows were pregnant (20.0%). The proportion of epithelial cells having collapsed nuclei in the cervical mucus of the non-pregnant cows was higher than that of pregnant cows (87.2% versus 70.0%,  $P < 0.05$ ). The pregnancy rates were 40.0% (2/5), 55.6% (5/9), 30.0% (3/10), 30.0% (3/10), 2.8% (1/36) for Patterns 1–5, respectively. Pattern 5 cows had lower pregnancy rates than the other groups ( $P < 0.05$ ). There was no relation between pH of cervical mucus and the pregnancy rates. In Experiment 2, 50 Holstein heifers and 27 Holstein cows were used. Forty-six Holstein heifers and 19 Holstein cows received fresh and frozen–thawed embryos 7 days after estrus by one technician. Twelve animals determined to have luteal hypoplasia were classified as “unacceptable” and were not used as recipients. All animals were divided into two groups according to the difference in the proportion of epithelial cells having collapsed nuclei in the cervical mucus (Low: 0–79%, High: 80–100%). The pregnancy rate of the Low group (69.0%, 20/29) was higher than that of High group (39.0%, 14/36) ( $P < 0.05$ ). The pregnancy rates were 62.5% (10/16), 58.8% (10/17), 72.7% (8/11), 35.3% (6/17), and 0.0% (0/4) for Patterns 1–5, respectively. Pattern 5 recipients had lower pregnancy rates than did the Pattern 3 recipients ( $P < 0.05$ ). There was no relation between pH of cervical mucus, type of embryo, and pregnancy rates. The proportion of the High group determined to be “unacceptable” recipients (91.7%, 11/12) was higher than of all recipients (55.4%, 36/65) ( $P < 0.05$ ), and the proportion of Pattern 5 that was determined to be “unacceptable” recipients (66.7%, 8/12) was higher than of all recipients (6.2%, 4/65) ( $P < 0.05$ ). However, morphology of epithelial cells within cervical mucus was associated with the presence or absence of a pregnancy. Further studies are needed to compare the use of the properties of cervical mucus with other methods to assess quality of embryo recipients.

EMBRYO TRANSFER IN THE SEMI-DOMESTICATED REINDEER  
(*RANGIFER TARANDUS FENNICUS*)

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The semi-domesticated reindeer was used as a model species in a project concentrating on developing assisted reproductive technology for conservation of wild forest reindeer (*Rangifer tarandus fennicus*). Reindeer are seasonal breeders exhibiting peak sexual activity during autumn from October to November. During the 1999 breeding season, oestrus cycles of three reindeer donors were synchronized with intravaginal progesterone devices (CIDR; type G, 9% progesterone, InterAg, New Zealand) for 15 days. The donors were mated with a vasectomized male during the following oestrus to confirm the success of the synchronization. Starting on Day 6 (Day 0 = day of mating) the donors were treated with FSH (Ovagen<sup>TM</sup>, Immuno Chemical Products [ICP], Auckland, New Zealand) twice daily over 4 days on a decreasing dose regimen (1 + 1, 1 + 1, 0.75 + 0.75, 0.5 + 0.5 NIH-FSH-SI units) to induce superovulation. Luteolysis was induced by cloprostenol (Estrumat<sup>®</sup> vet. 0.25 mg/ml, Pitman Moore, Germany) at 72 h (0.25 mg) and at 84 h (0.25 mg) after commencing superovulation treatment. The donors were mated with a fertile male and embryos were recovered under anaesthesia on Day 8 or 9 (Day 0 = day of mating with a fertile male). A flushing catheter (Ch 15, Wörrlein, Germany) was guided into the uterine horn through cervix with help of a surgeon's hand inserted into abdominal cavity through an incision (7 cm) in linea alba. The uterine horn was filled 10 times with 10–30 ml of Emcare<sup>TM</sup> flushing solution (ICP, New Zealand) supplemented with BSA (1 g/l). The recovered embryos were washed twice in flushing solution and loaded into 0.25 ml straws for transfer. The recipients were treated with intravaginal CIDR devices for 13 days and mated with a vasectomized male during the following oestrus to confirm the success of the synchronization. The embryos were transferred under anaesthesia on Day 7 or 8 with a Cassou pistolette as described with flushing. The ovaries of one donor contained no corpora lutea (CL) and this donor was not flushed. Two other donors had 3/3 (right/left ovary) and 2/1 CL and yielded two blastocysts + 2 unfertilized ova and one blastocyst, respectively. Two blastocysts were transferred into one recipient and one blastocyst into another recipient. The recipient with one blastocyst was diagnosed pregnant at ultrasound examination 60 days after transfer and delivered a male calf 212 days after transfer operation. To our knowledge, this is the first report describing a successful embryo transfer in the semi-domesticated reindeer.

INCREASED PREGNANCY RATES IN *BOS TAURUS* × *BOS INDICUS* EMBRYO  
RECIPIENTS WITH TREATMENTS TO INCREASE PLASMA  
PROGESTERONE CONCENTRATIONS

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It has been shown that plasma progesterone (P4) concentrations are positively correlated to embryo development, interferon- $\tau$  production by the conceptus and pregnancy recognition in cattle. In a previous work [Marques et al., *Theriogenology* 2002;57:548 (abstract)], we demonstrated that treatments with 1500 IU of hCG on Day 7 of the estrous cycle induced formation of accessory CL, improved plasma P4 concentration and increased the length of the estrous cycle in *Bos taurus* × *Bos indicus* heifers. The aim of the present experiment was to evaluate the effect of hCG, GnRH, LH or P4 treatments given at the time of direct embryo transfer on pregnancy rates in *Bos taurus* × *Bos indicus* cross-bred recipient heifers. Animals were kept on pasture regimen at commercial farms in Brazil and were synchronized with one i.m. injection of a prostaglandin F2 $\alpha$  analog. Heifers detected in estrus were examined by rectal palpation or ultrasonography 6–8 days after estrus. Those with a detectable CL ( $n = 485$ ) received a Grade 1 or 2 frozen/thawed embryo (in 1.5 M ethylene glycol) by direct transfer performed by one of five veterinarians. Immediately after the embryo transfer, recipients were randomly assigned to receive one of five treatments: Control (2 ml of saline i.m.;  $n = 98$ ), GnRH (10  $\mu$ g of Buserelin i.m., Conceptal, Hoechst, Brazil;  $n = 99$ ), hCG (1500 IU i.m., Chorulon, Intervet, The Netherlands;  $n = 96$ ), LH (25 mg Armour pLH i.m., Lutropin-V, Vetrepharm Canada Inc.;  $n = 97$ ) or a CIDR-B device for 13 days (1.9 g of P4, Pharmacia, Brazil;  $n = 95$ ). Pregnancy diagnosis was determined by real-time ultrasonography (Aloka SSD 500, Aloka Corp., Japan) 40–60 days after embryo transfer. Conception rates were analyzed by non-parametric one-way ANOVA (SAS System). The pregnancy rate in heifers treated with GnRH (53/99, 53.5%) was higher ( $P < 0.05$ ) than that of heifers treated with CIDR devices (39/95, 41.1%), but they did not differ from those of hCG (49/96, 51.0%) or LH (44/97, 45.4%) treated heifers. Furthermore, pregnancy rate in heifers in the untreated control group was lower than expected (28/98, 28.6%) and was significantly lower ( $P < 0.05$ ) than all treated groups. Effects of veterinarian ( $P = 0.28$ ), embryo quality ( $P = 0.68$ ), day of the recipient's estrous cycle at the time of embryo transfer ( $P = 0.67$ ) and farm ( $P = 0.13$ ) did not influence conception rates. We concluded that treatments aiming at the increase of P4 after embryo transfer can improve conception rates in *Bos taurus* × *Bos indicus* cross-bred heifers receiving frozen/thawed embryos. Further experiments are needed to evaluate if these treatments would increase pregnancy rates in recipients with normal conception rates (around 50%).

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FIXED-TIME EMBRYO TRANSFER IN COWS TREATED WITH  
PROGESTERONE VAGINAL DEVICES AND INDUCED TO  
OVULATE WITH ESTRADIOL BENZOATE OR HCG

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Estrus detection efficiency has been an important factor affecting the profitability of embryo transfer programs in cattle. It has been shown recently that treatments with progesterone (P4) releasing devices and estradiol benzoate (EB) plus P4 (to control follicle wave emergence) and the subsequent application of EB (to synchronize ovulation) result in acceptable pregnancy rates after embryo transfer without estrus detection. An experiment was designed to compare pregnancy rates in cows treated with P4 releasing devices and EB plus P4, and induced to ovulate with either EB or hCG. The experiment was performed in non-lactating *Bos taurus* × *Bos indicus* crossbred beef cows with a body condition score between 2.5 and 3.5 (1–5 scale) in three replicates (Replicate 1,  $n = 59$ ; Replicate 2,  $n = 72$  and Replicate 3,  $n = 84$ ). At the beginning of each replicate (Day 0) all cows received a P4 device (1 g of P4, DIB, Syntex, Argentina) and 2 mg EB (Syntex, Argentina) plus 50 mg P4 i.m. (Lab. Río de Janeiro, Argentina). All cows received 400 IU of eCG i.m. (Novormón 5000, Syntex) plus 500 µg cloprostenol (Estroplan, Syntex) on Day 5 and DIB devices were removed on Day 8. Cows were randomly divided to receive 1 mg EB i.m. on Day 9 or 1500 IU hCG (Ovusyn, Syntex) on Day 10. Cows were not observed for signs of estrus and Day 10 was arbitrarily considered as the day of estrus. On Day 17, all recipients were examined by ultrasonography and those with a CL  $\geq 10$  mm in diameter received fresh embryos in replicate 1 and fresh or frozen/thawed Direct Transfer embryos in replicates 2 and 3. In all replicates, embryos were transferred non-surgically into the horn ipsilateral to the CL by two technicians. Ovarian ultrasonography (Concept MC with a 7.5 MHz transducer) was performed on Day 0, to determine ovarian status (only cows with a CL or a follicle  $>10$  mm and uterine tone were used), on Day 17, to measure CL diameter, and 60 days after embryo transfer, to determine pregnancy status. Mean CL diameters on Day 17 were compared between groups by Student's *t*-test and the proportions of recipients selected and pregnant in all replicates were analyzed by logistic regression (Infostat<sup>®</sup>). Mean ( $\pm$ S.E.M.) CL diameters on Day 17 were not different ( $P > 0.1$ ) between recipients induced to ovulate with EB ( $21.1 \pm 0.6$  mm) or hCG ( $21.3 \pm 0.5$  mm). There were no significant effects of replicate, fresh versus frozen embryos, embryo quality, or technician on pregnancy rates ( $P > 0.1$ ). Furthermore, pregnancy rates were not different between recipients induced to ovulate with EB or hCG ( $P > 0.1$ ). It was concluded that EB and hCG were equally efficacious in synchronizing ovulation of a dominant follicle of an EB-induced wave in *Bos taurus* × *Bos indicus* recipients. Results also support previous studies demonstrating that treatments with P4 releasing devices and EB can be used successfully to transfer bovine embryos at a self-appointed time, without the necessity of estrus detection.

Group	<i>n</i>	Transferred/ treated (%)	Pregnant/ transferred (%)	Pregnant/ treated (%)
EB	106	91/106 (85.8)	52/91 (57.1)	52/106 (49.1)
hCG	109	90/109 (82.5)	48/90 (53.3)	48/109 (44.0)

Proportions did not differ ( $P > 0.5$ ).

## ESTRADIOL PRODUCTION BY PORCINE PARTHENOGENETIC EMBRYOS DURING EARLY PREGNANCY

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Estradiol production by embryos during Days 12–16 after fertilization is an essential signal for establishment of the early phase of pregnancy in the pig. The presence of a minimum of four embryos, equally distributed in the uterine horns, is also requested for the maintenance of pregnancy. Embryos resulting from parthenogenetic activation successfully implanted after embryo transfer and developed to at least the stage of limb-bud formation [Kure-Bayashi et al., *Theriogenology* 2000;53:1105–1119] and were often used as “carriers” when embryos manipulated in vitro were transferred [King et al., *Reproduction* 2002;123:507–515]. However, the ability of parthenotes to initiate pregnancy has not been determined. Therefore, estradiol production by parthenotes was investigated and compared with normally fertilized embryos on Day 12. Oocytes matured (with a first polar body) in vitro were electrostimulated by two DC pulses (100 V/mm, 62  $\mu$ s) 1 s apart in a fusion medium (0.3 M mannitol, 0.1 mM MgSO<sub>4</sub>, 0.05 mM CaCl<sub>2</sub> and 0.1% PVA). After several washings, oocytes were transferred to NCSU 23 containing 10  $\mu$ g/ml cytochalasin B and incubated for 6 h. These oocytes were cultured in NCSU 23 without cytochalasin B for additional 22–24 h until transfer. Embryo transfers were performed surgically approximately 48 h after hCG injection. Twenty-two to forty-two cleaved embryos were transferred to both fallopian tubes through the infundibulum. The day of embryo transfer was assigned as Day 1. Recipients were slaughtered on Day 12 and both uterine horns were flushed with 50 ml of PBS. Recovered embryos were cultured individually with 500  $\mu$ l of 10% FBS DMEM/F12 (1:1) in 4-well dish for 24 h. At the end of embryo culture, spent media was collected and stored at –20 °C. Estradiol-17 $\beta$  concentrations in the spent media were analyzed by competitive EIA. In a second experiment, the recipient animals were housed and the estrous cycles were observed. Blood from ear veins was collected weekly from 1 to 15 weeks after transfer for the determination of plasma progesterone concentrations. In a preliminary experiment, 70.9% of oocytes had a detectable first polar body at the end of IVM. After electroactivation, 43.8% of oocytes developed to morphologically normal 2-cell embryos. Two recipients which received a total of 120 parthenotes were slaughtered on Day 12 of gestation and a total of 10 ovoid/spherical parthenotes was recovered. As a control, one artificially inseminated gilt was slaughtered on Day 12 of gestation, and eight spherical embryos were recovered. There was no difference in the amounts of estradiol between parthenotes and fertilized embryos (2.746  $\pm$  1.116 ng/embryo/day versus 2.679  $\pm$  1.000 ng/embryo/day). In the second experiment, three recipients were used. One recipient, in which 48 parthenotes were transferred, returned to estrus on Day 34 after transfer. The remaining two recipients, with 100 parthenotes transferred in each recipient, were diagnosed as pseudopregnant by high plasma progesterone ( $\geq$ 7 pg/ml) and expanded uterine lumen, detected by using an echo apparatus until Day 103. In conclusion, porcine parthenotes produced similar amounts of estradiol as fertilized embryos during early pregnancy (Day 12). Results in this study will be helpful in determining the number of parthenotes co-transferred with target embryos.

## ENDOSCOPIC COLLECTION OF BOVINE EMBRYOS AT DIFFERENT TUBAL STAGES WITH RESPECT TO FSH AND ECG TREATMENT

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The aim of our study was the use of different gonadotropins for superovulation in cattle followed by endoscopic embryo collection at different time intervals. A total of 109 heifers and cows were prepared for superovulation by the removal of dominant follicles on Day 7 after heat. Thirty-six hours later, 53 animals were stimulated by administration of 400 mg FSH (Folltropin-V<sup>®</sup>, Vetrepharm) over a period of 4 days, and 66 animals received a single injection of 2000 IU eCG (Intergonan 6000<sup>®</sup>, Intervet). On Day 12, luteolysis was induced by PGF2alpha (Estrumate<sup>®</sup>, Essex) application. On Day 13, 3000 IU hCG (Ovogest 5000<sup>®</sup>, Intervet) was administered intravenously. The animals were inseminated 0, 12 and 24 h after hCG treatment. Endoscopic embryo flushings were done 24–108 h after the second insemination. During laparoscopy, the number of ovulation sites and non-ovulated follicles (cysts) was counted. Superovulation occurred more frequently after the administration of FSH versus eCG (51 of 53 versus 54 of 66, respectively;  $P \leq 0.05$ ; Chi-square test). A total of 1394 ovulation sites and 418 cysts were observed. Animals administered FSH versus eCG had more ovulation sites (means of 17.8 and 9.7 per animal, respectively;  $P \leq 0.001$ ; *t*-test) and more cysts (means: 6.0 and 2.3;  $P \leq 0.001$ ; *t*-test). A total of 1267 embryos was flushed from 105 animals. In FSH-treated animals, an average of 16.0 embryos was collected per animal (12.6 of which were graded as excellent quality); this was almost double the average number of embryos collected from eCG-treated animals (8.4 per animal with 7.1 not degenerated;  $P \leq 0.001$ ; *t*-test). FSH-treated animals yielded a higher percentage of degenerated embryos than animals treated with eCG (21.4% versus 15.0%;  $P \leq 0.01$ ; Chi-square test). Developmental stages of embryos did not differ between the two groups. Mostly zygotes were observed at 24–40 h, 2-cell-embryos at 45–55 h, 4-cell-embryos at 50–60 h, 8-cell-embryos at 75–85 h, and 16-cell-embryos at 95–105 h. Our results clearly demonstrate that the overall efficiency of stimulation with FSH is nearly twice as high compared to eCG, despite the fact that FSH results in more follicles that fail to ovulate and a slightly more degenerated embryos. Laparoscopic investigation of the eCG-group revealed a tremendous variation in ovarian structures. Corpora lutea and cysts on the same ovary were different in size, and cysts were often filled with blood, which was not observed in the FSH-group. We concluded that animals treated with FSH versus hCG responded to treatment in a more physiological manner and produced a greater number of embryos.

## EFFECT OF EMBRYO AGE ON VIABILITY OF EQUINE EMBRYOS AFTER COLD STORAGE IN TWO SYSTEMS

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Embryo age is one factor that could affect pregnancy rates after cold storage. The aim of this study was to compare viability of equine embryos collected on Day 7 or 8 after ovulation and maintained for 6 and 24 h in two systems of storage. Equine embryos ( $n = 100$ ) were recovered on Day 7 or 8 after ovulation using 1.5 l of warmed embryo flushing solution with albumin (0.1% w/v) and kanamycine sulphate (25 mg/l) (Emcare Flushing Solution, ICP, Auckland, NZ). Embryo scores ranged between 1 and 4, and diameters ranged from 200 to 1700  $\mu\text{m}$ . Embryos, collected on Day 7 or 8, were assigned to five treatments ( $n = 10/\text{treatment}$  for Day 7 or 8): (1) evaluated immediately after collection (Group-0 h); (2) stored for 6 h (free-floating) in a 0.5 l bottle of Emcare Flushing Solution (EFS) (Group-B-6 h) in a refrigerator at 5 °C; (3) stored for 24 h in a 0.5 l bottle of EFS (Group-B-24 h) in a refrigerator at 5 °C; (4) stored in 5 ml Emcare Holding Solution (EHS) in an Equitainer Hamilton-Thorn, S. Hamilton, MA) (Group-E-24 h); or (5) stored in 5 ml Ham's F-10 (Group-H-24 h) in an Equitainer. After collection (Treatment 1) or storage, embryos were washed in EHS then placed in EHS with 1  $\mu\text{g}/\text{ml}$  DAPI (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature. The number of dead cells (DAPI positive, fluorescent cells) per embryo was determined using an inverted fluorescence microscope. Differences between groups were analyzed using Kruskal–Wallis test (significance at  $P \leq 0.05$ ). The mean diameter of embryos for all groups, the mean ( $\pm$ S.E.M.) number of fluorescent dead cells per embryo and the mean ( $\pm$ S.E.M.) number of dead cells/ $\text{mm}^2$  for embryos collected on Day 7 or 8 are summarized in the table. To compensate for differences in size, the ratio between the number of dead cells and total number of cells per embryo was estimated by calculating the outer surface of the embryo and correlating the number of dead cells to a unit of surface. Within each collection day, the mean number of dead cells (per embryo and per unit of surface) in Groups-0 h and B-6 h was similar but was significantly lower than for embryos stored for 24 h in Groups B-24 h, E-24 h and H-24 h. The mean number of dead cells (per embryo and per unit of surface) was similar for Groups E-24 h and H-24 h, but was significantly lower than for embryos stored in a bottle at 5 °C for 24 h. Within each storage system (0, B-6, B-24, E-24 and H-24 h) no significant difference in the number of dead cells/ $\text{mm}^2$  between embryos collected on Day 7 versus Day 8 was observed. In the present experiment, embryos collected on Day 7 versus Day 8 responded in a similar fashion to cold storage; and the number of dead cells was not different for embryos immediately after collection or stored in a 0.5 l bottle of EFS at 5 °C for 6 h. This system offers an alternative to storage in an Equitainer.

	Diameter of embryos		Number of dead cells/embryos		Number of dead cells/ $\text{mm}^2$	
	Day 7	Day 8	Day 7	Day 8	Day 7	Day 8
Group-0 h	387 $\pm$ 56.3 <sup>a</sup>	940 $\pm$ 79 <sup>b</sup>	2 $\pm$ 0.9 <sup>a</sup>	46 $\pm$ 28.7 <sup>d</sup>	12.47 $\pm$ 6.6 <sup>a</sup>	24 $\pm$ 15.6 <sup>a</sup>
Group-B-6 h	388 $\pm$ 33.1 <sup>a</sup>	966 $\pm$ 92 <sup>b</sup>	6.6 $\pm$ 1.3 <sup>a</sup>	105.2 $\pm$ 51.8 <sup>d</sup>	25.44 $\pm$ 4.6 <sup>a</sup>	33.1 $\pm$ 15 <sup>a</sup>
Group-B-24 h	374 $\pm$ 37.6 <sup>a</sup>	863 $\pm$ 130 <sup>b</sup>	71.9 $\pm$ 21.6 <sup>b</sup>	400 $\pm$ 125 <sup>e</sup>	140 $\pm$ 48.3 <sup>b</sup>	196 $\pm$ 67.5 <sup>b</sup>
Group-E-24 h	418 $\pm$ 40.8 <sup>a</sup>	1018 $\pm$ 124 <sup>b</sup>	29.9 $\pm$ 10.1 <sup>c</sup>	175.2 $\pm$ 52.3 <sup>f</sup>	64.2 $\pm$ 25.3 <sup>c</sup>	45.6 $\pm$ 12.6 <sup>c</sup>
Group-H-24 h	390 $\pm$ 43.5 <sup>a</sup>	1080 $\pm$ 82.9 <sup>b</sup>	22.7 $\pm$ 8.2 <sup>c</sup>	272.4 $\pm$ 53.2 <sup>f</sup>	77.5 $\pm$ 32.6 <sup>c</sup>	64.2 $\pm$ 15.6 <sup>c</sup>

Different superscripts indicate significant difference ( $P \leq 0.05$ ).

FIRST PREGNANCIES ESTABLISHED FROM VITRIFIED BLASTOCYSTS  
ENTIRELY PRODUCED IN VITRO IN MEDITERRANEAN ITALIAN  
BUFFALO COWS (*BUBALUS BUBALIS*)

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The Ovum Pick-Up technique represents a valid procedure to produce a great number of embryos from live donors. The aim of this study was to assess the capability of buffalo embryos, entirely produced in vitro, to establish pregnancies. In March 2002, 12 lactating pluriparous buffalo cows underwent repeated transvaginal follicular aspiration bi-weekly for seven sessions. The cumulus-oocyte complexes (COCs) recovered were stored in hepes buffered TCM199 supplemented with 0.5 µg/ml FSH, 5 µg/ml LH, 1 µg/ml 17-β-estradiol, 50 µM cysteamine [Gasparrini et al., *Theriogenology* 2000;54:1537–1542] and 10% fetal calf serum (FCS) at 39 °C in a portable incubator and moved to the lab within 4–6 h. Maturation was carried out in bicarbonate buffered TCM199 supplemented with hormones, FCS and cysteamine in the same concentration previously described under 5% CO<sub>2</sub> in humidified air at 38.5 °C. After 22–24 h, matured COCs were in vitro fertilized in a modified TALP supplemented with 0.2 mM penicillamine, 0.1 mM hypotaurine and 0.01 mM heparin. Sperm was treated by swim-up procedure for a final sperm fertilizing concentration of  $2 \times 10^6$  ml<sup>-1</sup>. Fertilizing drops were incubated under the same gas atmosphere as for in vitro maturation. After 20–22 h of co-incubation presumptive zygotes were moved into SOF medium, supplemented with essential and nonessential aminoacids, in a gas atmosphere of 5% CO<sub>2</sub>, 7% O<sub>2</sub>, and 88% N<sub>2</sub>. At Day 5 (Day 0 = IVF day), embryos were transferred into fresh droplets until Day 7, when final embryo yield was evaluated. Embryos were transferred either after vitrification [Naitana et al., *Theriogenology* 46:813–824] or fresh; fresh embryos were transported to the farm in SOF medium in a CO<sub>2</sub> portable incubator. Embryo transfer was performed at Day 7 of estrus cycle of recipients, that were synchronized by two following injections of Prostaglandin (Gellini, Italy) 11 days apart. Pregnancy rate was assessed at 40 days and confirmed at 90 days by clinical examination. A total of 121 good quality oocytes was recovered and 19.8% total blastocyst rate was obtained. Seven blastocysts were transferred fresh and eight after vitrification and warming. The total blastocyst rate (19.8%) was lower than that previously reported (33.1%) in our laboratory [Neglia et al., *Theriogenology*, in press], probably because a hemorrhagic diarrhea was found in donor animals. Three pregnancies were established after transfer of vitrified embryos whereas no pregnancies were obtained from fresh embryos. The pregnant recipients, which are currently monitored, should deliver in January 2003. This is the first report of pregnancy from vitrified buffalo embryos entirely produced in vitro. In 1997, Boni et al. [Boni et al., *Proceedings World Buffalo Congress* 787–792] reported two pregnancies (interrupted at 3 and 5 months) obtained after transfer of two fresh morulae. In buffalo species only three calves have been produced from frozen embryos [Galli et al., *Theriogenology* 49:400], which were cultured in ligated ewe oviduct. The failure in pregnancies with fresh transferred embryos may be due to an improper transport technique and hence to pH changes in the culture medium.

## ENHANCED VIABILITY TO OBTAIN BOVINE BLASTOCYST AFTER RETINOL ADDITION TO AN EMBRYONIC CULTURE MEDIUM

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Vitamin A fulfills essential roles in the physiology of vertebrates, being involved in cell growth and differentiation, as well as in embryonic development. In vitro, retinol increased blastocyst development rates for cows when used in maturation medium [BORTOLOTO, 2000/Thesis M.Sc. UFSM-RS/Brazil, 76; DUQUE et al., 2002/Theriogenology 57:364 (abstract)]. Retinol has also had a beneficial effect when added to an embryonic culture medium with an oviductal cell monolayer [MONTAGNER, 1999/Thesis M.Sc. UFSM-RS/Brazil 1999;82]; however, the effect of this metabolite is not equivalent in the absence of a cell monolayer [LIMA et al., Rev Bras Reprod Anim 2002;26:163–165]. Therefore, the effect of retinol (0.28 µg/ml) on the development of bovine embryos was studied in cultures with KSOM and SOF in presence or absence of oviductal co-culture epithelial cells (MCO). Ovaries were transported to the laboratory in a thermal container at 30 °C. Oocytes were obtained from follicles measuring 2 to 5 mm in diameter. A total of 2448 oocytes were selected for in vitro maturation and incubated in TCM 199/25 mM Hepes in 39 °C in air with 5% CO<sub>2</sub> and 95% of humidity for 24 h. In vitro fertilization was performed in modified DM medium. At the end of an 18-h culture period, cumulus cells were removed and presumptive zygotes were randomly allocated to eight treatments (KSOM + Retinol, SOF + Retinol, KSOM/Control, SOF/Control, KSOM + Retinol + MCO, SOF + Retinol + MCO, KSOM + MCO/Control, SOF + MCO/Control). Incubations in the absence of MCO were carried out at 39 °C in a moist 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> atmosphere. Co-cultured embryos with MCO were incubated at 39 °C in a moist 5% CO<sub>2</sub> atmosphere. Culture media were changed (30%) after 48 h with removal of non-cleaved oocytes, and the embryos were maintained in culture for additional 7 days. Blastocyst formation rates in the absence of MCO were 11% (KSOM + Retinol), 13% (SOF + Retinol), 12% (KSOM/Control) and 13% (SOF/Control). In the presence of MCO, the blastocyst formation rates were 21% (KSOM + Retinol + MCO), 20% (SOF + Retinol + MCO), 13% (KSOM + MCO/Control) and 12% (SOF + MCO/Control). These results demonstrated that retinol associated with MCO had a significant ( $P < 0.001$ ) effect on bovine early embryonic development, under the conditions tested, and potentially could be used to enhance in vitro embryonic production.

EFFECT OF CO-TRANSFER OF BLASTOCYSTS DERIVED FROM  
PARTHENOGENESIS WITH RECONSTRUCTED EMBRYOS  
FROM BOVINE SOMATIC CELLS

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In the production of calves by nuclear transfer, low conception rates are one of the problems needed to be solved. Conception of split embryos was improved by co-transfer with trophoblastic vesicles (TB) in one report [Hashiyada et al., *Theriogenology* 2002;57:543]. Parthenogenetically activated oocytes can develop to blastocysts. Although these blastocysts do not develop into fetuses, they could have a luteotrophic effect similar to TB when introduced into the bovine uterus. In this study, we co-transferred parthenogenetic blastocysts (PB) with reconstructed embryos from bovine somatic cells, and studied if the co-transfer improved the incidence of reconstructed embryos to produce cloned calves. Reconstructed embryos were produced using cumulus-cells (Holstein and Japanese Black) cultured for 3–5 passages as donor cells [Goto et al., *Anim Sci J* 1999;70:243–245]. PBs were produced using a method previously reported [Aoyagi et al., *J Reprod Dev* 1994;40:j5–j11]. Cumulus–oocyte complexes were collected from slaughtered ovaries and were incubated for maturation in TCM-199 + 5% calf serum (CS). After 24 h of incubation, the oocytes were exposed to 5  $\mu$ M calcium ionophore in PBS for 5 min, then electrically stimulated twice using a single pulse of 75 for 50  $\mu$ s in Zimmerman medium. The incubation was continued in 10  $\mu$ M cycloheximide + 2.5  $\mu$ g/ml cytochalasin D in TCM-199 + 5% CS until 30 h of incubation, in 2.5  $\mu$ g/ml cytochalasin D in TCM-199 + 5% CS until 42 h, then in CR1aa + 5% CS until the co-transfer. Both the reconstructed embryos and the PBs were produced at the same time and incubated for 7–8 days after maturation. Twenty-four, non-lactating Holstein cows between Days 7 and 9 (Day 0 = estrus) were divided into two groups: co-transfer group and control group. PBs were co-transferred fresh or after storage by vitrification [Saito et al., *Cryobiol Cryotech* 1997;43:34–39]. Thirteen cows in the co-transfer group received 1 (fresh) to 2 (vitrified) reconstructed blastocysts (7 fresh and 6 vitrified) and 1–3 PBs. And, 11 cows in the control group received 1 (fresh) to 2 (vitrified) re-constructed blastocysts (five fresh and six vitrified). Pregnancies were diagnosed using ultrasonography at Days 40 and 60. In the co-transfer group, seven recipients (three fresh and four vitrified) become pregnant, and four recipients (two fresh and two vitrified) completed gestation and delivered calves. In the control group, three (two fresh and one vitrified) recipients become pregnant, and one recipient (vitrified) completed gestation and delivered a calf. These results indicate that co-transfer of PBs could be used favorably for the conception of reconstructed embryos and production of cloned calves. Further studies are needed to validate the usefulness of co-transfer.

## PREGNANCY RATES AFTER THE TRANSFER OF EMBRYOS INTO RECIPIENT MARES OF DIFFERENT REPRODUCTIVE STATUS

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Embryo transfer has the potential to improve reproductive efficiency in mares and to help accelerate genetic improvement in less time than required for natural improvement. However, the selection of recipient mares, a critical factor which may limit the efficiency of embryo transfer [Taveiros et al., *Rev Brasil Reprod Anim* 1999;23:391–393], continues to be a common topic for research, particularly in relation to reproductive status. Ultrasonography has established new parameters for the control of various reproductive events and has contributed to increase the effectiveness of ET, since early diagnosis of reproductive problems, embryonic loss, twin gestation and monitoring of embryonic and fetal development can be performed. Thus, the objectives of this study were to evaluate the effectiveness of equine embryo transfer using recipient mares of different reproductive status and to compare the development of transferred embryos in the recipients. Ten pluriparous donors, aged from 7 to 10 years, and 103 recipients (31 being nuliparous, 34 lactating pluriparous, and 38 non-lactating pluriparous) were used in this study. Embryo recovery was performed on Day 8 after ovulation and pregnancy was confirmed by ultrasound 6 days after transfer. Pregnancy monitoring was performed on the 10th, 12th, 14th, 16th, 18th, 20th, 25th and 30th days after embryo transfer. From 200 flushes performed, 115 (53.2%) provided embryos, 12 being degenerate and 103 (89.5%) in excellent, good or fair conditions. From the 103 embryo transfers carried out, 49.5% ( $n = 51$ ) pregnancies were confirmed by ultrasound within 30 days, 51.6% ( $n = 16$ ) in the nuliparous recipients, 47.0% ( $n = 16$ ) in the lactating pluriparous recipients and 50.0% ( $n = 19$ ) in the non-lactating pluriparous recipients. These results demonstrated that differences in reproductive status of recipient mares did not affect pregnancy status and embryo development during the first 30 days of pregnancy.

## NON-SURGICAL TRANSFER OF PORCINE EMBRYOS

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Recent progress in porcine biotechniques resulted in piglets following non-surgical embryo transfer. For practical applications, however, the efficiency of the process needs to be increased. Purposes of this study were to determine the optimal volume of transfer medium in non-surgical porcine embryo transfer using conventional artificial insemination catheter (Experiment 1), and to investigate the effect of anesthesia treatment to recipient gilts at embryo transfer (Experiment 2). In Experiment 1, prepubertal gilts (100–110 kg body weight, mainly Landrace × Yorkshire × Duroc) were used as donors and recipients. The donors were administered 1500 IU of eCG intramuscularly, followed by intramuscular injection of 750 IU of hCG 72 h later. The donors were then inseminated at 24, 36 and 48 h after the injection of the hCG. Donors were slaughtered on Day 5 of the estrous cycle (Day 0 = onset of estrus), and embryos were recovered by uterine flushing. The recipients were hormonally synchronized similarly to the donors, but 1000 IU of eCG and 500 IU of hCG were administered. On Day 4 of estrous cycle, 14 morulae and blastocysts-stage embryos were transferred with 5, 15 or 30 ml of transfer medium (m-KRB with antibiotics) [J Anim Sci 1978;46:1043–1053] into each recipient (10 transfers/group). During embryo transfer the recipients were kept in a standing position by belts without anesthesia. The transfer was performed with a conventional artificial insemination catheter, a 3-way stopcock and plastic syringes. All recipients were slaughtered on Days 33–37 after hCG injection in order to examine the number and position of fetuses. In Experiment 2, effect of anesthesia during embryo transfer was investigated. Twenty morulae and blastocysts in 15-ml of transfer medium were transferred as above, with or without anesthesia (9 transfers/treatment). For the anesthesia treatment, ketamine hydrochloride (5.76 mg/kg body weight) was administered intramuscularly. Pregnant recipients were followed to term, and litter size and body weight of piglets were examined. In Experiment 1, pregnancy rates for the 5-, 15-, and 30-ml groups as 80% (8/10), 70% (7/10) and 50% (5/10), respectively, without significant differences. The average number of fetuses for the 5-, 15-, and 30-ml groups was 5.0, 5.3 and 3.4, respectively. The number of fetuses for the 15-ml group was higher ( $P < 0.05$ ;  $t$ -test) than for the 30-ml group. In Experiment 2, the farrowing rates were 67% (6/9) and 56% (5/9) for groups with or without anesthesia, respectively, without a significant difference. In contrast, the average litter size for the anesthesia group (7.5) was significantly higher ( $P < 0.01$ ;  $t$ -test) than for the no-anesthesia group (4.4). In conclusion, high pregnancy rates and large number of fetuses were obtained when embryos were transferred non-surgically, with a conventional artificial insemination catheter and in 15-ml transfer medium, into recipients in standing position under anesthesia.

# Embryonic Stem Cells

## PRODUCTION OF CHIMERIC CHICKENS USING EMBRYONIC STEM CELLS

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The area pellucida was isolated from stage X (E-G&K) Barred Rock embryos. The blastodermal cells were mechanically dispersed into a single-cell suspension and seeded at a concentration of 30,000 cells/cm<sup>2</sup> or as single embryos on either polyester coated inserts (transwell#, Costar) or mitotically inactivated STO cells. STO cells were grown to confluency, treated with 10 µg/ml mitomycin for 3–4 h, washed, trypsinized and seeded on gelatin coated dishes. The cells were cultured according to Petite and Yang, USA patent 5,340,740, NCSU, 1994. Chicken embryonic stem (ES) cells became visible 3–7 days after seeding the blastodermal cells. The cells are small with a large nucleus and a pronounced nucleolus. Chicken ES cells grow in single layer colonies with clearly visible individual cells. The cells stain positively for the markers of pluripotency: MA-1, SS A-1 and alkaline phosphatase. Chicken ES cells were derived from single (10 cell lines out of 101 cultures set up) and pooled embryos (9 out of 90) on either polyester coated inserts (9 out of 62) and on mitotically inactivated STO cells (10 out of 129). When confluent, cultures are briefly treated with trypsin and passaged 1:2 in small clumps. A total of 19 separate S cell cultures was derived of which 14 were tested for their developmental potential by injection into recipient embryos. The Barred Rock S cells were injected into White Leghorn embryos, which are homozygous dominant at the dominant, white locus. A chimeric chicken will display black feathers from the S cells and white feathers from the recipient embryo. One to five microliter of an S cell suspension, containing 2000–5000 embryonic stem cells, was injected into the subgerminal cavity of the recipient embryos. Eleven of the 14 tested cultures contributed to recipient embryos as determined by feather pigmentation. Some of the cell lines have been kept in culture for over 9 months and 80 population doublings without loss of developmental potential. The cells can be cryopreserved and when injected into compromised recipient embryos have the potential to substantially contribute to somatic tissues. Chimeras are hatched that are indistinguishable from pure Barred Rock chicks.

EFFECTS OF THE CULTURE PERIOD AND PASSAGE NUMBER ON THE  
CAPACITY OF CHIMERA PARTICIPATION OF INNER CELL MASS  
DERIVING CELLS FROM PORCINE EMBRYOS

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Mammalian embryonic stem (ES) cells are pluripotent cells derived from inner cell mass (ICM) of the blastocyst [Evans and Kaufman, *Nature* 1981;292:154–156]. Under suitable conditions, S cells are able to proliferate continuously without differentiation in vitro. Their capacity of pluripotency in differentiation will be resumed when they are reintroduced into embryos, and then they will contribute to the embryonic development to form a chimeric individual. This ex vivo manipulation of S cells mainly established from the studies of the mouse. However, porcine ICM-derived cell lines, even possessed similar cellular morphology and in vitro behavior to those of murine S cells, had a less efficiency in chimera formation when reintroduced into the embryos [Anderson et al., *Theriogenology* 1994;42:204–212; Chen et al., *Theriogenology* 1999;52:195–212]. This study was undertaken to determine the influences of the passage number and the duration of in vitro culture on the capacity of porcine ICM-derived cells from Meisan pigs in participation of chimeric embryo formation with host blastocysts of the same breed. Clumps of ICM-derived cells with 10–15 cells each at different culturing periods (2, 4, and 6 days after passage) of different passages (0, 6th, 9th, 12th, and 15th) were labeled with 0.6 mg/ml fluorescein isothiocyanate (FITC, Butcher C and Weissman IL, *J Immunol Methods* 1980;37:97–108] and then subjected to blastocyst injection. The integration of FITC-labeled ICM-derived cells into the ICM of the host blastocysts was determined under a fluorescence microscope 48 h after injection. The results showed that the number of passage had no detrimental effects on the integration ability of porcine ICM-derived cells up to the 15th passage ( $P > 0.05$ ). However, elongating the culture period up to 6 days in each passage would impair the capacity of porcine ICM-derived cells to integrate into the ICM of the host blastocyst ( $P < 0.05$ ). Therefore, the culture period of the porcine ICM-derived cells in each passage should not be longer than 4 days if high efficiency of chimera production was to be achieved.

# Epidemiology/Diseases

## TRICHOMONAS FETUS IN IN VITRO FERTILIZATION SYSTEM

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*Trichomonas fetus* (*T. fetus*), a world-wide distributed parasitic protozoan, is a cause of infertility and abortion. There is no documented information on the susceptibility of bovine embryos to the parasite. Here we report on the embryonic development of preimplantation embryos and their sanitary status after experimental exposure to *T. fetus* during fertilization. Briefly, in vitro matured COC were fertilized with approximately  $1 \times 10^6 \text{ ml}^{-1}$  motile frozen-thawed sperm cells obtained after the swim up procedure in 50  $\mu\text{l}$  of modified Thyrode medium (TALP). In the experimental group, *T. fetus* at approximately  $1 \times 10^5 \text{ ml}^{-1}$  (ATCC strain) was added to the fertilization droplets. After 18 h, presumptive zygotes were freed of cumulus cells, transferred to 500  $\mu\text{l}$  of modified synthetic oviductal fluid (SOF) and incubated for 7 days under silicone oil at 38.5 °C in 5% CO<sub>2</sub>, 5% oxygen and 90% nitrogen [Holm et al., Theriogenology 1999;52:683–700]. Cultures were examined daily for the presence or absence of motile parasites. After multiple washing (trypsin not included), as recommended by IETS, the resulting embryos were examined with a light microscope, a scanning electron microscope (SEM) and a transmission electron microscope (TEM) for the presence of *T. fetus*. The percentages of cleaved zygotes, blastocysts and hatched embryos resulting from culture of experimental and uninfected control groups were 57% ( $n = 140$ ), 35% ( $n = 28$ ) and 46% ( $n = 13$ ) compared to 60% ( $n = 151$ ), 32% ( $n = 29$ ) and 45% ( $n = 13$ ) respectively ( $P > 0.05$ , Chi-square test). No motile parasites were observed in SOF culture drops beyond 72 h post IVF. Exposure of hatched blastocysts ( $n = 25$ ) to *T. fetus* did not result in degeneration of trophoblastic cells or death of embryos. *Trichomonas fetus* was not detected in embryonic cells of ZP-intact or hatched embryos by TEM ( $n = 10$ ). However, S.E.M. revealed a single or small groups of parasites on the surface of unwashed, as well as washed, ZP-intact ( $n = 15$ ) and ZP-free ( $n = 15$ ) embryos. It is concluded that *T. fetus* has no effect on the development of IVF embryos and the potential risk of transmission of trichomoniasis is unlikely due to the limited survival of the parasite in IVF culture conditions.

PREGNANCIES ESTABLISHED AFTER TREATMENT OF IVF BOVINE  
EMBRYOS WITH AN ANTIVIRAL AGENT

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Bovine viral diarrhea virus (BVDV) replicates in embryo culture systems and remains associated with developing IVF bovine embryos despite washing and trypsin treatment. Previous research [Givens et al., *Theriogenology* 2002;57:572 (abstract)] determined that 2-(4-[2-imidazoliny]phenyl)-5-(4-methoxyphenyl) furan (DB606) can inhibit replication of BVDV in primary cultures of uterine tubal cells. The objective of this study was to evaluate the capability of IVF embryos to initiate and maintain pregnancy after exposure to similar antiviral concentrations of DB606 during in vitro culture. During 22 replicates, oocytes were obtained from cows via transvaginal, ultrasound-guided follicular aspiration. Presumptive zygotes that resulted from fertilization of these oocytes were cultured for 7 days in medium supplemented with 0.4  $\mu$ M DB606 or medium lacking antiviral agent. All blastocysts were individually transferred non-surgically into the uterus of a synchronized recipient. The pregnancy status of recipients was evaluated by transrectal ultrasonography at 21–23 days after embryo transfer (gestational Days 28–30) and subsequently every 27–34 days. Pregnancy status was determined on initial examination by visualization of fetal heartbeat. Rates of blastocyst development, pregnancy per cultured zygote and pregnancy per transferred embryo were compared using the  $\chi^2$ -test statistic. The development of blastocysts and establishment and maintenance of pregnancies are documented in the table. Of pregnancies resulting from IVF embryos exposed to DB606, 3 of 20 expired prior to gestational Day 64. Of pregnancies resulting from negative control embryos, four expired prior to gestational Day 57 and an additional two expired prior to gestational Day 112. Differences in rates of blastocyst development, pregnancy per cultured zygote and pregnancy per transferred embryo were not significant ( $P = 0.05$ ). Pending birth of normal offspring, preliminary results indicate that bovine embryo cultures might be safely supplemented with effective concentrations of antiviral agent. Addition of this antiviral agent might prevent viral transmission if BVDV were inadvertently introduced into the embryo culture system.

Treatment	Presumptive zygotes cultured	Blastocysts transferred	Pregnancies established	Pregnancies maintained
DB606	425	61	21	18
Negative control	424	49	24	18

## THE IMPACTS OF XENOESTROGENS ON MOUSE EMBRYOS AND HUMAN ENDOMETRIAL EPITHELIAL CELLS IN VITRO

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Previous studies on the effects of xenoestrogens on pre-implantation embryos and endometrial epithelial cells have suggested that xenoestrogens have estrogenicity and detrimental effects on embryos and endometrial epithelial cell line in vitro. However, information about the impacts of xenoestrogens on development of mouse embryos and proliferation of epithelial cells in human endometrium is lacking. Embryos are highly susceptible to xenoestrogens, which highlights the developmental toxicity of the compounds. Endometrial epithelial cells are vital to important biological processes such as implantation and supporting early embryonic development. Therefore, disturbances in the functions of these cells can lead to reduced fertility. This study was carried out to examine the impacts of xenoestrogens on development of mouse embryos, and on proliferation and ability to support embryonic development of human endometrial epithelial cells in vitro. Bisphenol A (BPA) and aroclor 1254 (a congener of polychlorinated biphenyls) were used as xenoestrogens in this study. Mouse 2-cell embryos were cultured with or without human endometrial epithelial cells in the medium containing xenoestrogens. Also, human endometrial epithelial cells were cultured in increasing xenoestrogen concentrations. The developmental stages of embryos, cell number, cell viability and estrogen receptor expression of the cells were examined at 24 h interval. Xenoestrogens reduced the rates of development to A8-cell at 24 h, ABlastocyst at 48 h and AHatching blastocyst at 72 h significantly at  $10^{-4}$  M (BPA) or 1  $\mu\text{g}/\text{ml}$  (aroclor 1254) in both with and without human endometrial epithelial cells ( $P < 0.05$ ). The blastulation observed at  $10^{-4}$  M (BPA) or 1  $\mu\text{g}/\text{ml}$  (aroclor 1254) in the co-culture was significantly increased compared to the culture with medium alone ( $P < 0.05$ ). Xenoestrogens inhibited the growth of human endometrial epithelial cells in dose dependent manner, which was statistically significant at  $10^{-6}$  (48 and 72 h),  $10^{-4}$  M (24, 48 and 72 h) in BPA-groups or 0.2 (48 and 72 h), 1  $\mu\text{g}/\text{ml}$  (24, 48 and 72 h) in aroclor 1254-groups ( $P < 0.05$ ). This inhibition was observed in the cells from the proliferative phase. However, cell viability had no difference between control and the treatments. Estrogen receptor was expressed independently of xenoestrogen concentrations. Xenoestrogens directly reduced the developmental rates of mouse embryos in dose dependent manner. Also, xenoestrogens inhibited the proliferation of human endometrial epithelial cell from proliferative phase while having no impact on viability and estrogen receptor expression. Thus, the delay of development and proliferation, due to the impacts of xenoestrogen, might result in the asynchrony between blastocyst and endometrium followed by decrease in fertility.

## TRYPsin ACTIVITY AFTER PROLONGED REFRIGERATED STORAGE

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According to the *Manual of the International Embryo Transfer Society* (Stringfellow and Seidel (Eds.), 3rd Edition, 1998), it is important to store trypsin as a frozen stock and to prepare working solutions immediately before their use for disinfecting embryos of specific pathogens. However, it is evident that many practitioners do not follow such guidelines and some are known to refrigerate and re-use trypsin solutions for prolonged periods. Since it is not possible to enforce or ensure that trypsin solutions are prepared properly, there is a potential risk that disease transmission will occur via embryo transfer if the embryos were processed using trypsin solutions that were unknowingly inactive. The objective of this practical study, therefore, was to test the activity of trypsin after prolonged refrigerated storage. Aliquots of 0.25% trypsin (T-4549, Sigma Chemical Co., St. Louis, MO, USA) were immediately frozen (to serve as a positive control) and the remaining volume was placed in a conventional refrigerator. Aliquots of the refrigerated and frozen (freshly thawed) working solutions were removed weekly, warmed at 38 °C for 30 min, and then used to dissociate confluent Buffalo rat liver (BRL) cell monolayers cultured in Falcon 35 mm 6-well plates. The time taken from the point BRL cells were initially affected (cells rounding and lifting) to the time that >90% were dissociated and detached from the surface of the culture dishes were recorded and compared between the refrigerated and frozen-thawed trypsin aliquots. After 5 months of the weekly exercise, the refrigerated trypsin solution would dissociate and detach the confluent BRL cell monolayers within the same timeframe from initial exposure as the freshly prepared (frozen-thawed) trypsin aliquot ( $P > 0.05$ ,  $t$ -test), [Table 1](#).

In conclusion, it is evident by the results of this study that trypsin activity, as measured by the time taken to dissociate and detach >90% of confluent BRL cell monolayers, is not depleted after prolonged refrigerated storage of up to 5 months. In practice, however, it is important to stress that proper aseptic handling be maintained to avoid the risk of contaminating refrigerated trypsin solutions with psychrophilic bacteria and fungi.

Table 1

Mean times for confluent BRL cell monolayers to detach after exposure 2.5% trypsin stored at -20 °C vs. 4 °C for 5 months

Trypsin storage (°C)	Initial time (s)	Final time (s)
-20	41.6 ± 4.6	238.4 ± 23.2
4	36 ± 3.3	236.2 ± 22.2

(Initial = first sign of cells rounding and lifting; final ≥90% cells detached).

## SUSCEPTIBILITY OF IN VITRO IMMATURED OOCYTES TO NON-CYTOPATHOGENIC BOVINE VIRAL DIARRHEA VIRUS

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Non-cytopathogenic bovine viral diarrhea virus (NCP-BVDV) may infect primary and secondary oocytes in persistently infected cattle [Brownlie et al., *Vet Rec* 1997;141:335–337, Fray et al., *Vet Pathol* 1998;35:253–259]. In an in vitro study reported by Vanroose et al. [*Bio Reprod* 1998;58:857–866] BVDV was not detected in matured oocytes after exposure of mature ZP-free bovine oocytes to the virus. However, it is still not known whether the NCP-BVDV can infect immatured oocytes (IO). Here we report the results of preliminary investigation of the replication of BVDV in ZP-free and ZP-damaged IO during incubation in vitro. Duplicate experiments were performed, each involving ZP-free and ZP-damaged IO. ZP removal was performed by treatment with 3% sodium citrate and 0.1% pronase. Multiple piercing of ZP with microneedle was used to damage the integrity of the ZP. Both groups of IO were then exposed to BVDV-NY1 strain with a titer ( $10^6$  TCID<sub>50</sub>/ml). Twenty to thirty oocytes were collected immediately (0 h) and 24 h ( $n = 40–60$ ) after virus exposure, then washed three times with TCM-199 medium supplemented with 2% fetal bovine serum (FBS) to remove the unbound free virus, and stored at  $-80\text{ }^\circ\text{C}$  until assayed for BVDV. The FBS used was free of BVDV and anti-BVDV antibody. Prior to viral assay of ZP-damaged IO group, the oocyte cumulus cells and ZP were removed using 0.1% hyaluronidase and 0.1% pronase. BVDV RNA of individual oocytes was measured by dilution of C-DNA with RT-PCR [Vilcek et al., *Arch Virol* 1994;136:309–323]. BVDV infected MDBK cells were used as positive controls. Neither the ZP-free immature oocytes nor the ZP-damaged immature oocytes showed BVDV-PCR products. MDBK cells showed the products on the cells collected at 24 h after incubation with BVDV. These results suggest that immature oocytes derived from cattle may be relatively resistant to infection with BVDV under in vitro conditions. Further study is needed to elucidate the relationship between NCP-BVDV infection and in vitro oocytes during maturation. This study was supported by OECD fellowship 2001, Cooperative research program.

# Exotic Species

## COMPUTERIZED ANALYSIS OF SPERM MOTILITY IN EUROPEAN BROWN BEAR (*URSUS ARCTOS*) SEMEN

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Development of assisted reproductive techniques in ursids is justified because six of the eight existent species are endangered. Concretely, in the Cantabric Mountains (north-Spain), there is a threatened sub-population of European brown bear (~80 animals) that probably constitutes the purest genetic line of *Ursus arctos*. Germplasm banks, and the assisted reproductive techniques associated, are not well developed in bears, so it is necessary to carry out studies to adapt these methodologies. Our objective is to adjust a computerized sperm analysis system for brown bear semen (electroejaculated and epididymal) and to contribute with basic data of kinetic spermatozoa parameters in this species. *Ejaculated semen*: nine urine-free fractions of semen were obtained by electroejaculation during breeding season (June–July) of five adult European brown bears (240–298 kg) housed in a half-freedom system in the Cabarceno Park (Cantabria, Spain). Electroejaculation, under general anaesthesia (tiletamine + zolazepan-Zoletil<sup>®</sup> 100-7 mg/kg and ketamine-Imalgene<sup>®</sup> 1000-2 mg/kg) was carried out with an electroejaculator (PT Electronics<sup>®</sup>, OR, USA) with a transrectal probe (three electrodes, 26 mm of diameter, 320 mm of longitude). Stimuli of increasing intensity (3 s stimulus-3 s hold) were applied until obtaining the seminal sample (maximum 10 V and 250 mA). *Epididymal semen*: the sample was obtained post-mortem at the beginning of breeding season (May), from the epididymal tail by means of cuts [Anel et al., Theriogenology 1999;55:277]. Due to the high concentration observed, it was necessary to dilute the sample (1:50 in PBS). *Analysis procedure*: 4 µl samples were placed on a slide thermostatically maintained (37 °C) and eight randomly-chosen fields were analyzed (negative phase contrast; 200×). Sperm motility assessment was made with a computerized analysis system (Motility Analyzer 7.4 G, Hamilton Thorn Research<sup>TM</sup>). Analyzed parameters were mobile spermatozoa (M), progressive mobile spermatozoa (P), path velocity (VAP), track speed (VCL), progressive velocity (VSL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat frequency (BCF). After several adjust tests (correct spermatozoa identification; static/mobile discrimination), we fixed the next setup: frames acquired 20, frame rate 25/s, minimum contrast 9, minimum size 8, lo/hi size gates 0.7/1.4, lo/hi intensity gates 0.5/1.2, non-mobile head size 10, non-mobile brightness 20, medium VAP valued 25, low VAP valued 10, slow cells mobile NO and threshold STR 80. Results of the analyzed parameters (Table 1) show that, as it was expected, motility and progressive motility rates are clearly better in samples obtained by electroejaculation than in epididymal samples. However, it is surprising to see that velocities are smaller in electroejaculated sperm. This could be due to the dilution made in the epididymal sample, as we have observed that diluted electroejaculated bear semen analyzed pre-freezing sensibly improved its motility characteristics. This work was supported in part by Cabarceno Park (Cantabria, Spain).

Table 1

Kinetic values of ejaculated (mean ± S.E.M.) and epididymal brown bear spermatozoa

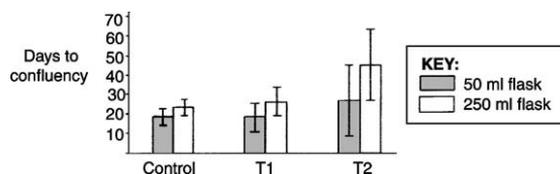
	<i>n</i>	M (%)	P (%)	VAP (µm/s)	VCL (µm/s)	
Ejaculated	9	79.2 ± 4.6	50.8 ± 3.5	62.8 ± 4.5	84.4 ± 5.1	
Epididymal	1	54	28	86	131	
		VSL (µm/s)	LIN (%)	STR (%)	ALH (µm)	BCF (MHz)
Ejaculated	52.9 ± 4.0	62.1 ± 2.9	81.7 ± 1.5	5.4 ± 0.3	12.8 ± 0.7	
Epididymal	68	55	79	6.3	5.6	

ESTABLISHING VIABLE CELL CULTURES FROM AFRICAN LIONS  
(*PANTHERA LEO*) FOLLOWING CRYOPRESERVATION OF SKIN  
BIOPSIES IN CBS STRAWS

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Live cells recovered from wildlife are a renewable genetic resource, useful for conservation genetics and nuclear transfer research. Freezing biopsies would allow cell lines to be established at a later date. The aim of this study was to determine whether viable fibroblast cultures could be established after cryopreservation of lion tissue biopsies in straws (CryoBioSystems, France). Ear skin notches,  $\sim 2.0 \text{ cm} \times 0.5 \text{ cm}$ , were surgically removed from six adolescent lions, chemically immobilized with 300 mg Zoletil 100 (Virbac, RSA). Biopsies were transported to the laboratory at  $4^\circ \text{C}$  within 2 h, cleaned with chlorohexidine gluconate (Hibiscrub, Zeneca, RSA), and sliced finely in Minimal Essential Medium (MEM; Highveld Biological, RSA), supplemented with 2.95% tryptose phosphate broth (Difco Laboratoria, RSA) and 10% foetal calf serum (FCS, Sterilab, RSA). The resultant tissue explants were divided equally into a control and two treatment groups. Control group: explants were incubated at  $37^\circ \text{C}$  for 24 h with 0.1% collagenase/hyaluronidase (Sigma, RSA) in MEM, gassed with humidified 5%  $\text{CO}_2$ , 20%  $\text{O}_2$  and 75%  $\text{N}_2$  in 10 ml Nunclon (AEC-Amisham, RSA) tubes. Thereafter, explants were transferred to 50 ml Nunc tissue culture flasks (AEC-Amisham, RSA) and re-gassed for cell culture. At confluency, cultures were treated with 0.125% trypsin (Gibco, RSA), transferred into 12 ml fresh medium in 250 ml Nunc tissue culture flasks, re-gassed and cultured until cells reached confluency. Treatment 1 (T1): tissue explants were placed in freezing medium consisting of MEM, supplemented with 20% FCS and 10% glycerol (Sigma, RSA), loaded into 0.5 ml CBS straws and frozen using a Mini DigitCool (IMV Technologies, France) at  $1^\circ \text{C}/\text{min}$  from 20 to  $100^\circ \text{C}$ , and then plunged into liquid nitrogen. Explants were thawed within 72 h of freezing by plunging into  $37^\circ \text{C}$  water. After thawing, explants were enzyme-treated and cultured as for the control group. Treatment 2 (T2): biopsies were pre-treated with enzyme for 24 h prior to cryopreservation in 0.5 ml CBS straws. Explants were thawed within 72 h, enzyme treated for 6 h, and cultured as for the control group. In each case, culture growth rates were estimated as time (days) taken to reach confluency. Percentage cell viability at confluency was estimated microscopically by staining with one part 0.4% Trypan blue (Sigma, RSA) to one part cell suspension. Growth rates and cell viability were compared between treatment groups by means of a Student's *t*-test or a Mann-Whitney rank sum test (in cases where the data were not normally distributed).



Cultures were successfully established in all treatment groups. Mean times to confluency in T1 50 and 250 ml flasks were  $18.2 \pm 3.0$  and  $26.3 \pm 7.4$  days, respectively, which did not differ significantly from the control (50 ml:  $18.6 \pm 3.1$  days; 250 ml:  $23.5 \pm 4.1$  days). Mean times to confluency in T2 50 and 250 ml flasks were  $26.9 \pm 9.5$  and  $45.1 \pm 18.3$  days, respectively. The former did not differ significantly from the control, but growth rates of T2 250 ml cultures were significantly slower than the control 250 ml flasks ( $t = -3.24$ , 7 d.f.,  $P = 0.014$ ). These initial results indicate viable lion fibroblast cultures can be established from biopsies cryopreserved in CBS straws. Furthermore, cryopreservation of biopsies prior to establishment of fibroblast cell cultures does not increase the time taken to establish the primary cultures, or the viability of resulting cells.

IN VITRO MATURATION OF OOCYTES COLLECTED FROM NON-STIMULATED COMMON WOMBATS (*VOMBATUS URSINUS*)M. Cleary<sup>1</sup>, M. West<sup>1</sup>, J. Shaw<sup>1</sup>, G. Jenkin<sup>1,2</sup>, and A. Trounson<sup>1</sup><sup>1</sup>Monash Institute of Reproduction and Development,<sup>2</sup>Department of Physiology, Monash University, Clayton, Vic., Australia

Artificial breeding technologies can be applied to rare, endangered and valuable species with the aim of increasing the population size. One method that may be used to generate oocytes for assisted breeding is in vitro oocyte maturation. A large number of oocytes are present within ovarian tissues; however, most of these are immature. In vitro oocyte maturation may allow these immature oocytes to develop. In vitro maturation has been successful in several marsupial species following hormonal stimulation; however, investigating oocyte maturation without prior stimulation has been less extensively studied. In the Common wombat mature MII oocytes can be collected following hormonal stimulation [West et al., 2002]. This study aims toward in vitro mature oocytes collected from Common wombats without prior hormonal stimulation.

Common wombat (*Vombatus ursinus*) ovarian tissue was collected from nine culled adult female wombats. Ovaries were immediately removed from the body cavity and stored in PBS supplemented with Penicillin and Streptomycin at 35–37 °C for 2–5 h. Ovaries were then transferred to Hepes buffered EMEM handling media at 35 °C where oocytes were liberated from all visible follicles present on the ovarian surface and classified into greater than 2 mm and less than 2 mm follicle groups. Collected oocytes were washed in handling media and either fixed at time 0 or after 60 h culture in EMEM at 35 °C in 5% CO<sub>2</sub> in air. The nuclear status of all oocytes was assessed under a fluorescent microscope following staining with 10 µg/ml Hoechst 33342 in PBS. Oocytes were classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), telophase (T), metaphase II (MII) or degenerate. Oocytes fixed at the time of collection T<sub>0</sub> ( $n = 41$ ) were degenerate (2%), GV or GVBD (78%) or at M1 (20%). After 60 h in culture ( $n = 68$ ) 12% were degenerate, 28% GV and GVBD, 25% of all oocytes were MI, 10% at T and 25% had reached MII. A higher proportion of the oocytes collected from follicles greater than 2 mm in diameter matured to T and MII. Maturation to T or MII was not dependent on the presence of cumulus cells.

This study demonstrated that oocytes could be matured in vitro after collection from Common wombats without prior hormonal stimulation. Further studies will investigate the influence of cycle stage on the developmental competence of Common wombat oocytes and the ability of these oocytes once fertilized to undergo further development.

MONITORING REPRODUCTIVE ACTIVITY IN THE TASMANIAN DEVIL  
(*SARCOPHILUS HARRISII*)

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Urine cytology, progesterone and estrogen concentrations and their relationship to breeding behavior were determined in the captive adult female Tasmanian devil (*Sarcophilus harrisii*,  $n = 2$ , aged 7–8 years). These animals were housed in separate indoor enclosures but had access to each other outdoors. A single adult male was introduced at various times when breeding behavior was suspected. Through two breeding seasons (late July–September, northern hemisphere), behavior was monitored five times weekly and rated on a scale of 1–5, with the highest activity (5: spinning, hind leg stance, air sniffing, scratching and anogenital dragging) being interpreted as breeding activity, based on behaviors indicative of estrus in marsupials [Fadem et al., *Horm Behav* 1996;30:44–49]. Urine (0.1–16.0 ml; 11–18 samples/animal/season) was collected opportunistically and delegated to two aliquots; the first was examined within 1 h by light microscopy for the presence of cornified epithelial cells and leukocytes; the second was frozen ( $-4^{\circ}\text{C}$ ) for subsequent hormone analysis. Enzyme immunoassays (EIAs) for estrogen and pregnenediol-3 $\alpha$ -glucuronide (PdG) were validated with a serially diluted devil pool and parallelism obtained, and assays were performed on all samples. The results were expressed as a function of creatinine concentrations. The results demonstrated that progesterone and estrogen can be measured by EIA in Tasmanian devil urine with up to 80- and 14-fold differences, respectively, measurable between low (baseline) and high concentrations. The presence of cornified cells in the urine often correlated with elevated estradiol levels. Breeding behavior (ratings of 4–5), recorded as occurring every 10–14 days in one animal, did not appear to correlate closely with peak estrogen levels. Observations made on both animals on September 16 of the second year of the study (animals failing to come indoors and reluctant to eat, suggestive of breeding behavior) occurred at a time of falling estrogen levels following elevated concentrations. Pairings, which took place at these times, did not result in pregnancies. In conclusion, this research has demonstrated that PdG and estrogen can be measured in female Tasmanian devil urine by IA, offering researchers a potentially valuable tool for non-invasively following reproductive activity in this species. Additionally, it has suggested that the presence of cornified cells in urine is a rapidly available method to monitor estrus in Tasmanian devils.

DEVELOPMENT OF GIANT ELAND ANTELOPE (*TAUROTRAGUS DERBIANUS*)  
EMBRYOS FOLLOWING NUCLEAR TRANSFER WITH COMMON ELAND  
(*TAUROTRAGUS ORYX*) AND BOVINE (*BOS TAURUS*) OOCYTES

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Nuclear transfer (NT) technology has been suggested as an assisted reproductive tool for endangered species. Unlike the cloning of domestic animals where there is a ready supply of oocytes for embryo production, the cloning of endangered species will require alternative methods, such as the use of oocytes from closely related domestic species. However, for NT to be accepted in a conservation program, collection of oocytes from the same species or closely related non-domestic species is warranted. Therefore experiments were designed to compare the embryonic development following NT of Giant eland antelope (Lord Derby eland) somatic cells into common eland and domestic cow oocytes. Common eland oocytes were recovered by transvaginal ultrasound-guided oocyte retrieval as described [Wirtu et al. *Theriogenology* 2002;57:595 (abstract)]. Mature eland oocytes were enucleated ( $n = 11/17$ ) and proliferating giant eland somatic cells used as donor nuclei. Couplets were fused and chemically activated with 5  $\mu\text{M}$  ionomycin and 2 mM 6-DMAP. Giant eland NT zygotes ( $n = 8$ ) were cultured in base medium BM3 [McKiernan et al., *Mol Reprod Dev* 1995;42:188–199], supplemented with 0.2 mM glucose, 1 mM glutamine, and 1% (v/v) MEM and BME, amino acids, respectively. On Day 2 cleaved embryos ( $n = 4$ ) were moved into BM3 supplemented with 10% FBS. Giant eland NT embryos reconstructed with common eland oocytes developed up to the 8-cell stage, confirmed by chromatin staining. Embryonic development following interspecies NT into domestic cow oocytes ( $n = 65$ ) was also evaluated. Resulting embryos ( $n = 42$ ) were allotted to two different embryo culture treatments: Treatment A BM3 +5% eland serum (ES) followed by BM3 +10% ES on Day 2 and Treatment B BM3 +5% ES + insulin-transferrin-selenite (ITS; insulin concentration 10  $\mu\text{g/ml}$ ) followed by BM3 + ITS +10% ES on Day 2. The interspecies NT embryos cultured in Treatment B had greater cleavage rates (77%) compared with embryos cultured in Treatment A (42%). In addition, development to the blastocyst stage was lower for NT embryos in Treatment A (19%) when compared to those in Treatment B (27%). A total of 6 blastocysts were non-surgically transferred to a cycling Common eland recipient. The animal was evaluated for pregnancy at Day 44 of gestation via ultrasonography. No fetal tissue was detected. In conclusion we have shown that Giant eland cells fused to common eland oocytes resulted in early embryonic development, up to the 8-cell stage. In addition, we have demonstrated that Giant eland embryos produced by interspecies NT can develop to the blastocyst stage resulting in late-stage embryos that could be used for embryo transfer. This research was supported in part by a grant from the Coypu Foundation.

OVARIAN FOLLICULAR SUPERSTIMULATION IN THE SOUTHERN  
HAIRY-NOSED WOMBAT, *LASIORHINUS LATIFRONS*

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The development of assisted reproductive techniques for the purpose of application to endangered species has attracted much interest in recent years. Ovarian follicular superstimulation protocols that may potentially increase the reproductive capacity of a number of Australia's seasonally breeding marsupials have been developed, based on the use of either PMSG or FSH to promote follicular growth (Table 1). The maturation of follicles and associated oocytes in the lead up to induced ovulation has been achieved using either GnRH or LH. The recovery of multiple MII oocytes has recently been reported in the monovular common wombat following superovulation using FSH and LH [Theriogenology 2002;57:594]. The southern hairy-nosed (SHN) wombat is the closest living relative to the critically endangered northern hairy-nosed wombat *Lasiorhinus krefftii*, and we have therefore chosen this as our model species to development assisted breeding techniques. The aim of this study was to investigate ovarian follicular response to treatment with exogenous FSH or PMSG in female SHN wombats prior to the natural breeding season. Twenty-four adult female SHN wombats were wild-caught and anoestrus confirmed by assessing pouch condition. Two groups ( $n = 6$ /group) were administered identical doses of porcine FSH (200 mg, Folltropin-V, Vetrepharm, Canada, Inc.) at 12 h intervals over either 4 or 7 days. Animals in FSH-treated groups were administered a single s.c. injection of porcine LH (25 mg, Lutropin-V, Vetrepharm, Canada, Inc.) 12 h after the final FSH injection. Timing of the LH injection was different due to the expected differences in follicular stimulation by FSH and PMSG. The third group ( $n = 6$ ) was administered a single i.m. injection of PMSG (150 IU, Vetrepharm, Canada, Inc.), followed 72 h later by a single s.c. injection of 25 mg porcine LH. Three control groups ( $n = 2$ /group) were administered saline injections on timescales identical to the three treatment groups. Reproductive tracts were recovered 24 h after the administration of LH or final saline dose, and assessed for response to treatment. Dissected ovaries were weighed and measured, and follicular response recorded. No ovulations were observed. Oocytes were recovered from recruited follicles ( $>1.5$  mm), prior to fixation in gluteraldehyde for analysis of stage of maturity. No difference between control animals was found and data were therefore pooled. Compared to control animals, animals that received treatment showed an obvious increase in follicular activity. One-way ANOVA showed a significant difference among treatments and a posteriori Tukey test distinguished the follicular response of the 7-day FSH treatment group from all other treatments at  $P < 0.05$ . In addition, this group also had increased uterine and ovarian weights compared to control animals. In summary, these results suggest that the 7-day FSH stimulation protocol results in ovarian follicular superstimulation in anoestrus SHN wombat females.

This study was sponsored by Dr. M. Jacobson and hormones were kindly supplied by Vetrepharm Pty Ltd.

Table 1  
Obtained results following ovarian follicular stimulation in SHN wombats

	Control	PMSG	4-day FSH	7-day FSH
Follicles/ovary	0.08 ± 0.083 <sup>a</sup>	2.17 ± 1.16 <sup>a</sup>	1.67 ± 0.62 <sup>a</sup>	5.91 ± 1.28 <sup>b</sup>
Uteri weight	0.92 ± 0.077 <sup>a</sup>	1.01 ± 0.12 <sup>a</sup>	1.23 ± 0.24 <sup>a,b</sup>	1.51 ± 0.13 <sup>b</sup>
Ovarian weight	0.44 ± 0.026 <sup>a</sup>	0.65 ± 0.11 <sup>a,b</sup>	0.56 ± 0.10 <sup>a,b</sup>	0.95 ± 0.12 <sup>b</sup>

Means with the same superscripts are not significantly different (Tukey test;  $P < 0.05$ ).

EFFECT OF BOVINE SEMINAL PLASMA ON THE ABILITY OF BUFFALO (*SYNCERUS CAFFER*) SPERMATOZOATO FERTILIZE BOVINE OOCYTES IN VITRO

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Oocytes from African buffalos (*Syncerus caffer*) are scarce and an attempt was therefore made to use bovine oocytes to characterize buffalo semen for the use in an in vitro fertilization system. Our previous work showed that the fertilization rate was between 0 and 3%.

The aim of this study was to test if bovine seminal plasma could improve the fertilization rate of bovine oocytes by buffalo spermatozoa in vitro.

Oocytes with an intact corona radiata and at least 3 layers of compact cumulus cells were selected from ovaries from a local abattoir and matured for 22 h under mineral oil in 50  $\mu$ l droplets (10 per droplet) of TCM 199 (Earle's salts) supplemented with 25 mg/ml gentamicin, 100  $\mu$ M 2-mercaptoethylamine, 25 mM HEPES and 5% steer serum at 39 °C in 5% CO<sub>2</sub> in air with 100% humidity. Matured oocytes were fertilized with  $1 \times 10^6$  spermatozoa per droplet in a modified TALP medium including 25  $\mu$ g/ml gentamicin, 0.6% essentially fatty acid free BSA, PHE (2.0 mM penicillamine, 1.0 mM hypotaurine and 25  $\mu$ M epinephrine) and 30  $\mu$ g/ml heparin. Epididymal and ejaculated sperm, each from 3 different African buffalo bulls (*Syncerus caffer*), frozen-thawed in 0.25 ml straws, was swim-up separated in either 1 ml fert-wash (same as IVM medium without PHE and heparin) or in 0.5 ml fert-wash and 0.5 ml seminal plasma from one of 10 bulls of different breeds. Sixty oocytes were used per treatment. A control bull was used with each run. After 20 h the oocytes were fixed for 48 h in 1:3 acetic acid:methanol, stained with 1% aceto orcein and viewed at 20–40 $\times$  using Nomarski optics. Oocytes were classified as degenerated (Deg); arrested at metaphase II (Met II); one pro-nucleus (PN); fertilized (2PN) or polyspermic (Pol). The effect of the seminal plasma was tested by repeated measure ANOVA with semen source (ejaculated or epididymal) and seminal plasma donor as main effects and with the buffalos as subjects (nested in semen source). All interactions were included. The Huynh-Feldt  $\epsilon$  corrections were applied to determine the level of significance ( $P < 0.05$  denoted significant differences).

There was no difference between ejaculated and epididymal sperm. The addition of seminal plasma had a significant effect on Deg, Met II, 2PN and Pol. Without and with the addition of seminal plasma there were  $5.5 \pm 2.39$  (mean  $\pm$  S.D.) and  $10.2 \pm 4.40$  Deg,  $50.8 \pm 3.19$  and  $10.5 \pm 5.00$  Met II,  $1.0 \pm 1.30$  and  $35.2 \pm 5.78$  2PN and  $0 \pm 0.00$  and  $2.1 \pm 1.55$  Pol, respectively. For the control bull there were  $13.8 \pm 3.13\%$  Met II and  $72.3 \pm 4.17\%$  2PN. The plasma donor had a significant effect on MII and Pol and the buffalo bull on Deg, 2PN and Pol.

There appears to be a "fertility factor" in bovine seminal plasma, which is absent in the tested buffalo bulls. The concentration and or the structure of this "fertility factor" may be different in individual beef and dairy bulls' seminal plasma. Buffalo appear unable to fertilize bovine oocytes without this "fertility factor". Further studies are needed to identify the "fertility factor" and to assess the mechanism by which it allows the fertilization of bovine oocytes by buffalo spermatozoa. This "fertility factor" could be responsible for the variable ability of individual bulls' spermatozoa to fertilize oocytes in vitro and it could further contribute to the difference in fertility in general.

COMPARISON OF THREE DIFFERENT MEDIA FOR FREEZING EPIDIDYMAL SPERM FROM AFRICAN BUFFALO (*SYNCERUS CAFFER*) AND INFLUENCE OF EQUILIBRATION TIME ON THE POST-THAW SPERM QUALITY

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Epidymal sperm is commonly collected under field conditions and frozen as a method of genetic resource banking. Little information exists with respect to the freezing of epididymal sperm from the African buffalo. The aim of this study was to compare the effects of three extenders and equilibration times of 2–9 h on the post-thaw motility and acrosomal integrity of epididymal sperm of African buffalo. The extenders are commercially available and easy to use in the field. The epididymides of six adult bulls were removed and flushed with air within 30 min after culling. Samples were split in three aliquots and diluted with either Triladyl<sup>TM</sup>, AndroMed<sup>®</sup> (both Minitüb, Germany) or Red Ovine Freezing Buffer (ROFB, IMV, France). Sperm was loaded into 0.25 ml French straws, cooled to 4 °C over 1 h and equilibrated for a total time of 2–9 h before being frozen. Straws of sperm were thawed in a 37 °C waterbath for 30 s. Sperm motility was evaluated immediately after flushing, and 0, 1 and 2 h after thawing. Post-thaw acrosomal integrity was assessed on FITC-PNA stained wet preparations. A one-way-repeated-measures-ANOVA with bulls as subjects was used to test for differences among extender-time combinations. Interactions between extender and time were not considered. Tukey's test was used to identify different groups.  $P \leq 0.05$  denoted significant differences. When extenders were viewed separately, post-thaw motility was similar among equilibration times of 4–9 h, whereas the motility was occasionally lower for shorter times. Data for shorter equilibration times were thus ignored and averages for 4–9 h were used for further analysis. Progressive motility immediately after thawing was higher for sperm frozen with ROFB ( $28.9 \pm 15.17$ ) than for sperm frozen with Triladyl<sup>TM</sup> ( $19.3 \pm 12.34$ ) or AndroMed<sup>®</sup> ( $14.1 \pm 13.85$ ). Progressive motility 1 and 2 h after thawing was higher for Triladyl<sup>TM</sup> ( $21.0 \pm 14.96$  and  $15.8 \pm 11.32$ ) than for the other extenders. Neither equilibration time nor extender influenced the acrosomal integrity (Table 1).

This study shows that any equilibration time between 4 and 9 h may be used before freezing epididymal sperm from African buffalo and that Triladyl<sup>TM</sup> is more suitable than AndroMed<sup>®</sup> or ROFB for the freezing of such sperm because Triladyl<sup>TM</sup> results in higher percentages of progressively motile sperm 1 or 2 h after thawing. Exposure to glycerol for more than 4 h is detrimental for ejaculated bull semen [Wiggin and Almquist, J Dairy Sci 1975;58:416–419]. This contrasts with the findings of the present study on epididymal buffalo sperm.

Table 1  
Total and progressive motility (%) and acrosomal integrity (%) of epididymal sperm from six bulls

Medium	Fresh		Time after thawing (h)						Intact acrosomes
	T	P	0		1		2		
			T	P	T	P	T	P	
AndroMed	57.2 (17.47)	31.0 (21.43)	46.1 <sup>a</sup> (13.15)	14.1 <sup>a</sup> (13.85)	48.3 <sup>a</sup> (12.07)	7.1 <sup>a</sup> (10.86)	44.7 (12.07)	2.5 <sup>a</sup> (5.79)	53.0 (6.83)
ROFB	58.0 (13.92)	24.0 (15.05)	56.9 <sup>b</sup> (11.91)	28.9 <sup>b</sup> (15.17)	58.3 <sup>b</sup> (15.40)	10.0 <sup>a</sup> (12.56)	51.4 (15.70)	2.4 <sup>a</sup> (6.38)	55.4 (5.91)
Triladyl	55.7 (13.37)	19.2 (10.85)	53.9 <sup>ab</sup> (13.37)	19.3 <sup>a</sup> (12.34)	55.6 <sup>ab</sup> (12.75)	21.0 <sup>b</sup> (14.96)	49.2 (12.04)	15.8 <sup>b</sup> (11.32)	56.5 (6.50)

Values are the averages (S.D.) for the equilibration of 4–9 h. Note: T = total motility, P = progressive motility. ROFB = Red Ovine Freezing Buffer. Values in a column with different superscripts differ ( $P < 0.05$ ).

COMPARISON OF TWO METHODS FOR THE CRYOPRESERVATION OF EPIDIDYMAL SPERMATOZOA FROM WILD AFRICAN BOVIDS

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In Southern Africa, wild animals are often culled for meat, trophies or to maintain the carrying capacity of parks and reserves. This practice provides the opportunity to collect epididymal spermatozoa for use in research and germplasm preservation. The objective of this experiment was to compare post-thaw motility of epididymal spermatozoa from African buffalo (*Syncerus caffer*), eland (*Tragelaphus oryx*), blesbok (*Damaliscus dorcas phillipsi*) and black wildebeest (*Connochaetes gnu*) cryopreserved using two methods (Biladyl versus EQ). Within 8 h of death (stored at 4 °C), the cauda epididymis and vas deferens were dissected from each testicle and flushed with air. Samples from both testes of individual males were pooled and divided into two treatment groups. The percentage of motile sperm (0–100%) and the rate of forward progression (0, no motility to 5, rapid forward movement) were evaluated after dilution in cryopreservation medium (Biladyl) or after washing in Hepes-buffered SOF (EQ). Samples were then diluted to 200 to 400 × 10<sup>6</sup> sperm/ml in Biladyl A (Minitüb, Germany; 20% egg yolk, 2.42% Tris, 1.38% citric acid, 1.00% fructose) or EQ (20% egg yolk, 5.5% lactose, 1.5% glucose, 0.09% citric acid) and allowed to cool to 4 °C by placing the tubes containing sperm in the refrigerator for 6 h (Biladyl) or in a water bath in a cold room (4 °C) for 1.5–2 h (EQ). An equal volume of cryopreservation medium containing 14% (Biladyl) or 10% (EQ) glycerol was then added in one step (Biladyl) or in three steps, 20 min apart (EQ; 0.25×, 0.25×, and 0.5× original volume). Samples in Biladyl were loaded into 0.25 ml straws and frozen 5 cm over liquid nitrogen vapor for 20 min before plunging into liquid nitrogen. Samples in EQ were equilibrated for 1 h, loaded into 0.25 ml straws, and frozen in one step by rapidly lowering to the bottom of a dry shipper. Straws from each sample were thawed in air for 5 s and then plunged into 38 °C water for 2 min. Immediately after thawing, aliquots were placed onto warm slides and assessed for motility and rate of progression. A sperm motility index [SMI; (percentage motile + (20 × rate))/2] was calculated for initial and post-thaw values (Table 1) to compare the efficiency of each method (paired *t*-test). There was no significant difference between initial or post-thaw SMI values between the two treatments (*P* > 0.05), but intra- and interspecies variability exists. Although both methods appear suitable for the cryopreservation of epididymal spermatozoa from these species, the use of a dry shipper instead of liquid nitrogen vapor makes the EQ method more adaptable for use under field conditions.

Table 1  
Sperm motility indexes for epididymal spermatozoa before and after cryopreservation using two methods

	Initial Biladyl	Post-thaw Biladyl	Initial EQ	Post-thaw EQ
Eland 1	97.5	85.0	70.0	37.5
Eland 2	67.5	45.0	52.5	40.0
Buffalo 1	30.0	0.0	55.0	27.5
Buffalo 2	42.5	30.0	57.5	30.0
Blesbok	85.0	70.0	82.5	42.5
Black Wildebeest	67.5	52.5	67.5	45.0
Mean ± S.E.M.	65.0 ± 10.3 <sup>a</sup>	47.1 ± 12.3 <sup>b</sup>	64.2 ± 4.6 <sup>a</sup>	37.1 ± 2.8 <sup>b</sup>

Similar superscripts indicate no significant difference (*P* > 0.05) between treatments within each time point.

OVARIAN FOLLICLE DEVELOPMENT IN WHITE-TAILED DEER  
(*ODOCOILEUS VIRGINIANUS*) IN THE ANESTROUS SEASON

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Very little is known about ovarian follicle development in white-tailed deer in the anestrus season. This information would be useful in the development of population- and breeding-control measures for both wild and captive herds of white-tailed deer. One of the greatest obstacles to the acquisition of this information is daily handling and examination of a species particularly susceptible to trauma and stress. The first objective of the study was to develop a method of handling white-tailed deer for the purposes of daily examination. The second objective was to gather ultrasound images of the ovaries to characterize follicular dynamics during the anestrus season. A herd of bottle-raised does was used in this trial. Initially, the does ( $n = 10$ ) were run through the chute system without examination on a daily basis for 2 weeks to become accustomed to the handling procedure. The animals were maintained in an outdoor corral and moved into an indoor chute system consisting of an enclosed tunnel that led to a drop-floor squeeze. Ultrasound examination of the ovaries was accomplished transrectally using an ALOKA 900 machine with a 7.5 MHz linear-array probe mounted on a rigid extension. For each ovary, follicles greater than 1 mm in diameter were measured and recorded. Examinations were done daily from June 11 to July 6 (26 days). The average duration of examination was approximately 15 min. The deer appeared to adapt well to daily handling; however, 2 does were removed from the study because of leg abrasions from struggling while in the drop-floor squeeze. Additional padding was added to the walls of the squeeze and to the back brace, resulting in a marked reduction in further injuries. Data from 8 does were used in the following analyses. Retrospective evaluation of ovarian sketches revealed that individual follicle diameter profiles could be traced back to an emerging diameter of  $2.0 \pm 0.1$  mm. The follicle destined to become the largest (apparent dominant follicle) emerged  $0.8 \pm 0.9$  days before the follicle destined to become the second largest (apparent subordinate follicle). The apparent dominant follicle was larger ( $P < 0.01$ ) than the largest subordinate follicle 2 days after emergence. The maximum diameter of the apparent dominant follicle was  $4.5 \pm 0.3$  mm, while that of the largest subordinate was  $2.9 \pm 0.1$  mm. From emergence to maximum diameter, the apparent dominant follicle grew  $0.6 \pm 0.1$  mm per day, while the largest subordinate follicle grew  $0.8 \pm 0.2$  mm per day. The lifespan (day of emergence until the last day the follicle could be uniquely identified) of the apparent dominant follicle was  $12.3 \pm 0.6$  days, compared to  $5.3 \pm 0.3$  days for the largest subordinate follicle. The interval between emergence of successive dominant follicles was  $5.3 \pm 0.3$  days. The success of serial data collection was attributed to the chute system specifically designed for white-tailed deer, the preconditioning period prior to data collection, and the relatively docile demeanor of this bottle-raised herd. Results of daily ovarian examination during the anestrus season supported the wave theory of follicular development in deer. These findings will contribute not only to the characterization of ovarian activity in white-tailed deer, but also to the development of assisted reproductive technologies (including estrus synchronization and embryo transfer).

RELATIONSHIP OF UMBILICAL BLOOD FLOW TO GROWTH PARAMETERS  
IN THE KOREAN BLACK GOAT FETUS

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The objectives of this study were (1) to assess the relative contributions of growth in umbilical vein diameter and of increased velocity to the increase in umbilical blood flow and (2) to determine the relationship of the umbilical vein blood flow to growth parameters in Korean black goat fetuses. Ultrasonographic scans were performed on eight Korean black goat fetuses at 60, 75, 90, 105, 120, and 135 days after breeding using SA 8800<sup>®</sup> (Medison Co., Korea) with a 4–9 MHz curved transducer. The internal diameter of the umbilical vein was measured by obtaining perpendicular views of the cord at maximum magnification. The umbilical vein mean velocity was calculated by maximum velocity method. Velocity values were reported as the mean of three different measurements. Absolute umbilical vein blood flow was calculated according to the following equation: umbilical vein flow = vessel cross-sectional area  $\times$  mean velocity  $\times$  60. Fetal growth was assessed by the diameter of the head and the body. The diameter of umbilical vein increased continuously during the 75 days of gestation and remained from then until birth at a relatively constant level (Day = 0.3073 value – 0.0449, R square = 0.7992). The mean blood velocity of umbilical blood veins increased constantly with fetal age (Day = 8.8889 value – 20.2844, R square = 0.8597). It is calculated that umbilical venous blood flow (Day = 0.1692 value + 50.3086, R square = 0.8789) increases with fetal age. However, the growth of umbilical venous diameter accounted for most of the growth in umbilical vein flow. There is a strong correlation between absolute umbilical vein flow and the fetal head (R square = 0.5975) and body diameter (R square = 0.7553). Therefore, it is concluded that there is high positive correlation between fetal growth and umbilical venous blood flow in Korean black goat.

## A RAPID TEST FOR UROSPERMIA DETECTION IN ELECTROEJACULATES FROM SEVERAL SPECIES

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In this work, we have used a quick and easy test to detect urine contamination in semen obtained by electroejaculation from bull, red deer, and brown bear. Electroejaculation is generally the best option to get semen samples from problematic and wild species. Urospermia is often a major problem in electroejaculation, as urine is deleterious for sperm. Electroejaculation may trigger urination; however, influence of the species factor is quite high, with felids, canids, and ursids the most problematic ones. This kind of contamination has been described according to changes in the color of the samples, but this is very subjective and inaccurate. Our objective is to quickly and objectively diagnose urospermia in electroejaculates of European brown bear (*Ursus arctos*), Iberian red deer (*Cervus elaphus hispanicus*) and bull (*Bos taurus*), by means of a rapid urea test. Semen samples were obtained by electroejaculation: 39 from brown bear, 79 from red deer, and 30 from bull (Sayaguesa breed). All samples were visually assessed for urine contamination (yellowish color). For each sample, pH and osmolality were measured with an electronic pH-meter (CG 837, Schott) and a cryoscopic osmometer (Osmomat-030, Gonotec), respectively, using 50  $\mu$ l. Urea presence was determined using Merckognost<sup>®</sup> Urea Rapid Screening test (Merck) for determination of urea concentration in serum or blood. The emergency diagnosis method described in its manual (semiquantitative) was carried out, slightly modified: 10  $\mu$ l of sample were pipetted onto the reaction zone of one test strip. The strip was read off 5 min later, noting if color change (yellow to blue) had reached 2 mm or further in the strip scale. Statistical analysis of the data was carried out with the SAS package. A nonparametric test (NPAR1WAY procedure, Wilcoxon test) was used to compare groups of data, considering urea presence as factor of variation. Table 1 shows results of statistical analysis of the data. Percentages of contaminated samples by species were: 74% for brown bear, 28% for red deer, and 0% for bull. This was expected as urine contamination during electroejaculation is not as frequent in ruminants as it is in other species (such as bears). The color of the samples was not a good indicator of urine contamination, as most contaminated samples were indistinguishable from uncontaminated ones. Urine has a lower pH and higher osmolality than sperm, so changes in these parameters are expected for contaminated samples. Indeed, osmolality was significantly higher for urea-positive samples. However, pH was significantly lower in bear but not in deer. Therefore, these indicators are either unreliable or difficult to use on the field (osmolality). The urea test that we have used in this experiment is a quick method, and it can be easily used in the field. This allows us to take immediate measures (e.g. diluting) if semen is contaminated with urine, helping to save valuable samples.

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Table 1  
pH and osmolality data grouped by urospermia positivity (mean  $\pm$  S.E.M.)

Species	Urospermia	n	pH	Osmolality (mOsm/kg)
Brown bear	Positive	31	8.14 $\pm$ 0.14 <sup>a</sup>	441 $\pm$ 7 <sup>a</sup>
	Negative	8	8.64 $\pm$ 0.07 <sup>b</sup>	328 $\pm$ 33 <sup>b</sup>
Red deer	Positive	22	8.03 $\pm$ 0.05 <sup>a</sup>	453 $\pm$ 31 <sup>a</sup>
	Negative	57	8.02 $\pm$ 0.07 <sup>a</sup>	333 $\pm$ 23 <sup>b</sup>
Bull	Positive	0	–	–
	Negative	30	7.79 $\pm$ 0.06	312 $\pm$ 4

Different superscripts (a, b) indicate significant differences between groups.

## FOLLICLE DEVELOPMENT IN WAPITI DURING THE ESTROUS CYCLE

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The need to control wild populations and interest in commercial farming of cervid species have led to a demand for methods to control estrus and the application of advanced reproductive technologies. However, the fundamental pattern of ovarian follicle development during the estrous season in wapiti (*Cervus elaphus*) is unknown. The purpose of this study was to characterize ovarian follicle dynamics in wapiti during the estrous season. The reproductive tract of 13 mature hinds, aged 18 months to 13 years, was examined by transrectal ultrasonography between the months of October and January. The hinds were maintained on a farm near Saskatoon, Saskatchewan (52°07'N, 106°38'W). The hinds were kept in a pen separate from, but within eyesight of the stag. The hinds were examined daily (>60 consecutive days per hind) in a squeeze chute in the standing position without tranquilization for one complete interovulatory interval (IOI). Follicle size and numbers were recorded, and individual follicles were identified serially using a B-mode scanner and a 7.5 MHz linear-array transducer. Differences among follicular patterns were compared by analysis of variance. The overall mean IOI was  $21.3 \pm 0.1$  day, but was shorter in hinds exhibiting 2 waves of follicular growth during the IOI than in hinds exhibiting three and 4 waves ( $20 \pm 0.2$  day,  $22.2 \pm 0.3$  day,  $23 \pm 0.7$  day, respectively;  $P < 0.05$ ). The inter-wave interval (IWI) and the maximum diameter of the dominant follicle were different among waves within and among 2-, 3-, and 4-wave cycles (Table). The maximum diameter of the CL did not differ ( $P = 0.45$ ) between 2-, 3-, and 4-wave cycles ( $15.5 \pm 0.5$ ,  $13.4 \pm 0.7$ , and  $16.5 \pm 2.5$  mm, respectively). The CL began to regress on day  $15.7 \pm 0.3$  in 2-wave, day  $16.4 \pm 0.3$  in 3-wave, and day  $18.5 \pm 1.8$  in 4-wave cycles, but differences were not significant ( $P = 0.24$ ). In conclusion, wapiti hinds have 2, 3, or 4 waves of follicle development during the estrous cycle. The anovulatory waves of 3- and 4-wave cycles were associated with a smaller dominant follicle and a shorter inter-wave interval; these characteristics may be attributed to the duration of luteal function. Further study is needed to test the hypothesis that luteal lifespan and circulating progesterone concentration are positively correlated with the number of follicular waves during the estrous cycle in wapiti.

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	Follicle wave	IWI	Maximum diameter dominant follicle
2-wave hinds, $n = 6$	1	$10 \pm 0.1^a$	$12.5 \pm 0.3^c$
		$210 \pm 0.2^a$	$11.7 \pm 0.2^c$
3-wave hinds, $n = 5$	1	$9.2 \pm 0.2^a$	$10.4 \pm 0.1^c$
	2	$6.2 \pm 0.2^b$	$9.4 \pm 0.1^d$
	3	$6.6 \pm 0.3^b$	$9.2 \pm 0.1^d$
4-wave hinds, $n = 2$	1	$7 \pm 0^b$	$11.5 \pm 0.2^c$
	2	$6.5 \pm 0.4^b$	$9.5 \pm 0.4^d$
	3	$4.0 \pm 0.7^b$	$8.5 \pm 0.4^d$
	4	$6.5 \pm 0.4^b$	$9.5 \pm 0.4^d$

Within columns, values with no common superscripts (a, b, c, d) are different ( $P < 0.05$ ).

## EFFECTS OF MEDIUM-TERM STORAGE OF JAGUAR SPERM PRIOR TO CRYOPRESERVATION

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Conditions for sperm cryopreservation in the laboratory setting have been defined for many felid species. To achieve gene flow between wild and captive populations, field conditions for sperm cryopreservation must be optimized. The initial step toward this goal is developing appropriate semen processing techniques for the field. This study examines the effect of storage media, temperature, and immediate removal of seminal plasma on jaguar sperm survival post-thaw. Semen was collected from six male jaguars using standard protocols [Howard JG et al., *J Androl* 1990;11:204–215]. Immediately after collection, ejaculates were divided into five aliquots: (1) diluted immediately 1:1 in Ham's F10 medium +5% fetal calf serum and 25 mM HEPES (H-HF10; fresh control) and centrifuged ( $200 \times g$ ) or 8 min. The supernatant removed and sperm resuspended in TEST egg yolk buffer with 0% glycerol to a concentration of  $50\text{--}100 \times 10^6/\text{ml}$ . This aliquot was then cooled slowly to  $4^\circ\text{C}$  over 3 h (control); (2) diluted 1:1 in H-HF10 and incubated for 3 h at room temperature ( $\sim 25^\circ\text{C}$ ) prior to centrifugation and subsequent treatment identical to the control; (3) diluted 1:1 in TEST 0% glycerol and incubated at room temperature prior to centrifugation and subsequent treatment identical to the control; (4) diluted 1:1 in H-HF10 and cooled slowly over 3 h, centrifuged and resuspended in TEST egg yolk buffer with 0% glycerol to a concentration of  $50\text{--}100 \times 10^6/\text{ml}$ ; (5) diluted 1:1 in TEST and cooled slowly over 3 h, centrifuged and resuspended in TEST egg yolk buffer with 0% glycerol to a concentration of  $50\text{--}100 \times 10^6/\text{ml}$ . After cooling, an equal volume of TEST with 8% glycerol was added over 30 min to each aliquot, and the sperm loaded into 0.25 ml straws. After loading, all straws were frozen using a standard 2-step method in liquid nitrogen vapor. Straws were placed 3 in. above, then 1 in. above liquid nitrogen vapor for 1 min each before being plunged into liquid nitrogen. Sperm straws were thawed by plunging into  $37^\circ\text{C}$  water bath and agitating for 30 s. Sperm was then diluted 1:1 with H-HF10, and percent motility, forward progressive status (1–5 scale) and acrosome integrity were assessed. The motility ( $55.0 + 10.1\%$ ), status ( $2.8 + 0.2$ ) and acrosome integrity ( $82.5 + 1.5\%$  normal) of fresh sperm decreased ( $P < 0.05$ ) following cryopreservation under control conditions (motility:  $26.7 + 7.9\%$ ; status:  $2.3 + 0.2$ ; acrosome integrity:  $62.8 + 3.1\%$  intact). Sperm motility and status were reduced to  $<5\%$  after storage in H-HF10 at room temperature for 3 h prior to cooling. There were no significant differences in sperm survival post-thaw between other treatments, although sperm motility ( $15.0 + 5.8\%$ ) and status ( $1.6 + 0.5$ ) after storage in H-HF10 at cool temperatures was slightly lower ( $P > 0.05$ ) than control. These results demonstrate that H-HF10 is inadequate for long-term storage of jaguar semen, particularly at room temperature. The immediate removal of seminal plasma does not appear to have an effect on sperm survival post-thaw, provided that TEST egg yolk buffer is used as the diluent. Finally, dilution and storage of semen in TEST egg yolk buffer at  $25^\circ\text{C}$  or lower is an appropriate procedure for prolonging the life of jaguar sperm prior to cryopreservation.

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EFFECT OF VARIOUS CRYOPROTECTANTS AND METHODS FOR THE SHORT- AND LONG-TERM PRESERVATION OF SPERM FROM THE DROMEDARY CAMEL (*CAMELUS DROMEDARIUS*)

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The goal of this study was to determine effective cryoprotectants and methods for the short-term (refrigerated) and long-term (frozen) preservation of dromedary camel sperm for use in assisted reproduction. The effect of different treatments using non-permeable and permeable cryoprotective agents was initially examined for maintaining spermatozoal motility after refrigeration (4–8 °C) for 48 h. Semen collected ( $n = 4$ ) from one sexually mature male by an artificial vagina (AV) was extended (1:4) using one of three diluents containing 8% (v/v) of either dimethyl sulfoxide (DMSO) or glycerol (Gly): 11% (v/v) sugar solution consisting of either (1) lactose (L + DMSO, L + Gly), (2) fructose (F + DMSO, F + Gly), or (3) glucose (G + DMSO, G + Gly); with 20% egg yolk and antibiotics (Minitube). The results indicated that sperm treated with F + Gly resulted in no reduction in motility 48 h post-collection at refrigeration and warmed at room temperature before evaluation, whereas all other treatment groups resulted in an average of a 15% reduction in motility from the original motility (45%) immediately after collection. Camel sperm cryopreservation trials were then conducted using two different concentrations of glycerol (8 and 12%, v/v) in the 11% (v/v) fructose solution (F + 8% Gly, F + 12% Gly). Each ejaculate ( $n = 4$ ) collected by AV from two males was subjected to each treatment. Semen was extended initially (1:4) with the fructose diluent (containing 8 or 12% glycerol) at room temperature. The extended sample was cooled slowly by placing the tube containing the sample into a beaker of room temperature water and then placing the beaker into a refrigerator where it was maintained for 1 h. Straws (0.25 and 0.5 ml) were loaded and allowed to cool for an additional hour, then frozen in liquid nitrogen (LN2) vapor (20 min). After plunging into LN2, straws were thawed using one of three methods: (1) 20 °C ambient air until thawed, (2) 32 °C water bath for 1 min, and (3) 50 °C water bath for 8 s. The thawed sperm were warmed at room temperature before evaluations immediately after post-thawing. In conclusion, fructose (11%, v/v) with glycerol (8%, v/v) was found to be effective for maintaining dromedary sperm survival after refrigeration for 48 h or after cryopreservation, with a tendency for higher post-thaw survival using 0.5 ml straws combined with a fast rate of thawing (50 °C water bath for 8 s) (Table 1).

Table 1

Mean dromedary camel sperm motility (%) immediately post-thawing using different glycerol concentrations, straw sizes, and thawing methods ( $n = 4$ )

	20 °C		32 °C		50 °C	
	0.25 ml	0.5 ml	0.25 ml	0.5 ml	0.25 ml	0.5 ml
F + 8% Gly	21.7	23.3 <sup>a</sup>	37.5	43	45	42.3
F + 12% Gly	5 <sup>a</sup>	18.3 <sup>a</sup>	16.7 <sup>a</sup>	31.7	0 <sup>a</sup>	22.5

<sup>a</sup> Within columns, superscripts indicate significant differences ( $t$ -test,  $P < 0.05$ ) from the original motility (38.75%).

## ANATOMICAL CONSIDERATIONS FOR THE DEVELOPMENT OF ARTIFICIAL INSEMINATION BY CATHETER IN TWO MARSUPIALS

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We recently reported the birth of a tammar wallaby produced by intrauterine AI [Paris DBBP et al., Australian Mammal Society Conference, July 2002]. The use of AI has great potential for the conservation of rare and endangered marsupials, but a non-surgical method of insemination is needed if such a technique is to be widely adopted. In this study we compared the anatomy of the reproductive tract of two monovular marsupials: the tammar wallaby (*Macropus eugenii*, family Macropodidae) and southern hairy-nosed wombat (*Lasiorhinus latifrons*, family Vombatidae), in relation to the ability to deposit semen non-surgically by catheter. These species are ideal models for endangered marsupials such as the brush-tailed rock wallaby (*Petrogale penicillata*), long-nosed potoroo (*Potorous tridactylus*) and critically endangered northern hairy-nosed wombat (*Lasiorhinus krefftii*). As in all marsupials studied, tammars and wombats have two completely separate uteri each opening into the anterior vaginal culs de sac (AVC) through two distinct cervixes. The vaginal canals, unique and highly variable in marsupials, consist of two lateral vaginae (LV) and a third median vagina (MV) connecting the AVC to the urogenital sinus and opening. Adult female reproductive tracts were examined from 13 tammars and 5 wombats. A silicon balloon HSG catheter (5 French 30 cm; Cook, Australia) was introduced at the urogenital opening and navigated through the MV to the AVC, proximal to the cervixes. The balloon was inflated to seal off the posterior end of the MV. Two milliliter (tammar) or 1 ml (wombat) of 0.1% Coomassie brilliant blue (Sigma, Australia) was injected into the AVC to stain and simulate the site of insemination. The balloon was defeated, catheter retracted and the extent of dye distribution in the tracts noted. In the tammar, as in all macropodids, the MV is a single open canal, allowing both cervixes to draw semen. However, a septum divides the MV in wombats and interestingly, the MV was partially fused at the caudal end. In the tammar, Coomassie blue penetrated the MV, the AVC and cervixes. These lie a distance of  $7.6 \pm 0.3$  cm from the urogenital opening (see table). In the wombat, the MV was fused caudally and so was difficult to penetrate. The cervixes lay  $13.3 \pm 0.3$  cm from the urogenital opening. Coomassie blue was localized to only one side of the MV and AVC, confirming that the median septum effectively separates the cervixes.

These results have important implications for AI using non-surgical catheters for semen deposition. In the tammar wallaby and potentially other macropodids, cervical insemination via the MV is feasible, requiring no ultrasonic guidance. However in the southern hairy-nosed wombat and other vombatids, cervical insemination is unlikely to be successful due to the fused MV. Presence of the septum would require the side of ovulation and thus catheter delivery to be determined by ultrasound.

We thank Cook Australia for the catheters and Dr. M. Jacobson for sponsoring this study.

Species	Catheter penetration				Median septum	MV, AVC and cervical staining
	UGS	LV	MV	Depth to cervixes <sup>a</sup> (cm)		
<i>M. eugenii</i>	Easy	None	Easy	$7.6 \pm 0.3$	Absent	All
<i>L. latifrons</i>	Easy	None	Difficult	$13.3 \pm 0.3$	Present	Right side only

UGS: urogenital sinus; LV: lateral vaginae; MV: median vagina; AVC: anterior vaginal culs de sac.

<sup>a</sup> Mean  $\pm$  S.E.M.

CRYOPRESERVATION OF EUROPEAN BISON (*BISON BONASUS*) SEMEN

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An experiment was designed to compare two different semen extenders, Triladyl (Minitube Spain; T) containing egg yolk, and synthetic extender (S) without egg yolk for the cryopreservation of European Bison (*Bison bonasus*) sperm cells. Ejaculates ( $n = 7$ ) were collected by rectal-probe electroejaculation under general anaesthesia from a 10-year-old, European Bison bull housed at the Madrid Zoo-Aquarium. Anaesthetic was administered by with darts containing 86 mg/100 kg PV Xilazine (Seton Dihapa S.A., Barcelona, Spain). The mean volume (ml) mass motility (0–5) and sperm number ( $\times 10^6$ ) of undiluted ejaculates and sperm concentration per ml were:  $4.9 \pm 1.5$ ,  $4.2 \pm 0.4$ ,  $2560.7 \pm 1530.7$ ,  $502.9 \pm 231.6$ , respectively. After preliminary evaluation, each ejaculate was diluted at 37 °C to a final concentration of  $200 \times 10^6$  sperms per ml with Triladyl containing 7% glycerol, antibiotics and 20% egg yolk, or S.E. based on the chemical formula of Triladyl, but in which egg yolk was replaced by 15% of phosphatidylcholine of soybean origin. Extended samples were maintained at 5 °C for 4 h, loaded into 0.25 ml French straws, frozen 10 cm above liquid nitrogen ( $\sim 166$  °C) for 10 min, then plunged into liquid nitrogen. After 2 weeks of storage, one straw from each extender and ejaculate was thawed in a water bath at 37 °C for 30 s. Post-thaw viability was evaluated by individual sperm motility, rate of sperm movement, numbers of sperm morphological abnormalities, intact acrosomes, functional integrity of the sperm membranes (hypoosmotic swelling test; HOST), sperm viability (live–dead, eosin–nigrosin stain), and a heterologous in vitro sperm penetration assay. A total of 600 in vitro matured bovine oocytes were inseminated with  $1 \times 10^6$  spermatozoa from Holstein semen frozen–thawed in Triladyl extender (Control;  $n = 158$ ) or of European Bison semen frozen in T ( $n = 221$ ) or S.E. ( $n = 221$ ). Nuclear status of the oocytes was determined after 18 h of sperm–oocyte co-incubation. All oocytes were denuded, fixed in acetic acid and stained with 1% lacmoid. Oocytes with two pronuclei were considered as fertilized. Data were compared by ANOVA or Kruskal–Wallis test. There were no differences between T and S in individual sperm motility after dilution ( $87.8 \pm 3.9$  versus  $85.7 \pm 5.3\%$ ), after 4 h ( $80.7 \pm 5.3\%$  versus  $75.7 \pm 9.3\%$ ) or after freeze/thaw ( $44.3 \pm 14.0\%$  versus  $32.8 \pm 12.0\%$ ); rate of movement after dilution ( $4.3 \pm 0.2$  versus  $4.3 \pm 0.3$ ), after 4 h ( $4.0 \pm 0.3$  versus  $3.8 \pm 0.4$ ) or after freeze/thaw ( $3.8 \pm 0.2$  versus  $3.8 \pm 0.4$ ); normal morphology after dilution ( $88.4 \pm 4.5\%$  versus  $88.4 \pm 4.5\%$ ), after 4 h ( $88.3 \pm 3.7\%$  versus  $87.6 \pm 4.6\%$ ) or after freeze/thaw ( $87.0 \pm 3.6\%$  versus  $89.4 \pm 2.7\%$ ). Although there was no difference between extenders in acrosome integrity after dilution ( $89.8 \pm 1.5\%$  versus  $89.8 \pm 1.5\%$ ) or after 4 h ( $85.5 \pm 2.8\%$  versus  $84.3 \pm 4.3\%$ ); hypoosmotic swelling test after dilution ( $89.5 \pm 7.0\%$  versus  $89.5 \pm 7\%$ ) or after 4 h ( $60.1 \pm 13.3$ ;  $52.7 \pm 15.1\%$ ); sperm viability after dilution ( $88.0 \pm 6.9\%$  versus  $88.0 \pm 6.9\%$ ) or after 4 h ( $82.6 \pm 5.7\%$  versus  $77.6 \pm 8.5\%$ ), there were differences ( $P < 0.05$ ) in acrosome integrity after freeze/thaw ( $56.7 \pm 12.2\%$  versus  $42.8 \pm 11.0\%$ ), hypoosmotic swelling test ( $55.1 \pm 8.1\%$  versus  $34.8 \pm 10.9\%$ ) and sperm viability ( $62.6 \pm 13.5\%$  versus  $36.3 \pm 8.7\%$ ). There was no difference in zona penetration assay between Controls ( $62.4 \pm 11.6$ ), T ( $43.0 \pm 24.2$ ) or S ( $63.1 \pm 16.0$ ). In summary, Triladyl extender and a synthetic extender in which phosphatidylcholine replaced egg yolk were used for the cryopreservation of European Bison semen.

NUCLEAR MATURATION OF EUROPEAN POLECAT (*MUSTELA PUTORIUS*)  
IN VIVO OOCYTES AND SUCCESS IN USING MUSTELID HYBRIDS  
AS RECIPIENTS FOR EMBRYO TRANSFER

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The endangered European mink (*Mustela lutreola*) is nearly extinct in Scandinavia. Last wild populations are considerably diminished in Russia, Belarus, France and Spain. Our long-term aim is to study the possibility to utilize cross-species nuclear transfer (NT) for rescuing the genetic material of suddenly deceased European mink. Farmed European polecat (*Mustela putorius*) oocytes may be used as appropriate cytoplasm recipients for rescued cells of European mink. Towards this aim, objectives of this study were to find out if hybrids of these two species are appropriate recipients for European mink embryos and to study the optimal time point for recovery of in vivo MII oocytes. European mink in vivo blastocysts ( $n = 26$ ) were surgically transferred into uteri of hybrid (European mink  $\times$  European polecat) recipients. To induce ovulation, the recipients were mated with vasectomized males during natural oestrus of the breeding season. Five embryo transfers resulted in birth of viable European mink pups ( $n = 15$ ) indicating that hybrids can serve as appropriate recipients for cross-species NT embryos. Nuclear maturation of European polecat in vivo oocytes was studied by mating females ( $n = 14$ ) with vasectomized males. The females were humanely killed and oocytes were recovered from developing follicles in every 3 h after mating. Oocytes collected from follicles of non-mated females ( $n = 2$ ) were used as a control. Cumulus cells were removed with hyaluronidase and oocytes were fixed and stained with Hoechst 33258. The average number of recovered oocytes per donor was 11. We found that 33–39 h after mating 80% of the in vivo oocytes were at MII stage (Table 1). These result in investigating in vivo nuclear maturation of European polecat oocytes combined with established IVC methods as described by Lindeberg et al. [Theriogenology 2001;55:429] encourage us to start modifying cross-species NT methods for endangered European mink.

Table 1  
Nuclear maturation of in vivo oocytes stained with Hoechst 33258 in the farmed European polecat (*Mustela putorius*)

Number of donors	Time after mating (h)	Nuclear maturation stage					Total number of oocytes
		GV	GVBD	MI	MI	NBA	
2	0 <sup>a</sup>	37	1				38
4	3–15	38	2			1	41
2	18–21	17	4		1	1	23
3	24–30	16	1	12	1	1	31
3	33–39			4	16		20

<sup>a</sup> Not mated NBA = not possible to analyze.

SUPEROVULATION IN WATER BUFFALO IS INFLUENCED BY PROTOCOL  
AND BREED DIFFERENCES

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Superovulation (SO) has been previously achieved in buffalo (*Bubalus bubalis*) using FSH or eCG. While treatment with eCG recruits a variable number of follicles from the current and subsequent follicular waves, follicles from the subsequent wave do not ovulate due to the lack of response to a low LH peak because of the effects of progesterone (PG). Also, the follicles that do not ovulate secrete oestradiol (E2) in abnormally large amounts that alters the PG:E2 ratio and results in improper completion of meiosis and maturation of the oocyte. For this reason FSH is the preferred gonadotropin for SO, but results in buffalo remain poor relative to other species. This study investigated the use of gonadotropin protocols for SO of swamp and riverine cross breed water buffalo. Semen collected from a riverine bull in the Northern Territory of Australia was cryopreserved in a Tris-based extender with 20 million spermatozoa per straw. Prior to implementation of the five SO treatments, each cow received prostaglandin (PGF, Estroplan, day 0). Groups 1–3 received PG (CIDR) on day 4 that was removed on day 15 along with PGF administration. Groups 2–5 received a GnRH agonist (Desorelin) from day 6 to day 12. All groups received a total of 14.4 ml of FSH (Folltropin) staggered from day 12 to day 16. Groups 3 and 5 were given LH (Lutropin) on the evening before AI on day 16 and 17 and group 4 received LH on the morning of AI on day 17. Embryos were collected non-surgically on day 24. Results were analyzed by ANOVA or GLM using Genstat with a significant difference at  $P < 0.05$ . The effect of gonadotropin protocol resulted in a significant difference in the number of CLs per cow for each treatment (2.29, 0.67, 3.33, 5.99, 0.25). Use of a GnRH agonist without PG and then LH on the morning of insemination resulted in the collection of 1.75 embryos per cow that tended to be greater than all other protocols. Use of LH on the morning of AI compared with LH in the evening or no LH resulted in a significantly ( $P < 0.05$ ) different number of viable embryos (1.75, 0.86, 0.0) and proportion of viable embryos from those collected (35.4, 26.4, and 0.0%). Riverine F1 compared with swamp females resulted in significantly greater embryo (0.6 versus 0.0) and unfertilized oocyte (0.31 versus 1.14) numbers and the proportion of viable embryos from those collected (45.0% versus 0.0%). The optimal SO treatment used initiation of synchronization using PGF, followed by a GnRH agonist to remove natural gonadotropin secretion. FSH recruited developing follicles and LH induced ovulation at the same time as a morning insemination. The use of LH to control ovulation helped overcome the inherent problem of buffalo sensitivity to E2 from non-ovulated follicles and aligned the timing of ovulation with AI, which enhanced embryo number and viability. This may be linked to the observation of greater reproductive behavior shown by buffalo in the morning and the ability of LH treatment to induce ovulation so that spermatozoa are in the reproductive tract when ovulation occurs. The shorter life of buffalo spermatozoa in the reproductive tract makes ovulation time a critical factor in the success of SO. Riverine F1 females may have been more successful embryo donors because of heterosis or selection for reproductive competence and productivity.

IN VITRO MATURATION (IVM) OF OOCYTES COLLECTED FOLLOWING  
HORMONAL OVARIAN STIMULATION OF COMMON WOMBATS  
(*VOMBATUS URSINUS*)

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Ovarian stimulation with gonadotropins can increase the number of mature MII oocytes that can be collected from the naturally monovular Common Wombat. However, following hormone stimulation and follicular aspiration, few oocytes collected from the Common Wombat are at the MII stage. If assisted reproduction is to be used to rescue endangered wombat species, we need an IVM protocol that allows the immature oocytes to develop into fertilizable MII oocytes. Eleven wombats were stimulated with eight injections of porcine FSH at 12 h intervals and one injection of porcine LH on day 6, 12 h after the last FSH injection. The ovaries were surgically harvested at the time of euthanasia and oocytes were aspirated from follicles >1 mm into Hepes buffered TCM 199. Cumulus cells were removed where possible with a finely pulled pipette to determine the oocytes maturation status. Oocytes with a polar body were defined as mature MII oocytes. All other oocytes were then placed in TCM 199 maturation media at 35 °C and 5% CO<sub>2</sub> in air. Oocytes were examined under a light microscope at 6–8 h intervals. After 96 h IVM all oocytes that had not extruded a polar body were stained with Hoechst 33342 to confirm the maturational status. A total of 244 oocytes was recovered from the 11 pFSH/pLH stimulated wombats. At the time of collection 33 (13.5%) were MII and 11 oocytes (4.4%) showed signs of degeneration. The remaining 204 immature oocytes lacked a polar body ( $n = 188$ ), or had cumulus cells ( $n = 16$ ) that could not be removed. After 24 h IVM, 29 (14.2%) of the 204 immature oocytes extruded a polar body. After 30 h IVM, another 4 (1.9%) immature oocytes had extruded a polar body. Despite maturation for up to 96 h, no further MII oocytes developed. A total of 33 MII oocytes (16.2%) was produced following IVM. IVM of immature oocytes following hormonal stimulation therefore doubled the yield of MII oocytes that could otherwise be collected following hormonal stimulation alone. Further research is still required to examine fertilization protocols and culture conditions. Porcine FSH and porcine LH were kindly supplied by Vetrepharm Pty Ltd.

## Folliculogenesis/Oogenesis

### OXYGEN AND CARBON DIOXIDE TENSION IN DAYS 14–15 DOMINANT BOVINE FOLLICLES MEASURED IN VIVO OR 4 HOURS POST-MORTEM

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The success of in vitro embryo production after OPU is generally lower compared with oocytes recovered after slaughter. [Hendriksen et al., *Theriogenology* 2000;53:11–20]. Increased developmental competence has been achieved by recovering the oocytes 4 h post-mortem and this effect has been attributed to the induction of follicular artesias [Blondin et al., *Theriogenology* 1997;47:1061–1075]. This study compared oxygen (O<sub>2</sub>), carbon dioxide (CO<sub>2</sub>) and pH in follicular fluid (FF) of known atretic and dominant follicles collected either in vivo or 4 h post-mortem. Twenty non-lactating cows (Friesian and Angus) were synchronized using a standard prostaglandin treatment. The largest follicle was ablated 18–24 h after the onset of observed estrus (Day 1). Follicular maps were constructed from daily ultrasound scanning starting at Day 7 until collection to distinguish between the first (DF1, atretic) and second wave (DF2, growing) dominant follicles. FF was sampled when the DF1 and DF2 were approximately the same size in diameter (10–14 mm), which occurred on Days 14–15. Animals that did not exhibit this follicular pattern were not sampled. Samples were obtained using modified ultrasound-guided OPU equipment and each sample was measured in a portable clinical gas tension analyzer. An arterial tail blood sample taken from each cow using an arterial sampler (PICO™) was similarly measured and served as a control. Sixteen of the cows were subsequently resynchronized, scanned/mapped and selected for slaughter on Day 14 or 15. Individual pairs of ovaries were recovered and held for 4 h in 30 °C saline before the FF was sampled and analyzed as previously described. Samples were successfully collected and measured from 14 DF1 and 13 DF2 in vivo follicles and 9 D1 and 13 D2 post-mortem. Data were analyzed using Residual Maximum Likelihood (Genstat V; Lawes Agricultural Trust, UK) to calculate the relevant contrasts and using *t*-tests to detect differences among the four follicle types. Results are presented as means (±S.E.M.). O<sub>2</sub> tension was lower for in vivo DF1 FF compared with the DF2 (67.0 ± 4.0 mmHg versus 86.4 ± 4.0 mmHg, *P* < 0.001). Holding the follicles at 30 °C for 4 h decreased DF2 O<sub>2</sub> tension to 65.8 ± 4.1 mmHg, but DF1 O<sub>2</sub> tension was not affected. Mean CO<sub>2</sub> tension differed between in vivo DF1 and DF2 (42.3 ± 1.0 and 39.8 ± 1.0 mmHg, *P* < 0.02) and when held for 4 h this increased to a significant difference of 158.4 ± 1.2 and 138.0 ± 1.5 mmHg, respectively (*P* < 0.002). There was no difference in FF pH (7.55 ± 0.02) for in vivo follicles but after being held for 4 h the FF pH significantly declined to 6.77 ± 0.02 (*P* < 0.001). Arterial mean O<sub>2</sub> tension was higher (97.1 ± 3.3 mmHg) than FF O<sub>2</sub> tension (*P* < 0.001) while CO<sub>2</sub> and pH levels were similar to in vivo FF. This is the first time that O<sub>2</sub>, CO<sub>2</sub> and pH have been measured in bovine FF collected in vivo and this procedure could be useful to characterize the follicular environment during follicle growth and decline. Results suggest that holding ovaries for 4 h at 30 °C creates a hypoxic environment in the follicle which may be related to the increased developmental competence of the oocyte. The authors thank FRST (C10X0018) for financial support.

## MOUSE OOCYTES DEVELOPED FROM FOLLICLE CULTURE HAVE PERTURBED METABOLISM AND COMPROMISED ENZYME ACTIVITY

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The quality of an embryo resulting from assisted reproductive technologies is dependent on the quality of the oocyte from which it is derived. Rates of blastocyst development in culture are reduced when oocytes are produced from IVM compared to in vivo maturation. Further reductions in development are observed when oocytes are grown in follicle culture and matured in vitro. Currently there are no markers of oocyte physiology that are predictive of subsequent developmental competence. The aim of this study was therefore to examine some metabolic characteristics of oocytes matured either in vivo, in vitro or from follicle culture. The long-term aim of this study is to determine a marker for oocyte quality.

Oocytes for this study were derived from CF1 mice. For follicle culture oocytes were collected from 12-day-old females by digesting the ovaries with collagenase. Oocytes were grown for 10 days to the GV stage on collagen inserts in Waymouth's medium supplemented with serum, BSA and ITS. For IVM, GV oocytes collected from both follicle culture and ovaries were matured in MEM medium supplemented with serum, FSH and EGF for 16 h. Rates of MII development were 76 and 70%, respectively. Metabolic parameters of oocytes were measured at the GV and MII stages. Pyruvate oxidation and glycolysis were assessed using radiolabelled substrates and lactate dehydrogenase (LDH) levels were determined using ultramicrofluorescence. Data were analyzed using one-way ANOVA and Bonferroni's post-test for multiple comparisons or Student's *t*-test.

GV oocytes that were collected and measured immediately had levels of pyruvate oxidation of  $2.14 \pm 0.2$  pmol/oocyte/h and levels of glycolysis of  $0.79 \pm 0.1$  pmol/oocyte/h. Oocytes that were grown from preantral follicles for 10 days to the GV stage had significantly lower rates of pyruvate oxidation ( $0.72 \pm 0.1$  pmol/oocyte/h,  $P < 0.05$ ) and significantly higher rates of glycolytic activity ( $3.55 \pm 0.4$  pmol/oocyte/h) compared to GV stage oocytes developed in vivo. Ovulated metaphase II oocytes had levels of pyruvate oxidation and glycolytic activity of  $2.2 \pm 0.2$  and  $1.5 \pm 0.2$  pmol/oocyte/h, respectively. Maturation of oocytes in vitro did not alter pyruvate oxidation or glycolytic activity ( $2.5 \pm 0.2$  and  $1.9 \pm 0.2$  pmol/oocyte/h) compared to ovulated MII oocytes. In contrast, MII oocytes that were derived from follicle culture had highly perturbed metabolic profiles. Oxidation was significantly reduced ( $0.71 \pm 0.2$  pmol/oocyte/h,  $P < 0.001$ ) and glycolysis significantly increased ( $5.3 \pm 0.9$  pmol/oocyte/h,  $P < 0.001$ ) compared to either in vivo ovulated oocytes or in vitro matured oocytes. MII oocytes derived from follicle oocytes that were grown and matured in vitro also had significantly lower levels of LDH activity ( $18.4 \pm 4.5$  nmol NADH oxidized/oocyte/h) compared to those that were matured in vitro ( $50.4 \pm 10.4$  nmol NADH oxidized/oocyte/h) or in vivo ( $55.4 \pm 7.4$  nmol NADH oxidized/oocyte/h).

Oocytes derived from follicle culture have a significantly reduced developmental capacity in culture. In this study such oocytes were found to have significantly perturbed metabolism and reduced LDH activity compared to in vitro matured or in vivo matured oocytes. Therefore, this work indicates that the metabolic profile of an oocyte may be of use in identifying viable oocytes and improving follicle culture conditions.

## DEVELOPMENT OF RABBIT PREANTRAL FOLLICLES ISOLATED FROM OVARIES OF SEXUALLY MATURED DOE IN THE SCID MICE

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*Objectives:* Xenogeneic transplantation of ovarian follicles into immunodeficient animal host may be an approach toward fertility preservation for young female patients undergoing cancer therapy and also female animals of endangered species. The present study was conducted to determine whether preantral follicles isolated from rabbit ovaries developed in kidney cortex of SCID mouse. *Materials and methods:* Male and female SCID mice (6–8 weeks of age) were obtained from local breeder. Cages were filtertopped, and animals had free access to sterilized food and water under 14L:10D condition. Preantral follicles (outer diameter of about 0.15–0.2 mm) were isolated from New Zealand white doe ovaries minced by scalpels. In group 1, 10 follicles were transplanted into each left kidney cortex of nine females and in group 2; the same number of follicles were transplanted into each of six males. Mice were anesthetized by i.p. injection of sodium pentobarbital (0.4 mg/BW). During surgery, mice were kept on a warming plate. Xenografting of ovarian follicles was performed within 2 h after removal from ovaries to minimize ischemic damage. The mice were killed every 2 weeks until 6th week after surgery, and grafts were dissected out from the kidney cortex. The recovered grafts were placed in Bouin's fixative overnight and fixed tissues were embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin. The sections were examined for presence of follicles and their developmental status was examined. Follicles were classified as follows: secondary follicles having up to two or three layers of cuboid granulosa cells but no antrum, and antral follicles with an antral cavity. Diameters of oocytes and follicles were recorded. *Results and discussion:* The 90 secondary follicles transplanted into nine female SCID mice at the time of isolation had an average diameter of  $165 \pm 12 \mu\text{m}$  and no antrum. After 2, 4, 6 weeks from transplantation, their average diameter increased to  $262 \pm 19$ ,  $380 \pm 84$ ,  $376 \pm 67 \mu\text{m}$  and 6/30, 11/30, 1/30 developed an antrum, respectively. Before transplantation, the average diameter of oocytes was  $80 \pm 4$  and after 2, 4, 6 weeks from transplantation, it increased to  $95 \pm 8$ ,  $98 \pm 9$ ,  $111 \pm 0 \mu\text{m}$ , respectively. In males, number of antral follicles observed were 13/30, 6/30 at 4, 6 weeks after transplantation. The diameter of follicles and oocytes was not measured, yet. At 6 weeks after transplantation, two follicles became corpus lutea in groups 1 and 4 bloody follicles were observed in group 2. Meiotic competence of oocytes in antral follicles was not confirmed yet. As shown in this experiment, rabbit secondary follicles could be developed to antral stage in kidney cortex of SCID mice.

## GROWTH OF PORCINE PRIMORDIAL FOLLICLES AND THEIR OOCYTE MATURATION BY XENOTRANSPLANTATION

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Primordial follicles are considered to be as the storage of ovarian follicles and can be a potential resource of oocytes that is used for agricultural and zoological purposes. However, the complete maturation of oocytes in preantral follicles using in vitro systems is difficult in large mammals. In this study, we investigated whether porcine oocytes in primordial follicles achieve full maturation by an in vivo grafting. Cortex of ovaries, collected from 20-day-old European breed piglets, was minced into small pieces of  $\sim 1 \text{ mm}^3$ . Three to five pieces of ovarian tissues were transplanted under the capsule of each kidney of ovariectomized athymic mice (CD-1(ICR)-nu/nu). Vaginal smears were taken daily, starting 10 days after surgery. After the recipient mice showed cornified epithelial cells in smear, the grafts were recovered from mice 48 h after treatment with 5 IU eCG ( $n = 6$ ) or without eCG treatment ( $n = 5$ ). Oocytes were isolated mechanically with a surgical blade from the grafts and diameter of the oocytes was measured. The oocytes were cultured in a modified North Carolina State University-37 solution (IVM media) for 48 h and fixed immediately for assessment of the nuclear status. Some IVM oocytes, which extruded a first polar body, were stimulated with an electric pulse and were fixed after the subsequent cultured for 10 h. To assess follicular development in the grafts, a piece of the graft was obtained from each three mice either with or without eCG treatment and was subjected to histological examination. The recipient mice showed presence of cornified epithelial cells between 45 and 77 days after transplantation ( $62.3 \pm 2.5$  days, mean  $\pm$  S.E.M.,  $n = 11$ ), suggesting the growth of estrogenic follicles at this time. Histological examination revealed formation of one to three antral follicles in each ovarian graft recovered from the three mice without eCG treatment. However, in 20-day-old piglets, primordial follicles accounted for more than 98% of the follicles in the cortex of ovary and the percentage of primary and secondary follicles was less than 2%. Without eCG treatment, isolation of porcine oocytes from five mice yielded 84 oocytes and diameter of the oocytes ranged from 75 to 141  $\mu\text{m}$  ( $102 \pm 1.6 \mu\text{m}$ ). Two of 42 oocytes (4.8%), obtained from three mice, were matured to metaphase-II (M-II) stage after the maturation culture. Treatment with eCG resulted in an enhanced growth of antral follicles in the ovarian grafts. Two hundred and forty-three porcine oocytes were recovered from six eCG primed-mice and their diameter was  $112 \pm 1.2 \mu\text{m}$  with a range of 80–148  $\mu\text{m}$ . Out of 243 recovered oocytes cultured in the IVM media, 38 oocytes (15.6%) were at M-II stage. Moreover, when IVM oocytes with first polar body were stimulated, all oocytes (3/3) were found to be activated with a female pronucleus and a second polar body. The present results indicate that xenotransplantation promotes the growth of primordial follicles from neonatal piglets and provides a full maturational competence to the oocytes in primordial follicles.

## EFFECT OF IGF-I AND EGF ON PORCINE PREANTRAL FOLLICULAR GROWTH AND DEVELOPMENT

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Follicular growth or atresia depends upon the balance between cell proliferation and death. Although there is little information on the regulation of early folliculogenesis, insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) are reported to have an important role. The objective of the current study was to determine the effect of IGF-I and EGF on preantral follicular growth, antrum formation, and proliferation, apoptosis of granulosa cells in cultured preantral follicles. In Experiment 1, 200 preantral follicles (mean diameter =  $254.7 \pm 24.5 \mu\text{m}$ ) were manually dissected from prepubertal gilt ovaries collected at an abattoir and cultured individually for 4 or 8 days in alpha-MEM medium supplemented with 2.5% BSA, 2 mIU/ml oFSH, 10  $\mu\text{g/ml}$  transferrin, 50  $\mu\text{g/ml}$  L-ascorbic acid/ml and growth factor [Control: no growth factor; IGF-I (100 ng/ml), EGF (10 ng/ml), E or I + E]. Half of the follicles on Day 4 of culture and the other half on Day 8 were fixed in 10% neutral buffered formalin (NBF) and processed for immunodetection of cell proliferation (PCNA) and apoptosis (TUNEL). There was a significant interaction between growth factor groups and day of culture on follicular diameter. Addition of either growth factor sustained follicular growth. Compared to the control group, IGF-I increased the percentage of proliferating granulosa cells (GCs) in follicles cultured for 4 days and EGF increased the proportion of antral follicles in EGF and I + E groups ( $P < 0.05$ ). Both IGF-I and EGF suppressed apoptosis of GCs; however, at the end of an 8-day culture, there was no difference among the four groups in the percentage of proliferating GCs. Granulosa cell proliferation decreased from a mean of  $24.6 \pm 1.5\%$  in freshly isolated preantral follicles to  $1.0 \pm 0.3\%$  in follicles cultured for 8 days. Since the proportion of proliferating GCs decreased dramatically in serum-free culture medium, the objective of the second experiment was to determine the effect of IGF-I and EGF on granulosa cell proliferation and apoptosis after shorter-term culture (1–4 days) in serum-supplemented medium. Preantral follicles (mean diameter =  $252.6 \pm 26.9 \mu\text{m}$ ) were cultured as described for Experiment 1 except that the culture medium was supplemented with 7.5% fetal calf serum. On Day 1, 2, 3, or 4, follicles were fixed in 10% NBF and processed for cell proliferation and apoptosis assays. There was a significant treatment  $\times$  day interaction on the proportion of proliferating GCs. IGF-I sustained a high percentage of cell proliferation from Day 1 to 4, however, EGF did not. There was a rapid decrease in cell proliferation from Day 3 to 4 in each of the four groups. EGF, IGF-I, and EGF + IGF-I suppressed ( $P < 0.05$ ) apoptosis of granulosa cells compared to the control group. In addition, EGF increased ( $P < 0.05$ ) the proportion of antral follicles, as described in Experiment 1. Our results indicate that IGF-I was effective in stimulating cell proliferation in both serum-free and serum-supplemented medium. EGF stimulated antrum formation in preantral follicles and both IGF-I and EGF inhibited apoptosis of granulosa cells. Both IGF-I and EGF may have an important role in regulating preantral follicular growth and differentiation, *in vitro*.

THE EXPRESSION AND LOCALIZATION OF TUMOR NECROSIS FACTOR  
(TNF) $\alpha$ , TNF $\alpha$  RECEPTOR (TNFR) 2 AND TNFR-ASSOCIATED FACTOR 2  
DURING FOLLICULAR ATRESIA IN PIG OVARIES

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Tumor necrosis factor (TNF) $\alpha$  can induce both cell death and cell proliferation and exerts its effects by binding to either TNF receptor (TNFR) 1 or 2. When TNF $\alpha$  bound TNFR2 interacts with TNF receptor-associated factor 2 (TRAF2), expression of survival genes is up-regulated; thus TRAF2 is a good indicator of TNF $\alpha$ -dependent cell proliferation. In the present study, we histochemically analyzed the changes in localization of TNF $\alpha$  and TRAF2 in granulosa cells during follicular atresia in pig ovaries. We examined the changes in localization and levels of expression of TNF $\alpha$  and TRAF2 mRNAs by in situ hybridization and by reverse transcription (RT)-polymerase chain reaction (PCR) analysis. Moreover, the changes in expression of TNFR2 in granulosa cells during follicular atresia were examined by Western blotting analysis. For immunohistochemistry and in situ hybridization for TRAF2 mRNA and TNF $\alpha$  mRNA, pig ovaries obtained at a local slaughterhouse were fixed with 20% buffered formalin. For Western blotting and RT-PCR analysis, individual antral follicles, 3–5 mm in diameter, were dissected from the ovaries. Based on morphological and endocrinological criteria, the antral follicles were divided into three categories as follows: healthy, early stage of atresia, progressed stage of atresia. In healthy follicles, intense signals for TNF $\alpha$  and TRAF2 and their mRNAs were demonstrated in the outer zone of the granulosa layer, where many proliferating cells and no apoptotic cells were seen. In atretic follicles, trace staining for TRAF2 and its mRNA and decreased expression of TNFR2 were observed in the granulosa layer, where many apoptotic cells were seen. Decreases in TNFR2 and TRAF2 expression with progression of follicular atresia were demonstrated in Western blotting analysis and RT-PCR analysis, respectively. The expression decreased in early atretic follicles compared with healthy follicles, and disappeared in progressed atretic follicles. The present findings suggested that the TNF $\alpha$ -receptor system plays critical roles in induction of survival signals in granulosa cells during follicular selection in porcine ovaries.

PROLACTIN RECEPTORS IN SHEEP OVARY: REGULATED EXPRESSION OF THE LONG BUT NOT THE SHORT PROLACTIN RECEPTOR ISOFORM THROUGHOUT THE ESTROUS CYCLE

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PRL might play an essential role on early follicular development and atresia in the ovary, for its proliferative and differentiative actions. Long (L) and short (S) isoforms of PRL receptor (PRLr) are expressed in ovine ovary, but only binding of PRL to homodimers of the LPRLr is able to induce proliferation and differentiation on responsive cells. Thus, the expression of LPRLr is correlated with being a real target of PRL actions. The aim of this study was to determine the variations in potential responsiveness to PRL in sheep ovary at three critical time-points during the estrous cycle, by comparing the expression of L and SPRLr. Estrous cycle was synchronized in adult Merino ewes with two cloprostenol injections (125 µg i.m., Estrumate, Essex Animal Health, Germany) given 10 days apart. Ovariectomies were performed on Day 0 ( $n = 4$ , day of estrus), Day 10 ( $n = 4$ , mid luteal phase) and Day 14 ( $n = 4$ , early follicular phase) of the cycle, and ovaries were immediately frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$ . Total RNA was extracted [Zabala and Garcia Ruiz, *Endocrinology* 1989;125:2587–2593] from the ovaries of each ewe, and 5 µg were reverse transcribed (AMV reverse transcriptase, Amersham) to synthesize cDNA used as template for PCR amplification. For PCR, forward and reverse primers were designed to amplify DNA fragments corresponding to L (342 bp) and S (265 bp) PRLr, using the reported mRNA sequences [Bignon et al., *J Mol Endocr* 1997;19:109–120]. Ovine actin was used as control for internal normalization. For each PCR, DNA (1 µl for L and SPRLr, 0.5 µl for actin), 1 µl of each primer, microliter dNTP (10 mM, Amersham), 5 µl of 10× PCR buffer, 6 µl MgCl<sub>2</sub> (25 mM), 0.5 µl of Taq polymerase (Promega) and water up to 50 µl were mixed and placed in a thermal cycler. Amplification was carried out for 35 cycles of 1 min at 94 °C, 2 min at 55 °C (SPRLr) or at 52 °C (for LPRLr), 3 min at 72 °C. PCR products (16 µl) were electrophoresed in 2% agarose gel that was then stained in EtBr to identify L and SPRLr DNA bands of predicted sizes, and photographed under UVA light. Optical density of each individual L and SPRLr DNA band was measured (GS-710, Calibrated Imaging Densitometer, Bio-Rad Labs, Hercules, CA, USA) and normalized with corresponding actin optical density. Significant effects of the day of cycle on the level of expression of each isoform were analyzed by ANOVA. Results showed that SPRLr expression remained at similar levels throughout the estrous cycle, suggesting its constitutive expression, whereas LPRLr mRNA levels markedly increased ( $P < 0.01$ ) at the time of estrus when compared with levels at mid luteal phase, and at early follicular phase, time at which expression of LPRLr was even lower ( $P < 0.05$ ) than in mid luteal phase. The expression of LPRLr was much greater ( $P < 0.01$ ) than that of SPRLr at the time of estrus, the expression of both forms was similar in mid luteal phase, and LPRLr expression was much lower ( $P < 0.05$ ) than that of SPRLr at the early follicular phase. These results indicate that the expression of the LPRLr is highly regulated during the estrous cycle in sheep, unlike the SPRLr, and that the possibilities for PRL to bind homodimers of LPRLr are markedly increased at the time of estrus and greatly reduced at early follicular phase. Therefore, the preovulatory phase of the estrous cycle in sheep might be time of maximum responsiveness to PRL.

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## FOLLICULAR WAVE SYNCHRONIZATION AND FIXED-TIME MATING IN LLAMAS

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The objective of the study was to compare the efficacy of treatments intended to induce follicular wave synchronization among lactating and non-lactating llamas (Experiment 1), and determine the effect of these treatments on pregnancy rates after fixed-time natural mating (Experiment 2). In Experiment 1, lactating ( $n = 40$ ) and non-lactating ( $n = 40$ ) adult llamas were allotted randomly to the following treatments groups: (1) 2 ml phosphate buffered saline i.m. (Control,  $n = 20$ ), 1 mg estradiol  $17\beta$  + 25 mg progesterone in safflower oil i.m. (E/P,  $n = 20$ ), (3) 5 mg Armour Standard LH i.m. (LH,  $n = 20$ ), (4) transvaginal ultrasound-guided ablation of follicles  $\geq 5$  mm in diameter (FA,  $n = 20$ ). Daily transrectal ultrasound examinations were performed using a 7.5 MHz linear-array transducer (Aloka, SSD500) starting 3 days before to 20 days after treatment. In Experiment 2, 90 adult lactating llamas at unknown stages of the follicular development were randomly assigned to the following treatments groups ( $n = 30$  per group): (1) Control, (2) E/P or (3) LH. A single, fixed-time natural mating was permitted 10–12 days after treatment. Different male was used for each female (90 males). Ultrasound examinations were done at 3, 8 and 20 days after mating to evaluate ovulation, the presence of CL, and pregnancy, respectively. Data from Experiment 1 were analyzed by two-way ANOVA (treatment and lactational status) and the degree of synchrony in wave emergence (variability) was estimated by first calculating the group mean. The group mean was then subtracted from each data point and the residual was squared. The final values were analyzed by two-way ANOVA to determine if the variability was different among groups. Chi-square analysis was used to evaluate ovulation and pregnancy rates in Experiment 2. In Experiment 1, the mean interval from treatment to follicular wave emergence did not differ between Control ( $5.6 \pm 1.2$  days) and E/P ( $4.5 \pm 0.8$  days) groups, but both were longer ( $P < 0.05$ ) than in the LH ( $2.1 \pm 0.3$ ) and FA groups ( $1.8 \pm 0.3$ ). There was a treatment  $\times$  lactation interaction ( $P < 0.01$ ) on the variability of the interval from treatment to follicular wave emergence. In lactating llamas, wave emergence was more variable ( $P < 0.01$ ) in the control group (range: 1–15 days) than in the E/P (2–9 days), LH (0–4 days) and FA (1–4 days) groups. The variation in the interval to wave emergence in non-lactating llamas was numerically lower in the LH (1–5 days) and FA (1–5 days) groups than in the control (0–8 days) and E/P (1–14 days) groups, but the difference was not significant. The interval to wave emergence was most variable ( $P < 0.01$ ) in the non-lactating E/P group. In Experiment 2, females from all groups were sexually receptive to mating. Ovulation rates did not differ among groups (Control, 93%; E/P, 90%; LH, 90%;  $P = 0.99$ ). A CL was detected 8 days after the mating in all females that ovulated. The pregnancy rate tended to be higher ( $P = 0.06$ ) in the E/P group (70%) than in the Control group (50%); the LH group was intermediate (66%). In conclusion, LH and FA treatments were most effective for inducing follicular wave synchronization, but synchronization treatments did not influence ovulation rate subsequent to natural mating. Apparent differences in pregnancy rate after fixed-time natural mating between synchronized versus non-synchronized llamas warrant critical evaluation of the effects of follicular status on the developmental competence of the contained oocyte.

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## BOVINE OOCYTES IN SECONDARY FOLLICLES GROW AND ACQUIRE THE MEIOTIC COMPETENCE IN SCID MICE

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Several culture systems have been developed to support the growth of oocytes, but the efficiency of the procedure was low. Xenografting of ovarian tissue into immunodeficient (SCID) mice has been used as a model to verify follicle viability and also to study the dynamics of follicular development. Although preantral follicles in the xenografts have been shown to develop to antral stages in some mammalian species, there is a little information about oocyte growth in the follicles developed in SCID mice, and no report describing the meiotic competence of these oocytes. In the present study, we transplanted bovine ovarian tissues which contained primordial/primary or secondary follicles, separately into SCID mice to determine the stage of follicles which have the ability to develop in the grafts. Then we examined the ability of grown oocytes in SCID mice to resume meiosis and mature to the second metaphase. In the first experiment, pieces of bovine ovarian cortex about  $1\text{ mm} \times 1\text{ mm} \times 0.5\text{ mm}$  which contained only primordial and primary follicles were dissected, and transplanted under the kidney capsule of female SCID mice. After 4 or 6 weeks of transplantation the kidneys were collected, and follicular development and oocyte growth were examined. In the second experiment, bovine secondary follicles were transplanted, and follicular development and oocyte growth were examined in the same manner. Then, oocytes were collected from the follicles which had developed to  $400\text{ }\mu\text{m}$  or more in the diameter after 6 weeks of transplantation and were subjected to *in vitro* maturation. Oocytes were cultured in  $10\text{ }\mu\text{l}$  drops of TCM199 containing  $2.2\text{ mg/ml}$   $\text{NaHCO}_3$ ,  $0.08\text{ mg/ml}$  kanamycin,  $0.1\text{ mg/ml}$  sodium pyruvate and 10% fetal calf serum for 24 h. Statistical differences in the mean diameters of the follicles and oocytes were analyzed by the Student's *t*-test. Other values were analyzed by the Chi-square analysis with Yates correction for continuity. In the first experiment, all grafts were well vascularized and contained surviving follicles. However, primordial and primary follicles did not develop and no follicles developed beyond the primary stage after transplantation. In the second experiment, surviving secondary follicles formed an antrum after 4 weeks of transplantation. Secondary follicles, whose mean diameter was  $165.2 \pm 17.0\text{ }\mu\text{m}$  ( $n = 42$ ), developed to  $442.9 \pm 77.9\text{ }\mu\text{m}$  ( $n = 37$ ) and  $592.9 \pm 116.0\text{ }\mu\text{m}$  ( $n = 45$ ) in diameter after 4 and 6 weeks of transplantation, respectively. The mean diameter of oocytes in secondary follicles was  $55.1 \pm 4.9\text{ }\mu\text{m}$  ( $n = 42$ ). The diameter of oocytes increased ( $P < 0.05$ ) after transplantation in SCID mice (after 4 weeks:  $105.6 \pm 6.3\text{ }\mu\text{m}$ , after 6 weeks:  $122.2 \pm 2.6\text{ }\mu\text{m}$ ). Of the 32 oocytes 34% resumed meiosis and 6% matured to the second metaphase. In contrast, no oocytes from secondary follicles before transplantation resumed meiosis. These results demonstrated that bovine secondary follicles develop to the antral stage in SCID mice, and that grown oocytes from these follicles acquire the meiotic competence.

EFFECT OF NORGESTOMET ON INTRAFOLLICULAR STEROIDOGENIC  
ACTIVITY, FOLLICULAR DYNAMICS AND ENDOCRINE PATTERNS  
OF ZEBU COWS UNDERGOING REPEATED OOCYTE PICK-UP

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Ultrasound-guided follicular puncture allows the recovery of a large number of cumulus–oocyte complexes (COCs) for in vitro embryo production. However, repeated aspiration of ovarian follicles may induce subluteal progesterone concentrations and interfere with follicular growth pattern. The aim of this study was to evaluate the effect of norgestomet treatment on intra-follicular and systemic steroid concentrations and on ovarian follicular dynamics. Pluriparous, non-lactating Gir breed (*Bos indicus*) cows ( $n = 10$ ) were randomly distributed between treatment and control groups, and had their ovarian follicles above 3 mm aspirated twice a week (at 3–4 days intervals) during eight consecutive weeks. Oocyte pick-up (OPU) was performed with a portable ultrasound device (Scanner 100S, Pie Medical, The Netherlands). Treatment cows received norgestomet ear implants (Crestar, Intervet, The Netherlands), replaced weekly, without concomitant administration of estradiol-valerate. Follicular dynamics was evaluated by ultrasonography every 12 h and blood samples were collected daily between OPU sessions. Follicles larger than 9 mm were characterized as dominant and were used to recover samples of follicular fluid. Plasma and follicular fluid of the emerging dominant follicles were stored at  $-20\text{ }^{\circ}\text{C}$  until hormone concentration analysis. Progesterone and estradiol were measured by RIA, using commercial Kits (MedLab and DSL, USA). Data was analyzed by ANOVA, and means were compared by Tukey's test. Percentages were compared by Chi-square method. Results are presented as means  $\pm$  S.E.M. The short OPU interval used prevented natural ovulations, and mean plasma progesterone was basal ( $143.3 \pm 28.1$  pg/ml) during the experimental period. However, progesterone concentration was lower in cows treated with norgestomet ( $36.3 \pm 14.0$  pg/ml versus  $250.3 \pm 49.3$  pg/ml,  $P < 0.0001$ ), probably due to a reduction in partial luteinization of punctured follicles. There was no difference ( $P > 0.05$ ) in estradiol plasma concentrations between groups, but norgestomet treatment reduced the incidence of follicles growing above 9 mm (30% versus 65%,  $P < 0.05$ ) and the presence of co-dominant follicles (0% versus 20%) 96 h after OPU sessions. Dominant follicles were also observed in both groups when 3-days intervals were analyzed (25% versus 45%). Intrafollicular estradiol and progesterone concentrations were lower in treated than in control cows ( $265.5 \pm 47.4$  and  $34.9 \pm 5.4$  ng/ml versus  $765.2 \pm 169.1$  and  $173.3 \pm 43.4$  ng/ml, respectively,  $P < 0.05$ ). Low steroidogenic activity ( $324.5 \pm 53.7$  and  $39.6 \pm 7.9$  ng/ml for estradiol and progesterone, respectively) was observed in the treated group even when the largest follicle present was larger than 9 mm (mean diameter of  $10.1 \pm 0.4$  mm). Estradiol/progesterone ratio was not different between control and treatment groups ( $10.2 \pm 1.6$  versus  $6.5 \pm 1.8$ ,  $P > 0.05$ ). Reduction in the steroidogenic activity of growing follicles is coherent with the expected inhibitory effect of the norgestomet on LH release pattern, and the use of norgestomet ear implants is an alternative for the management of cows undergoing oocyte pick-up.

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# Gene Expression

## RELATIVE PATTERN OF GENE EXPRESSION IN PLACENTAL AND FETAL TISSUES BY IN VIVO- AND IN VITRO-PRODUCED DAY-90 AND DAY-180 BOVINE CONCEPTI

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The effects of the embryo production system on growth and the transcriptional activity of genes associated with embryo development and metabolism by the end of the first and second trimesters of gestation were investigated. Bovine females were superovulated to obtain in vivo-produced embryos, whereas in vitro-produced embryos were derived from established in vitro maturation, fertilization, and culture procedures [Bertolini et al., *Theriogenology* 2002;58:973–994]. Blastocysts were transferred to recipients, and pregnant animals from each group ( $n = 4$  in vivo- and  $n = 5$  in vitro-derived pregnancies for each day) were slaughtered on Day 90 or 180 of gestation. Uterine, placental, and fetal traits were weighed and measured; sample tissues were snap-frozen and stored at  $-80^{\circ}\text{C}$ . A sensitive real-time TaqMan PCR was optimized according to Bertolini et al. [*Mol Reprod Dev* 2002, in press] to quantify transcripts for insulin-like growth factors-1 and -2 (IGF-1 and IGF-2), their receptors (IGF-1r and IGF-2r), and facilitative glucose transporters-1 and -3 (Glut-1 and Glut-3) in placentomes, fetal skeletal muscle (*Longissimus dorsi*) and fetal liver. Data comprising physical traits and gene expression were analyzed by a  $2 \times 2$  ANOVA considering group (in vivo, in vitro) and Day (90, 180) as factors in the analyses (Minitab Inc., State College, PA). Pairwise comparisons were scrutinized by the Bonferroni test. A simple correlation test was used for the analysis of the relationship between traits. Overall, in vitro-derived conceptus traits tended to be heavier and larger than controls, but as male fetuses are also normally heavier and larger, differences between groups were not as pronounced due to the inclusion of male concepti in both groups. Consequently, data analyses were also performed considering only females ( $n = 3$  per group, except Day 90 in vitro,  $n = 4$ ). In vitro-produced pregnancies carrying females exhibited greater crown-rump lengths and fetal organ weights, larger total placentome gross surface areas on Day 90 ( $P < 0.05$ ), and larger uteri, concepti, and fetal livers on Day 180 of gestation ( $P < 0.05$ ). Irrespective of the fetal gender, in vitro-derived Day-90 placentomes displayed 2.6-fold lower IGF-2r mRNA levels than controls ( $P = 0.031$ ). A temporal four-fold increase in hepatic IGF-1 relative transcription ( $P\text{-day} = 0.002$ ) was observed in male and female fetuses from Day 90 to Day 180 of gestation. Physical traits (uterine, placental, and fetal and organ traits for both sexes) were highly correlated with one another ( $r > 0.970$ ,  $P < 0.0001$ ) and were also correlated with the levels of hepatic IGF-1 transcripts in the fetuses ( $r > 0.720$ ,  $P < 0.001$ ). No differences were detected in the relative transcription of IGF-1 in placentomes and skeletal muscle, IGF-1r, Glut-1, and Glut-3 genes in the placentomes, and IGF-2 in the liver, muscle and placentomes, regarding group and/or day of development, irrespective of the gender. The relative IGF-2 transcription levels positively correlated with the relative abundance of IGF-2r ( $r = 0.718$ ,  $P = 0.002$ ) and IGF-1r ( $r = 0.628$ ;  $P = 0.029$ ) transcripts in the placentomes, and both IGF-1r and -2r mRNA amounts correlated with one another ( $r = 0.736$ ,  $P = 0.010$ ). The decrease in IGF-2r gene expression in in vitro-derived placentomes on Day 90 of gestation may be associated with conceptus development, specifically the early embryo and fetal growth retardation seen after in vitro embryo production [Bertolini et al., *Theriogenology* 2002;58:973–994].

## GENOMIC IMPRINTING OF THE BOVINE IGF2, GTL2 AND XIST LOCI

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Genomic imprinting, the parent-of-origin control over the expression of genes, is theorized to exist in all placental mammals. Imprinting creates a functionally haploid state by silencing an allele depending on its paternal or maternal origin. In imprinted genes transcriptional regulation is achieved by the addition of methyl groups onto CpG nucleotide islands located within the promoter, resulting in repression of transcriptional activity from the methylated allele. To date, approximately 40 imprinted genes have been identified between humans and mice. The effects of imprinting are widespread and include involvement in tumor formation, Qualitative Trait Loci (QTL), fetal, placental and neurological development, X-chromosome inactivation (dosage compensation mechanism in females) and recently have been implicated as a potential factor in the abnormalities associated with nuclear transfer.

We have developed an interspecies model that allows the identification of the maternal or paternal origin of a transcript based on coding single nucleotide polymorphisms (cSNPs). This model will allow for the comparative analysis of imprinting in mammals as well as facilitate the understanding of the nuclear reprogramming process in nuclear transfer offspring.

To examine the allelic expression of candidate imprinted genes, informative cSNPs between *Bos gaurus* (seledang) and *Bos taurus* (angus) cattle were identified at three loci: Xist (X inactivation transcript), Igf2 (insulin-like growth factor 2) and Gtl2 (gene trap locus 2). *Bos gaurus* semen was used to artificially inseminate six *Bos taurus* heifers. Pregnancy was determined at Day 25 and development of fetuses was monitored until Day 72 of gestation at which time reproductive tracts were removed and placental and somatic tissues isolated. RNA was extracted (TriReagent), treated with DNase-1 (Ambion) and converted to cDNA (Stratagene). RT-PCR was used to amplify the three loci and each amplicon was sequenced (ABI 3700) directly. Allelic expression profiles of tissues were assessed by the presence or absence of cSNPs analyzed on chromatograms obtained from sequences. Primers flanking introns identified samples containing genomic contamination.

Paternal imprinting of the Gtl2 locus is observed in all tissues examined, whereas the Igf2 and Xist exhibit tissue-specific imprinting patterns with Igf2 being maternally imprinted in all tissues tested except liver, and Xist being maternally imprinted only in placenta-derived tissues (Table 1). These results confirm genomic imprinting and X-chromosome inactivation in the bovine in a manner that is conserved among other placental mammals investigated. It also supports the validity of the interspecies model for studying imprinting in the bovine.

Through utilization of this interspecies model, the status of candidate imprinted genes can be analyzed in pregnancies from normal and nuclear transfer-derived pregnancies.

Table 1  
Analysis of imprinting status at the Igf2, Gtl2, and Xist loci in Day 72 interspecies fetuses

	Brain	Liver	Lung	Fibroblast	Cotyledon	Chorion
Igf2	Mat	Bi	Mat	Mat	NA	NA
Gtl2	Pat	Pat	Pat	Pat	Pat	NA
Xist	Bi	Bi	Bi	Bi	Mat	Mat

Pat: paternally imprinted, Mat: maternally imprinted, Bi: biallelic, not imprinted; NA: results not available.

DETECTION OF SRY GENE EXPRESSION IN PREIMPLANTATION  
PORCINE EMBRYO PRODUCED IN VITRO

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Quality of in vitro-produced (IVP; in vitro matured, fertilized and cultured) blastocysts has been improved and resulted in live birth of piglets after the transfer to recipients [Kikuchi et al., *Biol Reprod* 2002;66:1033–1041], promising the progress of in vitro analysis of porcine embryos. However, it is considerable that gene expression in porcine IVP embryos is different from that in embryos obtained in vivo, because previous studies in other mammals suggest that embryos cultured in vitro have different pattern of gene expression from in vivo-developed embryos. Therefore, the comparison of gene expression pattern of in vitro and in vivo-produced embryos is important for evaluation of the suitable in vitro culture system. In the present study, SRY gene expression, as an example of embryonic gene expressions, in porcine preimplantation embryos was analyzed in both IVP and in vivo-derived embryos.

IVP embryos were obtained each day from Day 0 (Day 0 = fertilization) to 8 after fertilization as described previously [Kikuchi et al., 2002]. In vivo embryos were collected from uterus of gilts on Day 4 (Day 0 = putative ovulation day) after artificial insemination. In the first experiment, we established the method of picking up normal developing IVP embryos, since we also observe fragmentation or abnormal cell division frequently in IVP embryos, but fragmentation cannot be distinguished from the normal cell division under a microscopic observation. To avoid contamination of samples with fragmented embryos, we have to pick up normal developing embryos. By observing under fluorescent microscope after staining with 0.1% Hoechst-33342, we could distinguish normal developing embryos, which have nuclei in every blastomere, from fragmented oocytes that lack a nucleus in some blastomeres. This procedure was found to be applicable for embryos from 2-cell to morula stages. In the Experiment 2, we investigated the expression of SRY gene by RT-PCR. Total RNA was isolated with ISOGEN (Wako, Japan) and were reverse-transcribed using random hexamer- and Rnase-free reverse transcriptase. PCR was performed using a set of following primers: 5'-GGATCGTGTCAAGCGACCCA-3' (sense) and 5'-CTTGCG-ACGAGGTCGGTATT-3' (antisense), which were designed from porcine SRY gene sequence (DDBJ: U49860). It can be performed with only half amount of Day 6 IVP blastocyst and after amplification expected PCR product of SRY was detected as a single band, which was confirmed by the generation of the expected size using the restriction fragment analysis. The expression of SRY was detected from Day 4 (4–8-cell) to Day 8 (Expanded Blastocyst) IVP embryos and also in vivo embryos at Day 4 (morula stage). The experiment was performed using a single pool from 30 embryos for each day and was repeated three times. At Day 4, no significant difference in SRY expression between in vivo and vitro derived embryo was observed. These results indicate that evaluation system detecting specific gene expression can be possible in porcine early embryos produced in vivo and in vitro. Although SRY expression in IVP embryos was detected after the 4-cell stage, at which the embryonic genome is considered to be activated in pigs, the functions of the gene before implantation remain unclear. Further experiments will enable the analysis of a function(s) of SRY products in porcine embryos produced in vivo and in vitro.

MESSENGER RNA EXPRESSION PATTERNS IN BOVINE OOCYTES  
DERIVED FROM FOLLICLES OF DIFFERENT SIZE

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During the growth phase, the bovine oocyte shows a high rate of transcription and translation resulting in accumulation of RNAs and proteins to sustain future meiotic maturation, fertilization and the initial early embryonic development up to the 8–16 cell stage. At the end of the growth phase corresponding to a follicle size of about 3 mm in diameter, transcription ceases within the oocyte. The objective of the present study was to investigate the expression pattern of selected gene transcripts in oocytes from follicles of different size. The analyzed gene transcripts are thought to play important roles in RNA transcription and further processing [RNA polymerase I (RNA pol I), upstream binding factor (UBF), poly(A) polymerase (PolyA)] as well as in adaptation to (oxidative) stress [heat shock protein (Hsp)]. Follicles were dissected from bovine slaughterhouse ovaries and divided in the following three categories: small (1.0–1.5 mm), middle (2.0–3.0 mm) and large (3.0–6.0 mm). Following classification, the follicles were ruptured and the cumulus–oocyte complexes (COCs) were removed. Subsequently the COC were mechanically denuded by pipetting [Pavlok et al., *Mol Reprod Dev* 1992;31:63–73]. After washing three times in PBS supplemented with 0.1% polyvinyl alcohol (PVA), single oocytes were frozen at  $-80^{\circ}\text{C}$  until use. A highly sensitive semi-quantitative RT-PCR assay [Wrenzycki et al., *Mol Reprod Dev* 1999;53:8–18] was used to determine the relative abundances of gene transcripts in single oocytes. Assays were repeated at least 10 times. Data were analyzed using ANOVA followed by multiple pairwise comparisons using a Tukey's test. Differences of  $P \leq 0.05$  were considered to be significant. The expression pattern for Hsp and UBF transcripts was similar in oocytes isolated from all follicular categories. However, RNA pol I expression was significantly higher in oocytes derived from small follicles, whereas the amount of PolyA transcripts significantly increased in oocytes from middle and large follicular category. These results indicate that the decrease in the relative abundance (RA) of RNA pol I transcripts and the increase in the RA of PolyA transcripts in oocytes from follicles of medium and large size might be due to the gradual termination of an active rRNA synthesis and the great demand of mRNA polyadenylation as an essential step for mRNA transport and stabilization.

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## CULTURE EFFECTS ON MOUSE EMBRYO GENE EXPRESSION ARE LIMITED TO THE FIRST THREE CLEAVAGE DIVISIONS

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It has been known for several decades that the culture of embryos in sub-optimal conditions results in perturbations in embryo physiology and metabolism. Recently it has been demonstrated that sub-optimal culture conditions also perturb gene expression in resultant blastocysts cultured from the zygote stage. Significantly, the effects of culture media on embryo physiology and metabolism are substantially reduced once the embryo has compacted. Therefore, the aim of this study was to assess the effects of culture media on embryo development and gene expression of embryos grown from both the zygote and from the 8-cell stage to the blastocyst.

Eight-cell embryos were collected from F1 hybrid mice (C57BL/6 × CBA) at 68 h post hCG, and cultured in one of the following media: Whitten's, KSOMAA or G2. As a negative control, zygotes were collected at 21 h post hCG and cultured in Whitten's medium to the blastocyst stage. In vivo developed blastocysts (Controls) were collected at 94 h post hCG. Eight-cell embryos were cultured at 37 °C in an atmosphere of 6% CO<sub>2</sub>, 5% O<sub>2</sub>, 89% N<sub>2</sub> for 26 h (94 h post hCG) and blastocyst development assessed. Blastocysts were subsequently analyzed for cell number or gene expression. Genes analyzed were β actin, Na/K ATPase, IGF-II, and H19. RNA extraction was performed on 50 embryos, and levels of gene expression assessed by semi-quantitative reverse-transcriptase PCR. Amplified DNA was electrophoretically analyzed in a 1.6% agarose gel to check for appropriate sized products. In vivo controls were set to 100% and the levels of mRNA in the cultured blastocysts expressed as a percentage of controls. The experiment was repeated five times. Differences between treatments were assessed using either generalized linear modeling or one-way ANOVA and Bonferroni's post-test for multiple comparisons.

Development of 8-cell embryos to the blastocyst stage at 94 h post hCG was equivalent in all 3 culture media (Whitten's, 86.9%; KSOMAA, 89.8%; G2, 91.7%). However, there were significantly more expanded and hatching blastocysts in the G2 (72.8%) compared to the Whitten's (52.9%) and KSOMAA (56.4%) ( $P < 0.05$ ). Similarly, blastocysts cultured in G2 had significantly higher trophoctoderm and inner cell mass cell numbers ( $33.8 \pm 1.0$ ,  $21.6 \pm 0.8$ ) compared to Whitten's ( $28.7 \pm 0.9$ ,  $13.6 \pm 0.6$ ,  $P < 0.01$ ) and KSOMAA ( $23.3 \pm 1.0$ ,  $12.1 \pm 0.7$ ,  $P < 0.01$ ). Of interest, significantly fewer embryos formed blastocysts when cultured from the zygote in Whitten's medium (68.7%;  $P < 0.01$ ). There was no significant difference in mRNA expression for the 4 genes studied in embryos cultured from the 8-cell stage to the blastocyst when compared to the in vivo control. In contrast when embryos were cultured from the zygote stage the expression of both β actin and H19 was reduced ( $P < 0.001$ ).

These data indicate that abnormal gene expression of mouse blastocysts is due to cellular trauma inflicted during the first 3 cleavage divisions. Culture of the later stage preimplantation embryo did not significantly impact gene expression.

## RNA INTERFERENCE OF GREEN FLUORESCENT PROTEIN GENE IN MOUSE EMBRYOS BY INJECTION OF LONG/SHORT DSRNAS INTO CYTOPLASM OR PRONUCLEUS

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Double-stranded RNA interference (dsRNAi) is a useful tool for interfering with gene function promoting the sequence-dependent degradation of the targeted mRNA. In the present study, in order to confirm and improve the effect of dsRNAi in the preimplantation mouse embryo, we investigated the inhibitory effect of the gene expression by dsRNA using both a transient and stable enhanced green fluorescence protein (EGFP) system in mouse embryos. In Experiment 1, an GFP expression vector fragment (10 ng/ $\mu$ l) was microinjected into the male pronucleus of zygotes with or without 600 bp EGFP dsRNAi (10 ng/ $\mu$ l) essentially generated by the procedure described in Wianny et al. [Nature Cell Biol 2000;2:70–75]. In Experiment 2, to investigate the effective injection site of dsRNA, the dsRNA was microinjected into the male pronucleus and/or cytoplasm of zygotes obtained from an EGFP transgenic strain. In Experiment 3, to investigate the effect of short interfering RNAs, 21 bp dsRNA produced by annealing the 21 nuclear transfer (nt) EGFP gene was injected into cytoplasm of zygotes collected from EGFP transgenic mice. After injection, embryos were cultured in M16 medium at 37.5 °C in a 5% CO<sub>2</sub> atmosphere. Expression of EGFP at morula and blastocyst stage embryos was observed by fluorescence microscopy. In case of mosaic fluorescence expression, these embryos were classified GFP positive embryos. Statistical analysis of each experiment was carried out by ANOVA. Experiment 1: when the EGFP expression vector fragment and dsRNA were co-injected into zygotes, the fraction of fluorescent blastocysts was significantly decreased ( $59.5 \pm 21.5\%$ ;  $n = 164$  versus  $3.7 \pm 3.7\%$ ;  $n = 145$ ,  $P < 0.01$ ). Experiment 2: while all control embryos from the transgenic mice expressed GFP in morula and blastocyst stages, embryos in which dsRNA was injected both into the pronucleus and cytoplasm showed a significant decrease of GFP expression. In morula stage embryos, a greater decrease was observed when the cytoplasm was injected when compared to the pronucleus ( $37.4 \pm 8.9\%$ ;  $n = 122$  versus  $59.8 \pm 9.8\%$ ;  $n = 124$ ,  $P < 0.01$ ). However, this difference was not observed in blastocysts ( $70.9 \pm 10.4\%$  versus  $80.8 \pm 7.4\%$ ). Experiment 3: expression of EGFP was significantly inhibited at the morula-stage embryos ( $15.7 \pm 13.4\%$ ,  $n = 79$ ), but not blastocysts ( $87.3 \pm 7.3\%$ ). These results indicate that both long and short interfering dsRNAs are efficient for RNAi in mouse embryos.

5'-AZA-2'-DEOXYCYTIDINE INDUCES EXPRESSION OF PLP1,  
AN X-LINKED GENE NORMALLY SILENT IN BOVINE ADULT FIBROBLASTS

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Epigenetic modifications such as DNA methylation and histone acetylation are involved in regulations of gene expression. Our recent study indicated that incomplete nuclear reprogramming might generate abnormal epigenetic marks on the X chromosomes of cloned cattle, affecting both random and imprinted X-linked genes [Xue et al., *Nature Genet*, 2002]. Thus, induction of epigenetic modifications in somatic donor cells may improve the ability of cloned embryos to reprogram the donor nuclei. We first screened the expression profile for 10 genes that are either developmentally important (Oct4, FGF4) or X-linked (ELF4, BGN, PP F1, SYP, SYN1, FACL4, TBL1, PLP1) in the skin fibroblast cells from a 13-year-old Holstein cow. Next, in an attempt to induce epigenetic modifications in the donor cells, we treated cells with either 5'-aza-2'-deoxycytidine (5-azadC) or trichostatin A (TSA) to inhibit the DNA methyltransferase (DMNT1) or the histone deacetylases, respectively. Briefly, cells were seeded in culture dishes in the presence of either 5-azadC (0, 0.25, 0.5, 1, 2, 4, 8, 16  $\mu\text{M}$ ) or TSA (0, 0.08, 0.16, 0.32, 0.64, 1.28  $\mu\text{M}$ ) and were cultured until the control groups (no drug treatments) reached confluency. Cells were then collected for the total RNA extraction followed by RT-PCR for the qualitative gene expression analyses experiments were repeated three times. Our results show that Oct4, FGF4 and PLP1 were not expressed in the adult fibroblast cells before treatment. Expression of PLP1, however, was induced by 5-azadC treatments after 72 h in all concentrations. Despite proliferation of cells slowed down in a dose-dependent manner upon treatments by either 5-azadC or TSA, TSA treatments had no effect on PLP1 expression. Furthermore, neither Oct4 nor FGF4 was induced by either drug. The facts that 5-azadC treatments for 48 h did not induce PLP1 and that 5-azadC treatments on confluent cells did not induce the gene expression even at higher concentrations (up to 125  $\mu\text{M}$ ) indicate several rounds of cell divisions might be necessary for PLP1 induction. Our results demonstrate that the successful induction of PLP1, an X-linked gene that is normally not expressed in somatic donor cells, may involve DNA demethylation. The possibility that monoallelically expressed X-linked genes were induced to express biallelically by 5-azadC or TSA treatments will be the objective for further studies.

## EXPRESSION OF THE IGF2 AND THE IGF2R PARTHENOGENETIC AND BIPARENTAL FETUSES IN THE PIG

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The insulin-like growth factor 2 (Igf2) and the insulin-like growth factor 2 receptor (Igf2r) genes were identified as imprinted genes in the mice. The aim of this study was to examine the gene expression of the Igf2 and Igf2r in porcine parthenogenetic fetuses that have two maternal genomes and no paternal genome and control biparental fetuses. Porcine cumulus-oocyte complexes were collected from slaughter house ovaries and matured in vitro for 44 h. Oocytes with a first polar body were stimulated by two dc pulses of 1500 V/cm for 60  $\mu$ s. The electro-stimulated oocytes subsequently cultured in NCSU-37 contained 5.0  $\mu$ g/ml cytochalasin B (Sigma) for 4 h in order to obtain diploid oocytes. Cultures were performed under humidified atmosphere of 5% CO<sub>2</sub> in air at 38.5 °C. The oocytes treated with cytochalasin B were surgically transferred to oviducts of synchronized recipient females. Parthenogenetic fetuses were surgically collected from uterine horns of the recipients at 28 days post electro-stimulation. As control, porcine fetuses through natural mating were also recovered surgically on day 28 of the gestation. Some of fetuses were fixed for histological preparations. From the other fetuses the total RNA was isolated using an SV Total Isolation System (Promega) for analysis of expressing the Igf2 and Igf2r. The expression of the Igf2 and the Igf2r was analyzed by RT-PCR. The first strand of cDNA was synthesized from the total RNA by oligo dT primers and SuperScript II reverse transcriptase (Life Technologies Inc.) according to the manufacturer's instructions. The cDNA was subjected to PCR using specific primers for porcine Igf2 and Igf2r genes. Twenty-eight parthenogenetic fetuses were recovered from two recipients that had received 400 IVM/ICSI oocytes. Although half of the parthenogenetic fetuses reached the stage of limb-bud formation, some of the fetuses externally showed abnormalities of organ formation, cleft of palate, and ungrowing placenta; these abnormalities were not observed in the non-parthenogenetic fetuses. In addition, the parthenogenetic fetuses were smaller than biparental ones. On the contrary, these abnormalities were not observed in the control fetuses; the biparental fetuses were larger than parthenogenetic fetuses. In the parthenogenetic fetuses, the expression of the Igf2 was not detected, and the Igf2r was expressed. In contrast, the expression of the Igf2 was expressed, and the Igf2r was slightly expressed in control biparental fetuses. The results suggested that the Igf2 gene was imprinted and paternally expressed in pigs as well as mice [DeChiara et al., *Cell* 1991;64:849–959] and sheep [Feil et al., *Mammalian Genome* 1998;9:831–834].

## STEROID RECEPTOR EXPRESSION IN THE EARLY PREIMPLANTATION BOVINE EMBRYO

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Steroid receptors have been identified in embryos of several mammalian species, including the mouse [Hou and Gorski, *Proc Natl Acad Sci USA* 1993;90:9460–9464; Hou et al., *Proc Natl Acad Sci USA* 1996;93:2376–2381]. Porcine embryos are also known to express estrogen receptor beta (ER $\beta$ ), but not alpha (ER $\alpha$ ), and steroids have been shown to regulate their development [Kowalski et al., *Biol Reprod* 2002;66:760–769]. Furthermore, treatment of porcine trophoblastic cell lines with estrogen is known to regulate growth of these cells [Kowalski et al., *Biol Reprod* 2001;64(Suppl 1):280]. Together, these data suggest that steroids may help regulate early embryonic development. However, the action of steroids and presence of steroid receptors during early bovine embryo development have not been established. The objectives of this investigation were to: (1) elucidate the presence of steroid receptors in the bovine in vitro-produced embryo and (2) determine the pattern of receptor protein expression during early preimplantation development. Bovine oocytes were collected from slaughterhouse ovaries, and matured and fertilized as previously described [Parrish et al., *Theriogenology* 1986;25:591–600]. Embryos were stripped of cummulus at 18–20 h post fertilization and cultured in KSOM for up to 7 days in a humidified environment of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub>, 39 °C. In Experiment 1 total RNA was isolated from embryos or corpora lutea (CL, positive control) using Absolutely RNA<sup>TM</sup> Nanoprep kit (Strata-gene, La Jolla, CA). Reverse transcription-polymerase chain reaction was performed with cDNA from embryos or CL using specific primers for ER $\alpha$ , ER $\beta$ , androgen receptor (AR) and progesterone receptor (PR). PR and ER $\beta$  were both detected in blastocyst stage embryos. Interestingly, ER $\beta$  and AR were not expressed by the Day 7 bovine embryo, but were present in the CL. In the second experiment, ER $\beta$  protein expression during early bovine embryo development was further characterized by immunofluorescence as previously described [Spector and Smith, *Exp Cell Res* 1986;163:87–94] using rabbit polyclonal anti-rat ER $\beta$  antibody (Upstate Biotechnology, Lake Placid, NY) or whole rabbit IgG (negative control; Sigma, St. Louis, MO). In vitro-produced embryos from the 2-cell to 8-cell stage were negative for ER $\beta$  protein. However, embryos from the 16-cell to blastocyst stages stained positively for ER $\beta$  protein. Incubation with immunizing peptide for ER $\beta$  (Upstate Biotechnology) was able to displace antibody signal, confirming specificity of the antibody. These results provide for the first time, evidence of steroid receptor expression in the bovine embryo and open the possibility of steroid regulation of preimplantation embryo development. Additional research will further characterize receptor expression throughout early preimplantation development and determine whether steroids have an impact on early bovine embryo development.

## INTERFERON-TAU SECRETION BY HYBRID CATTLE X BISON BLASTOCYSTS AND BLASTOCYST OUTGROWTHS

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Interferon-tau (IFN- $\tau$ ) is the major secretory protein produced by the ruminant conceptus around the time of implantation and is essential for the establishment of pregnancy. More than one locus is thought to be expressed in each embryo and as most of the bovine IFN- $\tau$  gene sequences appear to have been identified, the use of hybrid embryos could facilitate a determination of the number of expressed paternal and maternal alleles. The purpose of this study, therefore, was (a) to assess development of bovine oocytes fertilized by bison semen and (b) to analyze the secretion of IFN- $\tau$  by resulting blastocysts and blastocyst outgrowths. A total of 600 cattle (*Bos taurus*) oocytes from two separate batches was inseminated by frozen-thawed American plains bison (*Bison bison*) semen. After 48 h, 244 (40.7%) embryos had cleaved at least once. A total of 89 inseminated oocytes proceeded to the blastocyst stage (14.8%) of which 45 (50.6%) subsequently hatched. Thirty-six hatched blastocysts were cultured individually in 500  $\mu$ l of TCM 199 in wells treated with matrigel. On Day 6, only five blastocysts had attached and formed small outgrowths while nine appeared degenerate. IFN- $\tau$  concentrations in the medium of individual blastocysts were determined after the medium had been replaced 24 h earlier. Samples were analyzed using a standard antiviral assay and values were subjected to log transformation, but are reported here as least squares means. On Day 6, the medium of outgrowths contained significantly higher concentrations of IFN- $\tau$  ( $15608.9 \pm 3342.6$  pM) than in those of non-attached blastocysts ( $6007.4 \pm 1645.3$  pM) or degenerate embryos ( $446.0 \pm 258.8$  pM). On Day 12, 22 outgrowths and 4 non-attached blastocysts were found to be viable and producing IFN- $\tau$ . PCR-based sex analysis revealed 15 and 11 of these to be male and female, respectively. Sex was a significant determinant of IFN- $\tau$  secretion on Day 6 ( $3278.5 \pm 2334.8$  and  $10642.7 \pm 2820.9$  pM, for male and female embryos, respectively), but not on Day 12, when the size of the outgrowth proved to be the most important determinant of IFN- $\tau$  secretion. mRNA was extracted from several outgrowths and RT/PCR was performed using primers annealing to highly conserved regions of known IFN- $\tau$  sequences from several species. Resulting fragments were cloned and sequenced. Several known bovine mRNA isoforms were identified as well as several clones with novel polymorphisms.

## EMBRYONIC TRANSCRIPTION IS REQUIRED FOR ALLOCATION OF NUCLEOLAR PROTEINS IN BOVINE EMBRYOS PRODUCED IN VITRO

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A panel of key nucleolar proteins involved in ribosomal RNA (rRNA) transcription (topoisomerase I, polymerase I, and upstream binding factor (UBF)), initial rRNA processing (fibrillarin) or later rRNA processing and/or nucleolar transport (nucleolin (C23) and nucleophosmin (B23)) are allocated to the developing nucleolus during the fourth cell cycle, i.e. the 8-cell stage, in bovine in vitro-produced embryos [Laurincik et al., Biol Reprod 2000;62:1024–1032]. It is, however, unknown whether these proteins are derived from de novo transcribed or existing messengers in the embryo. Hence, the aim of the present study was to examine the allocation of nucleolar proteins in bovine in vitro-produced embryos that were cultured with or without the presence of  $\alpha$ -amanitin, which blocks the action of RNA polymerase II and, thus, the synthesis of messenger RNA. Bovine embryos were produced by oocyte maturation, fertilization and further culture in vitro [Avery et al., Theriogenology 2000;54:1259–1268]. An experimental group of embryos was cultured in B2-medium supplemented with 25 g/ml  $\alpha$ -amanitin from the 2-cell stage while a control-group was cultured continuously in  $\alpha$ -amanitin free medium. Embryos in both groups were fixed in 4% paraformaldehyde in 0.1 M PBS at 3–6 h post cleavage (hpc) to the 4-cell stage (Control:  $N = 53$ ; amanitin:  $N = 49$ ) and 7–12 hpc (early 8-cell; Control:  $N = 54$ ; amanitin:  $N = 49$ ) and 37–41 hpc (late 8-cell; Control: 56; amanitin:  $N = 49$ ) to the 8-cell stage. The six nucleolar proteins mentioned above were localized by fluorescence immunocytochemistry and confocal laser scanning microscopy. The nuclei were counterstained with propidium iodide. In the control group, a few of the 4-cell embryos showed a diffuse nucleoplasmic labeling for topoisomerase I and nucleophosmin while all showed labeling for nucleolin to nuclear foci. No labeling was observed for the remaining three proteins. All early 8-cell embryos displayed a diffuse nucleoplasmic labeling for topoisomerase I, and focal nuclear labeling for nucleophosmin and nucleolin was detected in about half and all of the embryos, respectively. No labeling was observed for the remaining three proteins. All late 8-cell embryos displayed a localized nuclear labeling for the six proteins compatible with the formation of functional nucleoli: Topoisomerase I, UBF, RNA polymerase I and fibrillarin were localized to more or less conuent clusters of foci while nucleophosmin and nucleolin were localized to shell-like structures appearing ring-like in the confocal sections. In the amanitin group, no labeling for any of the proteins was observed in 4-cell embryos. All early 8-cell embryos displayed diffuse labeling for nucleophosmin and a few displayed focal nuclear labeling for nucleolin. The same applied for the late 8-cell embryos. The results indicate that nucleolar allocation of at least topoisomerase I, UBF, RNA polymerase and fibrillarin during the fourth cell cycle in bovine in vitro-produced embryos is based on de novo transcription from the embryonic genome. The nucleoplasmic presence of nucleophosmin is apparently less dependent on de novo transcription, although its spatial localization to the developing nucleolus is. Nucleolin may in some cases be allocated to the developing nucleolus independent of de novo transcription.

IDENTIFICATION AND CHROMOSOMAL ALLOCATION OF ESTS DERIVED  
FROM IN VITRO-PRODUCED CATTLE EMBRYO DEVELOPMENTAL  
STAGE RANGES

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The various features of gene mapping have proved themselves as a potential tool to facilitate identification of candidate genes. With objectives to identify and characterize genes expressed at the two extremes of pre-implantation development stage, eight ESTs (Expressed Sequence Tags) derived from oocyte and blastocyst stage embryo cDNA library [Ponsuksili et al., *Theriogenology* 2001;55:415 (abstract)] were mapped using radiation hybrid panel [Womack et al., *Mammalian Genome* 1997;8:854–856]. One primer pair was designed per EST for product amplification from radiation hybrid panel clones whose identity was confirmed by sequencing on a Licor automatic sequencer following standard procedures. To evaluate the retention pattern and allocate the loci of genes, RHMAPP R 1.22 software was used. Comparative mapping by annotation and sequence similarity (COMPASS) program was also used for loci prediction [Ma et al., *Mammalian Genome* 1998;9:545–549]. All primer pairs amplified the expected product, ranging in size from 111 to 225 bp. Marker retention frequency, calculated as the percentage of clones retained a given marker, was on average 19% with extreme ranges being 11–29%. All markers represented various developmentally important genes with average of greater than 92% similarity (Table). The function of these genes include, but are not limited to, cholesterol biosynthesis, calcium binding protein, intracellular protein transport, control cell shape and size, cell adhesion, migration processes, embryogenesis, protein degradation and metabolic nitrogen oxidation. With this study, these genes are allocated to six different chromosomes, fortifying the already existing ordered genes list and facilitating further the search for developmentally important genes at pre-implantation stage. Apart from these major benefits, this study in conjunction with the previous allocations of other loci will pave the way to study the contribution of these genes to embryo quality and developmental stage hierarchies, which should be a complement of this study.

Marker name (length in bp)	Source	Gene symbol	<i>P</i> ( <i>N</i> ) <sup>a</sup>	Match		Cattle chromosome location
				% <sup>b</sup>	Length <sup>b</sup>	
ESTBb003 (169)	Oocyte	SC5DL	1e–60	92	168	15
ESTBb007 (129)	Oocyte	HNKmRNA <sup>c</sup>	1e–13	88	81	14
ESTBb008 (225)	Oocyte	NUP160	1e–30	88	134	15
ESTBoo11 (111)	Oocyte	LCP	8e–24	89	113	10
ESTBblc148 (218)	Blastocyst	SPUVE	4e–65	90	204	29
ESTBblc231 (215)	Blastocyst	CK 8	6e–99	97	217	10
ESTBblc245 (206)	Blastocyst	FN1	e–104	98	206	2
ESTPBL178 (142)	Blastocyst	FMO1	6e–38	89	142	16

<sup>a</sup> Smallest sum of poisson probability reported with BLASTN.

<sup>b</sup> Columns showing similarity (%) and the length (bp) of the overlap.

<sup>c</sup> Human normal keratinocyte mRNA.

MOLECULAR CLONING AND CHARACTERIZATION OF A NOVEL  
GENE SPECIFICALLY EXPRESSED IN GONAD

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The differentiation of germ cells in morphological and biochemical properties are determined by changes of gene expression. It requires a strict program of stage- and cell-specific gene expression in germ cells as well as in surrounding somatic cell. Therefore, it is necessary to study this cell- and stage-specific expression of gene for understanding the molecular mechanisms of spermatogenesis and oogenesis. We identified a novel gene, termed GS (gonad specific expression gene). Nucleotide sequence analysis of GS cDNA revealed that the open reading frame of 763 bps encodes a protein of 247 amino acids with a predicted molecular mass of 27.6 kDa. The deduced amino acid sequence indicated that GS protein might be a soluble protein without a signal peptide. Two microgram of Poly(A)<sup>+</sup> RNA from various tissues of adult mice were subjected to Northern blot analysis using the mouse GSE cDNA probe. It was shown that this gene was abundantly expressed in mouse testis and slightly expressed in mouse ovary. The transcript of this gene was not detected in other mouse tissues (heart, liver, kidney, lung, brain, skeletal muscle and spleen). To elucidate the relationship of GS expression and gamatogenesis, mRNA (0.5 µg each) from mouse testes and ovaries on different postnatal days was analyzed by RT-PCR with specific primers for GSE cDNA. The GSE mRNA in the testis of mouse was first detected at Day 14 postpartum, when cells at mid-pachytene are likely to appear. To investigate furthermore whether GSE is specially expressed in restricted testicular germ cells, GS mRNA in the testis was examined by in situ hybridization. The GS mRNA expression was confirmed to localize at late spermatocytes and round spermatids. On the other hand, Northern blot analysis indicated that the GS mRNA in the ovaries already appeared at birth, when germ cells are in meiosis. These findings suggest that GS may associate with meiosis during gametogenesis.

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## OCT-4 EXPRESSION IN RHESUS MONKEY IVF OR SOMATIC CELL NUCLEAR TRANSFER (NT) EMBRYOS AND EMBRYONIC STEM (ES) CELLS

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The POU-domain transcription factor, Oct-4, plays an essential role during early mammalian embryogenesis and serves as a marker for pluripotent cells. In the mouse, Oct-4 expression is detectable throughout oogenesis. During preimplantation development, expression is initiated at the 4–8-cell stage after embryonic genome activation and becomes restricted to the inner cell mass (ICM) of blastocysts and later to developing germ cells [Palmieri et al., *Dev Biol* 1994;166:259–267]. Oct-4 expression is also a characteristic of mouse and human ES cells. Here, we determined the expression and distribution of Oct-4 in monkey IVF or somatic cell NT embryos and S cells in an effort to monitor nuclear reprogramming and establish markers for pluripotent cells. Rhesus monkey embryos produced by IVF or intracytoplasmic sperm injection (ICSI) were fixed in 4% paraformaldehyde at various stages of preimplantation development: on Day 3 at 8–16 cell stage (Day 0 = day of fertilization;  $n = 6$ ); on Day 5 at morula/compact morula stage ( $n = 10$ ); and on Day 7 at the expanded blastocyst stage ( $n = 11$ ). NT embryos reconstructed from adult skin fibroblasts were fixed on Day 5 at the morula/compact morula stage ( $n = 16$ ). After permeabilization with 0.2% Triton X-100 and 0.1% Tween-20, non-specific reactions were blocked with 10% normal goat serum. Embryos were then incubated in affinity-purified rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1–134 mapping at the terminus of Oct-4 of human origin for 40 min followed by exposure to a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488. Embryos were then co-stained with bisbenzimidazole (Hoechst 33342), whole-mounted onto slides and examined with epifluorescence microscopy. Monkey S cells were plated on Lab-Tek II chamber slides, fixed and stained for Oct-4 as outlined above. Oct-4 protein was present in all sampled IVF or ICSI embryos except controls in which the primary antibody had been omitted. In cleavage stage embryos (Days 3 and 5), the signal was diffuse and localized primarily in the cytoplasm. The signal was detectable in both ICM and trophectoderm of expanded IVF or ICSI blastocysts; however, localization to the cytoplasm of trophectoderm cells was observed, while in the ICM the signal was confined primarily to nuclei. ES cells also expressed Oct-4 and the protein was restricted to nuclei, similar to that seen in the ICM of expanded blastocysts. Somatic cell NT embryos analyzed at the morula/compact morula stage also initiated Oct-4 expression indicative of reprogramming. In summary, we demonstrated expression of Oct-4 in preimplantation monkey embryos and S cells. The expression pattern observed in monkey blastocysts with both ICM and trophectoderm localization was similar to that observed by others previously in the bovine and pig but in contrast to findings in the mouse [Kirchhof, *Biol Reprod* 2000;63:1698–1705]. Detection of Oct-4 in somatic cell NT embryos suggests that this crucial developmental gene is appropriately expressed and that it may serve as a marker of nuclear reprogramming. Supported by NIH grant RR12804, Core Grant 007198 and a product donation from Ares Advanced Technology, Inc.

CHROMOSOME ABNORMALITIES IN IN VIVO DEVELOPED, IN VITRO PRODUCED OR PARTHENOGENETICALLY ACTIVATED PORCINE EMBRYOS

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Developmental capacity of embryos is intimately linked to a normal chromosome constitution, and cytogenetic analysis of interphase nuclei by fluorescent in situ hybridization (FISH) may therefore be used to monitor embryo quality. The aim of this study was to extend previous studies on cattle embryos to include domestic pigs by estimating the frequency of chromosome abnormalities in porcine embryos produced by in vitro techniques. We therefore determined the frequency of numerical chromosome abnormalities in embryos produced by in vitro fertilization of in vitro-matured oocytes or following parthenogenetic activation of in vitro-matured oocytes and compared it to the corresponding frequency in in vivo produced porcine embryos. In vivo produced embryos ( $n = 84$ ) were recovered from seven Danish Landrace sows and fixed 4–5 days after expected ovulation. In vitro ( $n = 9$ ) and parthenogenetically ( $n = 16$ ) produced embryos were fixed on Day 5 after fertilization or activation, respectively. Embryo developmental stages ranged from the 4-cell stage to blastocyst. A total of 1761, 290 and 325 interphase nuclei from the in vivo, in vitro and parthenogenetically produced embryos, respectively was analyzed by FISH, using probes for pig chromosomes 1 and 10. Only 10% (8 of 84) of the in vivo produced embryos, but 89% (8 of 9) of the in vitro produced and 25% (4 of 16) of the parthenogenetically activated embryos contained polyploid cells. Preliminary results of the data analysis indicate that there was a lower proportion of mean chromosomal abnormalities (sum of percentages of polyploid nuclei in the individual embryos divided by total number of embryos within a group) in the embryos developed in vivo (1.2%) compared to the in vitro produced (3.8%) and to the parthenogenetically activated embryos (32%). Thus, most of the chromosomally abnormal embryos contained only few polyploid cells, i.e. were mixoploid, and embryos, where all cells were polyploid, were present only in the group of parthenogenetically activated embryos. These preliminary data indicate that in vitro procedures, be it fertilization or parthenogenetical activation of porcine oocytes, increase the frequency of mixoploid embryos.

BOVINE CUMULUS-GRANULOSA CELLS EXPRESS INCREASED MIDKINE BUT NOT IGF-I IN RESPONSE TO 9-*CIS*-RETINOIC ACID DURING IN VITRO MATURATION

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The growth factor midkine (MK) enhances embryo development of bovine oocytes. The effect of MK mainly mediated by cumulus-granulosa (CG) cells during in vitro maturation (IVM) (Ikeda et al., Mol Reprod Dev 2000; 57:99–107; Ikeda et al., Biol Reprod 2000; 63:1067–1074). However, the development promoting effect of IGF-I and its expression in granulosa cells are controversial. Despite both gene products show increased levels in dependence on retinoids, probably it is MK and not IGF-I a retinoic acid (RA) directly responsive gene. In the present work we analyze MK and IGF-I expression in CG cells in response to: (1) the presence of 9-*cis*-RA as a promoter of embryo development during IVM; and (2) various concentrations of ethanol, a specific inhibitor of RA synthesis from retinol (ROH), as a vehicle for RA. Cumulus-oocyte complexes (COCs) from slaughterhouse ovaries matured in TCM-199, 0.5 mg/ml PVA, FSH, LH and E<sub>2</sub> (MM) at 39 °C and 5% CO<sub>2</sub> in air for 24 h. For dose-response analysis, MM received 9-*cis*-RA (5, 50 and 500 nM), ethanol or no additives. Matured COCs were fertilized and cultured up to Day 8 in simple medium at 39 °C, 5% CO<sub>2</sub> and 5% O<sub>2</sub>. Comparable blastocyst rates were obtained with 9-*cis*-RA 5 and 50 nM, while 9-*cis*-RA 500 nM was detrimental. To analyze MK and IGF-I expression, groups of 10 COCs were treated both immature and matured in MM containing 9-*cis*-RA 5 nM, ethanol (1% vehicle; 3 and 5%) and no additives. CG cells were detached from oocytes with 0.5% hyaluronidase and pipetting. RNA was isolated, treated with DNase-I and samples balanced in concentration by measuring absorbance at 260 nm expression of β-actin was analyzed as a constitutive control. RT-PCR lectures were carried out in exponential phase at 37 cycles for β-actin and 40 cycles for MK and IGF-I. After identification of MK amplicon with the restriction enzyme HpaII, PCR products were checked in agarose gel and bands submitted to densitometric analysis. The expression level of each gene was quantified as a proportion of β-actin amplified in each group. PCR lectures (N) were analyzed by means of ANOVA and Duncan's test. Treatment and number of samples of CG cells (R) were fitted as fixed effects. Results are shown as LSM ± S.E.M. (significant differences: <sup>x,y</sup>(*P* < 0.01); <sup>a,b</sup>(*P* < 0.10)). Expression of MK was detected in all groups and showed to be RA dependent. However, IGF-I expression, as reported by other authors, was not detected at all. As expected, ethanol inhibited MK expression, suggesting endogenous RA synthesis, and this effect tended to be dose-dependent. The effect of RA during bovine in vitro maturation is probably mediated by MK gene but not by IGF-I (Table 1).

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Table 1  
Relative midkine expression in CG cells matured and immature

Treatment	N	R	Midkine (%)
9- <i>cis</i> -RA	12	5	83.7 ± 4.8 <sup>a</sup>
Control (-)	17	6	70.5 ± 3.9 <sup>ab</sup>
Ethanol 1% (vehicle)	13	5	67.9 ± 4.6 <sup>b</sup>
Ethanol 3%	9	3	66.4 ± 5.7 <sup>b</sup>
Ethanol 5%	9	3	61.6 ± 5.7 <sup>bc</sup>
Immature	17	6	63.3 ± 3.9 <sup>b</sup>

EXPRESSION OF 8 IMPRINTED GENES IN CDNA POOLS PRODUCED FROM  
SINGLE BOVINE OOCYTES AND BLASTOCYSTS

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The correct expression of imprinted (monoallelically expressed) genes has proven to be crucial for proper development of the foetus and adult. These genes have been shown through disease and knockout models in the mouse to be key in the regulation of growth of the placenta and foetus and proper functioning of the brain and other organs. The only imprinted gene to have been investigated in cattle is IGF2R, known to be critical for growth regulation. The expression of eight genes imprinted in either the human or mouse was investigated in bovine cDNA pools created from single IVM oocytes and single IVM/IVF blastocysts (four single oocytes and four single blastocysts). Bovine ovaries were collected from the abattoir, and oocytes aspirated. Oocytes with multiple layers of cumulus cells were selected for maturation in modified TCM199 for 20–22 h. At this time oocytes were either denuded and checked for maturation, or fertilized and cultured for 7 days in modified SOFaa, before scoring and selection of expanded blastocysts. Single IVM oocytes and IVM/IVF blastocysts were snap frozen with liquid N<sub>2</sub> in 5 µl lysis buffer (0.8% Igepal, 5 mM DTT, and 1 IU/µl RNAsin). Dynabeads (Dynal, Inc.) were used to isolate mRNA, and SMART cDNA synthesis kits (Clontech) were used to produce amplified pools of cDNA. Products were then diluted 1:10 and used for gene specific PCR. PCR primers were designed from human and mouse sequence, as well as bovine EST sequence when available. Amplicons were subcloned and sequenced for product confirmation. Expression results are given in the table below. These preliminary results indicated that Dlk1 is not expressed in either the bovine oocyte or blastocyst. Gnas, Mest, Obph1 and Ube3a are expressed at both stages, Sgce and Zac1 are expressed only in the oocyte, and Grb10 is expressed only in the blastocyst. Further studies are underway to confirm these results and to determine the expression during the 2-, 4-, 8-cell and morula stages of development.

Gene	Oocyte expression	Blastocyst expression
Poly (A) polymerase	4/4	4/4
GDF9	4/4	0/4
IFN-τ	1/4	4/4
Oct4	4/4	4/4
Dlk1	0/0	0/0
Gnas	3/4	4/4
Grb10	0/4	1/4
Mest	3/4	2/4
Obph1	3/4	2/4
Sgce	2/4	0/4
Ube3a	2/4	1/4
Zac1	2/4	0/4

QUANTIFICATION OF MESSENGER RNA IN BOVINE NUCLEAR TRANSFER  
EMBRYOS RECONSTRUCTED WITH FETAL FIBROBLAST CELLS  
AND EMBRYO BLASTOMERS

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It is probable that the gene transcription is an important factor in development of embryos and production of normal offsprings derived from nuclear transfer. The objective of the study was to determine the abundance of gene transcripts in cloned bovine embryos reconstructed with somatic cells and blastomeres. Five specific mRNAs in undifferentiation (octamer-binding transcription factor, OCT-4), development of early embryonic stage (interleukin-6, IL-6), DNA methylation (DNA methyltransferase, DNMT), early differentiation and trophoblastic function (interferon- $\tau$ , IF- $\tau$ ), metabolism (glucose transporter-1, GLUT-1) and housekeeping gene (glyceraldehydes-3-phosphate dehydrogenase, GAPDH) were selected for the study. Bovine embryos at blastocyst stage were obtained from in vitro fertilization (IVF), uterus of recipient cows at Day 8 after estrus (Vivo), nuclear transfer used fetal fibroblast cell (NT-FC) or blastomere at morula stage (NT-EM). Single embryos were added to 5  $\mu$ l lysis buffer, frozen in LN<sub>2</sub>, and storage at  $-80^{\circ}$  C. Total RNA was reverse transcribed with SuperScript (Invitrogen) according to the manufacturer's instructions for RT-PCR. After RT reaction, samples were diluted to 20  $\mu$ l. Quantification of mRNA abundance was performed by real-time PCR detection using a LightCycler (Roche). Real-time PCR amplification carried out in final volume of 20  $\mu$ l PCR mix contained 2  $\mu$ l of the RT product, 1 $\times$  QuantiTect SYBR Green PCR mix (QIAGEN) and 0.5  $\mu$ M each primer. Template was amplified by 50 cycles of denaturation for 15 s at  $94^{\circ}$  C, annealing of primers at  $52^{\circ}$  C (OCT-4 and IL-6),  $57^{\circ}$  C (IF- $\tau$ ) or  $60^{\circ}$  C (DNMT, GLUT-1 and GAPDH) for 30 s and extension  $72^{\circ}$  C for 30 s. Fluorescence data were acquired at every step of extension. Plot of melting curves and agarose gel electrophoresis were used to determine PCR product identity. The standard curves for quantification of mRNA were generated by amplifying serial dilution of a known quantity of purified PCR products of each gene. Ten embryos in each group were used for RT-PCR analysis. Except for DNMT and IF- $\tau$ , all embryos were with positive signals of each gene by real-time PCR. The rates of embryo with positive signals from DNMT and IF- $\tau$  were 40–90 and 80–90%, respectively. Quantities of IL-6, DNMT, GLUT-1 and GAPDH mRNA ( $2.99 \pm 0.72$  to  $9.65 \pm 2.19$ ,  $5.66 \pm 2.99$  to  $7.20 \pm 1.51$ ,  $5.93 \pm 1.66$  to  $9.61 \pm 3.46$  and  $622.40 \pm 107.90$  to  $1114.40 \pm 140.80$ , respectively) were not significantly different in embryos derived from NT-FC, NT-EM, IVF and Vivo. OCT-4 mRNA in IVF embryos ( $20.14 \pm 2.83$ ) was significantly ( $P < 0.05$ ) higher than NT-FC ( $8.03 \pm 1.41$ ) and Vivo ( $8.31 \pm 1.10$ ). In addition, mRNA content of IF- $\tau$  in NT-EM embryos ( $240.88 \pm 53.03$ ) was higher ( $P < 0.01$ ) than that in other embryos ( $61.88 \pm 22.49$  to  $121.33 \pm 17.47$ ). These results suggest that there are differences in the gene expression levels among the bovine embryos produced using nuclear transfer, IVF and in vivo fertilization. In further studies, we are intending to clarify the characteristics of bovine cloned embryos by detection of gene transcripts in single embryos at various developmental stages using presented method.

## DIFFERENTIAL SCREENING OF EMBRYONIC ESTS DERIVED FROM DIFFERENTIAL DISPLAY USING REAL-TIME PCR TECHNIQUE

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Differential expression of developmentally important genes is known to regulate the various morphological events that occur during the course of development of in vitro matured and fertilized oocytes to transferable blastocysts. Besides identification, accurate quantification of these transcripts favors advances in functional genomics in embryogenesis. In this study we aimed to quantify transcripts derived from Differential Display PCR (DD-PCR) using fluorescence monitored real-time PCR system throughout the preimplantation stage. RNA samples, isolated from pools of in vitro produced blastocysts, morula, 16- and 8-cell embryos, were reverse transcribed and subjected to DD-PCR using 2.5  $\mu$ M oligo(dT)12N and 26 upstream primers [Bauer et al., *Nucleic Acids Res* 1993;21:4272–4280]. Real-time PCR for selected differentially expressed clones was performed using mRNA, isolated from pooled matured oocytes, 2-, 4-, 8-, and 16-cell, morula and blastocysts, with SYBR PCR master mix in an ABI prism 7000 SDS instrument (Applied Biosystems, USA). Primer pairs were designed to amplify 176 and 167 bp long product for clone 2C14 and 2C4, respectively throughout preimplantation stage. A standard curve was generated for each amplicon by amplifying serial dilutions of known quantity amplicons to interpolate the copy number of unknown samples. Beta actin gene was used to normalize real-time PCR results. Applying 20 primer combinations in DD-PCR a total of 305, 205, 162 and 239 cDNA bands was conserved in 8-cell, 16-cell, morula and blastocyst stages, respectively. Of the potential 40 differentially expressed cDNA bands, 16 transcripts were cloned and sequenced. Of these, while clone 2C14 (295 bp) was found to be differentially expressed in 8- and 16-cell stages, clone 2C4 (440 bp) was found in morula stage. The clone 2C14 share sequence homology with *H. sapiens* PSCD2 mRNA (Acc. No. XM-009110) and clone 2C4 showed significant sequence similarity with *H. sapiens* mRNA for KIAA1764 protein (Acc. No. AB051551). The blastocyst stage was taken as a calibrator for both clones to compare the relative expression level between samples. For clone 2C14, expression levels of  $5.5\times$  in 8-cell,  $17.4\times$  in 16-cell and  $10.4\times$  in morula stage embryos were found as compared to blastocysts stage. This clone was also found as much as  $4.7\times$  in 2-cell and  $7.8\times$  in 4-cell stage embryos. For clone 2C4, higher expression was observed in morula stage with  $49.7\times$  more expression than in blastocyst stage. This clone was expressed  $3.4\times$  in matured oocytes,  $20.5\times$  in 4-cell and  $18.8\times$  in 16-cell embryos compared to the blastocyst stage. Real-time PCR results for clone 2C4 were found to be in agreement with the DD-PCR, as higher expression was observed in morula stage. However, the results for clone 2C14, whose expression is relatively higher in morula stage compared to the 8-cell stage, are not in support of our DD-PCR. Relatively higher expression of clone 2C4 in morula stage may indicate the potential role of this clone in the process of compaction and cell-to-cell interaction. Quantification of these clones at single embryo level in in vitro versus in vivo derived embryos of different qualities may supplement the results of this study.

## SERIAL ANALYSIS OF GENE EXPRESSION (SAGE) COMPARISONS BETWEEN DAY 11 AND DAY 12 PORCINE EMBRYOS

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Mammalian embryo development represents a continuum of molecular and cellular interactions whereby early events dictate or influence subsequent developmental outcomes. Swine often exhibit high rates (>30%) of early embryonic mortality, and the efficiency of producing swine embryos *in vitro* is poor compared to methods applied in other livestock species. The maternal recognition of pregnancy combined with the dramatic embryo elongation that occurs between Day 11 (D11) and Day 12 (D12) of gestation denote critical stages in porcine development. Serial Analysis of Gene Expression (SAGE) enables both qualitative and quantitative analysis of gene expression on a whole-transcriptome level [Velculescu et al., *Science* 1995;270:484–487]. We performed SAGE on *in vivo*-derived D11 and D12 porcine embryos to identify and characterize critical gene expression events that occur during and between these key stages of early porcine embryo development.

Total RNA prepared from *in vivo*-derived D11 and D12 stage porcine embryos was used to construct each respective SAGE tag library as per the I-SAGE Kit (Invitrogen, Carlsbad, CA) protocol. A total of 23,179 and 24,346 SAGE tags representing 9209 and 8945 unique putative genes was sequenced from the D11 and D12 libraries, respectively. Comparative statistical analysis of SAGE tag frequencies between D11 and D12 libraries was performed with chi-square analysis with Monte-Carlo Simulations (SAGE 2000 Analysis Software Package, Invitrogen). At  $P < 0.05$  significance, 246 tags were differentially expressed between D11 and D12; 135 tags were more frequent and 111 tags were less frequent on D12 than D11. At  $P < 0.001$  significance, 54 tags were differentially expressed between D11 and D12; 31 tags were more frequent and 23 tags were less frequent on D12 than D11. Due to the paucity of porcine three expressed sequence tag (EST) data publicly available for BLAST analysis, the nucleotide sequences from the 54 ( $P < 0.001$ ) differentially expressed tags were compared to the NCBI human SAGEmap database (<http://www.ncbi.nlm.nih.gov/SAGE>). Sequence similarities between the porcine embryo SAGE tags and the NCBI data indicated these 54 ( $P < 0.001$ ) tags were clustered into the following putative functional groupings: metabolic enzymes (8 tags); heat-shock proteins (3 tags); transcription factors (3 tags); represented EST or hypothetical proteins (19 tags); or unknown (including novel) genes (21 tags).

We constructed SAGE libraries representing *in vivo*-derived D11 and D12 stage embryos as part of an ongoing project to develop and characterize gene expression profiles during comparative stages of *in vivo* and *in vitro* development of porcine embryos. Comparative analysis of these D11 and D12 embryo SAGE libraries confirmed significant levels of differential gene expression between these stages of porcine embryo development. SAGE enabled the identification of genes associated with putative functional pathways and demonstrated its potential utility facilitating the discovery of new genes in a poorly characterized system for future elucidation of the most critical differentially expressed genes.

## IVF/IVP

### THE ONSET OF PUBERTY IN PIG IMMEDIATELY CHANGES OVARIAN MORPHOLOGY BUT NOT OOCYTE IN VITRO DEVELOPMENTAL COMPETENCE

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Oocytes from adult animals display higher developmental competence than those from prepubertal animals when used for in vitro fertilization or other reproductive techniques [Armstrong, *Theriogenology* 2001;55(6):1303–1322]. This has been described also in pig and since these animals are often slaughtered around the time of puberty, this species is an ideal model to study the changes that occur at the onset of puberty. The objective of this study was to determine how the initial onset of puberty affects the morphology of different ovarian structures and whether it is immediately accompanied by an increased oocyte developmental competence. A total of 190 pigs of Large White, Landrace and Large White–Landrace cross breeds at  $232 \pm 14$  days of age, weighing  $160 \pm 4$  kg were used for this experiment. Pig ovaries were collected from the abattoir in saline solution and maintained at  $37^\circ\text{C}$ . Allocation of ovaries to two groups was based on presence (CL) or absence (NCL) of corpora lutea. A representative sample from each group was measured at the time of aspiration (summarized in Table 1). Oocyte maturation, activation and culture were performed as previously described [Grupen et al., *Mol Reprod Dev* 2002;62(3):387–396]. Briefly, cumulus–oocyte complexes (COCs) aspirated from antral follicles (3–8 mm diameter), with evenly granulated cytoplasm, and more than three layers of compact cumulus cells were matured for 44 h in 50  $\mu\text{l}$  droplets of medium 199 supplemented with antibiotics, 0.1 mg/ml sodium pyruvate, 10  $\mu\text{g}/\text{ml}$  FSH, 5  $\mu\text{g}/\text{ml}$  LH, 1  $\mu\text{g}/\text{ml}$   $17\beta$ -oestradiol, 0.5 mM cysteamine, 1.0 mM dibutryl cAMP (dcAMP), 10 ng/ml EGF and 25% porcine follicular fluid. After 24 h oocytes were washed in the above maturation medium (MM) without dcAMP and transferred to 50  $\mu\text{l}$  MM droplets without dcAMP. At 46 h COCs were denuded and oocytes were activated in 5  $\mu\text{M}$  ionomycin diluted in TALP-PVA medium supplemented with 3.0 mM calcium lactate and 2.0 mM caffeine-sodium benzoate. They were incubated in 2 mM 6-DMAP for 3 h then cultured in NCSU 23 medium for 7 days. Each experiment was replicated at least three times on separate days. Data were subjected to ANOVA. Morphological differences were found between CL and NCL ovaries. CL ovaries were significantly larger, had fewer but larger follicles and larger oocytes than NCL ovaries. However, no differences were found between oocytes from CL and NCL ovaries for both nuclear maturation and blastocyst formation rates. These results indicate that the initial onset of puberty immediately alters ovary, follicle and oocyte morphology, but does not affect oocyte developmental competence. Our results are in agreement with those obtained by Archibong et al. [*Biol Reprod* 1992;47:1026–1030] on in vivo generated embryos and extends them to IVM oocytes from earlier stage follicles. It confirms that oocytes must be exposed to more than one oestrus cycle in order to attain their full developmental competence. Work supported by MIUR MM07155389

Table 1  
Morphological and functional differences between NCL and CL ovaries (mean  $\pm$  S.D.)

Source	Variable	NCL	<i>n</i>	CL	<i>n</i>	<i>P</i> -value
Ovary	Width (cm)	1.59 $\pm$ 0.29	49	2.23 $\pm$ 0.66	49	0.0005
	Length (cm)	2.41 $\pm$ 0.3	51	3.32 $\pm$ 0.68	51	0.0003
Follicle	Total per ovary ( <i>n</i> )	47 $\pm$ 12	29	21 $\pm$ 9	25	0.007
	Diameter (mm)	3.28 $\pm$ 0.5	681	4.52 $\pm$ 1.4	459	0.006
Oocyte	Size ( $\mu$ )	146.1 $\pm$ 10.9	54	159 $\pm$ 9.35	50	0.0005
	Size zona-free ( $\mu$ )	113.1 $\pm$ 11.6	54	124.7 $\pm$ 12.8	50	0.004
Developmental competence	Metaphase II	92.4 $\pm$ 6.37	590	88.4 $\pm$ 21.0	358	0.86
	Blastocyst (%)	23.7 $\pm$ 11.3		21 $\pm$ 7.5		0.7

## IN VITRO FERTILIZATION OF PORCINE OOCYTES MATURED IN PRESENCE OF NERVE GROWTH FACTOR

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The aim of the present study was to examine the effect of different concentration of nerve growth factor during IVM on embryo development. Participation of the NGF in the ovulatory process appears to provide a unique example of the neuroendocrine integration.

Cumulus–oocyte complexes were harvested from slaughterhouse ovaries, selected and matured in modified TCM 199. Oocytes (45–50) were transferred into 4-well plates, containing 500 µl of culture medium. The oocytes were matured in the presence of 1, 5, 10 ng/ml of NGF (Sigma N 2393). After the completion of the maturation oocytes was denuded by vortexing, 25–30 oocytes were placed in 50 µl drops of mTBM medium covered with oil. Preparation of spermatozoa was carried out by thawing two 0.25 ml straw washed in 8 ml TCM 199 by centrifugation for 2 min, 2000 rpm. The pellet was resuspended in 40 µl mTBM, and later on 50 µl of semen suspension was added to drops of oocytes to give the final sperm concentration  $2 \times 10^5$  cells/ml. Fertilization was performed at 38.5 °C for 4 h. Presumptive zygotes were then cultured in NCSU 23 medium with 0.4% BSA for 6 days. The experiments were repeated three times. Data (mean ± S.E.M.) were pooled and analyzed by one-way ANOVA.

It seems that the completion of maturation with NGF takes the maturation processes faster. It was the reason why the fertilization time point was 42 h. No difference was found in cleavage rate ( $P \leq 0.05$ ) for oocytes matured in control (37%), or 1 ng/ml NGF (47%), 5 ng/ml NGF (41%), and 10 ng/ml NGF (31%). However, compared to that in control (3%), a higher ( $P \leq 0.05$ ) proportion of putative IVM-IVF zygotes developed to the blastocyst stage when oocytes were matured in the presence of 1 ng/ml NGF (11%), 5 ng/ml NGF (6%) or 10 ng/ml NGF (4%), with no difference between them.

The results show that some NGF (1.5 ng/ml) concentrations during maturation enhanced the early oocyte maturation and embryo development, but the differences were not significant. The IVP zygotes' viability will be demonstrated by full-term development following the embryo transfer in the near future. Financial support: OTKA (T 031758).

NUCLEOLAR PROTEINS AND ULTRASTRUCTURE IN EARLY PORCINE  
EMBRYOS PRODUCED IN VITRO

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The major transcriptional activation of the porcine embryonic genome occurs during the third post-fertilization cell cycle (4-cell stage) in vivo [Hyttel et al., *Biol Reprod* 2000;63:1848–1856]. It includes formation of a fibrillo-granular nucleolus indicating activation of the ribosomal RNA (rRNA) genes. Accordingly, key nucleolar proteins involved in rRNA transcription (upstream binding factor (UBF), RNA polymerase I, and topoisomerase I), initial rRNA processing (fibrillarin) or later processing and/or nucleolar transport (nucleolin and nucleophosmin) can be immunocytochemically localized to the nucleolus towards the end of the third cell cycle or initially during the fourth (8-cell stage). The aim of the present study was to reveal the chronology of the major transcriptional activation of the genome in relation to ultrastructural features of the nucleolus and the allocation of the key nucleolar proteins in porcine embryos produced in vitro. Porcine embryos were produced by oocyte maturation, fertilization and further culture in vitro as previously described [Rath et al., *Theriogenology* 1997;47:795–800]. Embryos were collected at the 2-, early 4-, late 4-, early 8-, late 8-, and 16-cell stage and processed for transmission electron microscopy (TEM), light microscopical autoradiography following 20 min of <sup>3</sup>H-uridine incubation ( $N = 60$ ), and for immunocytochemical localization by confocal laser scanning microscopy of the above mentioned key nucleolar proteins ( $N = 107$ ). Autoradiographic labelling did not occur until the fifth cell cycle (16-cell stage). In one-fourth of the embryos, labeling of both nucleoplasm and nucleoli was observed while other embryos only displayed nucleoplasmic labeling. From zygote until the 16-cell stage the structurally most prominent nuclear entities were the electron-dense fibrillar spheres referred to as nuclear precursor bodies (NPBs). At the 16-cell stage, the nuclei of embryos with autoradiographic labeling of the nucleoli displayed different stages of nucleolus formation, ranging from inactive NPBs to fibrillo-granular nucleoli with predominantly dense fibrillar components (DFC), fibrillar centers (FCs) and a granular component GC on the surface of the NPBs. Fibrillarin and nucleolin were localized to the NPBs in about half the embryos from the late 4-cell stage. We were unable to detect topoisomerase I, UBF and nucleophosmin at any stage. However, RNA polymerase I was localized to discrete nucleoplasmic foci in the majority of the embryos at the 16-cell stage. The lack of detectable levels of the nucleolar proteins is consistent with the absence of autoradiographic labelling and of fibrillo-granular nucleoli. The results indicate that the onset of embryonic genome activation is severely compromised in porcine in vitro produced embryos. Genome activation was delayed one to two cell cycles compared to in vivo developed embryos, and some of the key nucleolar proteins were absent or only present in undetectable levels in accordance with impaired development of nucleoli. The absence or delayed formation of nucleoli and, hence, rRNA and protein synthesis may be a key factor in the compromised developmental competence of in vitro produced porcine embryos. Therefore, the rRNA gene activation and the associated nucleolus formation may be used as a marker for activation of the embryonic genome and, thus, serve to evaluate the developmental competence of porcine embryos originating from different embryo technological procedures.

## SPERM CHROMATIN STRUCTURE AND IVF IN BULLS WITH LOW FERTILITY IN VIVO

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The objective of this study was to relate sperm chromatin structure from bulls with low 56-day non-return rates (NRR) to in vitro embryo development in a standard IVP system. Cumulus oocyte complexes (COCs), collected from abattoir ovaries, were in vitro matured, fertilized and cultured until Day 8 after insemination. The chromatin structure of the frozen-thawed semen from four bulls used for IVF was assessed using the Sperm Chromatin Structure Assay (SCSA) [Evenson and Jost, *Methods Cell Sci* 2000;22:169–189]. The integrity of the sperm chromatin structure was expressed as DNA fragmentation index (DFI%) and evaluated (i) immediately post-thaw (37 °C in 30 s), (ii) after 2 × 5 min centrifugation at 300 × *g* in TALP medium (modified Tyrode medium with albumin, pyruvate and lactate), and (iii) after incubation for 4 h at 38 °C and 5% CO<sub>2</sub> in IVF-TALP (TALP with heparin, penicillamine, hypotaurine, epinephrine). The results are shown in Table 1. After IVF with bull B a higher proportion of unfertilized oocytes and lower cleavage, morula and blastocyst rates were seen compared to the three other bulls ( $P < 0.05$ ). The results from the SCSA analysis showed that after centrifugation and dilution in TALP, bull B sperm had significantly more chromatin damage than bulls A, C and D ( $P < 0.05$ ). After incubation, bulls A and B each had significantly more chromatin damage than bulls C and D ( $P < 0.05$ ). The NRR for bulls C and D are low, but the oocyte penetration and development until Day 8 do not seem affected. The relatively low cleavage and morula rates seen for bull B could be due to bull Bs decline in chromatin integrity, when tested after preparation of the sperm for IVF. Our data suggest that when attempting to extrapolate between the in vitro and in vivo fertility of a bull, sperm parameters should be assessed with respect to chromatin integrity and the steps in the preparation of the semen for IVF carefully evaluated.

Table 1  
In vitro embryo development of bovine oocytes and SCSA analysis of the semen

	Bull			
	A	B	C	D
NRR	61.6%	61.7%	37.7%	42.9%
<2PN	16 ± 7% <sup>a</sup> (13/79)	62 ± 18% <sup>b</sup> (44/72)	22 ± 21% <sup>a</sup> (17/78)	23 ± 10% <sup>a</sup> (17/74)
2PN	81 ± 8% <sup>a</sup> (64/79)	37 ± 20% <sup>b</sup> (27/72)	70 ± 16% <sup>a</sup> (54/78)	71 ± 14% <sup>a</sup> (53/74)
Day 2CL	85 ± 5% <sup>a</sup> (411/478)	48 ± 10% <sup>b</sup> (238/496)	75 ± 11% <sup>a</sup> (238/477)	74 ± 16% <sup>a</sup> (336/464)
Day 5CM	40 ± 4% <sup>a</sup> (192/478)	10 ± 4% <sup>b</sup> (46/496)	36 ± 9% <sup>a</sup> (170/477)	40 ± 12% <sup>a</sup> (182/464)
Day 8BL	32 ± 9% <sup>a</sup> (150/478)	8 ± 5% <sup>b</sup> (37/496)	25 ± 10% <sup>a</sup> (113/477)	27 ± 6% <sup>a</sup> (124/464)
DFI Thawing	4.3 ± 0.2%	4.6 ± 0.3%	2.3 ± 0.3%	3.1 ± 0.1%
DFI IVF Preparation	12.0 ± 2.4% <sup>a</sup>	29.1 ± 0.1% <sup>b</sup>	6.9 ± 0.1% <sup>c</sup>	5.1 ± 0.8% <sup>c</sup>
IVF Incubation	38.0 ± 1.0% <sup>a</sup>	40.6 ± 1.4% <sup>a</sup>	21.5 ± 1.4% <sup>b</sup>	19.9 ± 1.2% <sup>b</sup>

Five replicates. Results expressed as mean ± S.D.; <2PN: unfertilized oocytes; 2PN: normal fertilization; CL: cleavage rate; CM: compact morula rate; BL: blastocyst rate; DFI: DNA fragmentation index. Rows with different superscripts (a and b) differ significantly ( $P < 0.05$ ). Data analyzed by ANOVA and post-tests.

FOLLICULAR FLUID CONCENTRATION DURING PIG IVM AFFECTS OOCYTE PARTHENOGENETIC DEVELOPMENT AND MITOCHONDRIA DISTRIBUTION

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The addition of follicular fluid in the maturation medium has been proposed as an efficient way to improve pig oocyte developmental competence following IVF and parthenogenetic activation [Abeydeera, Theriogenology 2002;57:257]. Mitochondria migration during in vitro maturation (IVM) has been described in several species including pig [Sun et al., BOR 2001;122:155] and their distribution has been correlated with developmental competence in cow [Stojkovic et al., BOR 2001;64:904]. In the present study, we exposed cumulus-oocyte complexes (COCs) to increasing concentrations of prepuberal follicular fluid (pff) during IVM, and we investigated whether this affected oocyte developmental competence after parthenogenetic activation as well as the mitochondria distribution pattern after maturation. Oocyte IVM, parthenogenetic activation and embryo culture were performed as previously described by Grupen et al. [Mol Reprod Dev 2002;62:387] with minor modi modifications. COC were collected from ovaries of prepuberal gilts (age:  $9.5 \pm 0.5$  months; weight:  $163 \pm 6.4$  kg, absence of corpora lutea) and matured in vitro for 46 h with either 0, 10 or 25% pff in TCM 199, in 5% CO<sub>2</sub> at 38.5 °C. At the end of IVM, nuclear morphology was assessed by orcein stain. COC were parthenogenetically activated by sequential exposure to 5 µM ionomycin and 2 mM 6DMAP. Embryos were cultured in 50 µl droplets of NCSU-23 for 7 days under mineral oil in an atmosphere of 5% CO<sub>2</sub> and 5% O<sub>2</sub> at 38.5 °C. At the beginning and at the end of IVM, mitochondria were stained with MitoTracker Orange CMTMRos (Molecular Probes), specific for active mitochondria, and meiotic stage was contemporarily assessed with DAPI. Specimens were examined with a Leica TCS-NT confocal microscope. While maturation rate was not different among treatments, the number of blastocyst was significantly higher in the 25% pff group. In immature oocytes mitochondria showed a polarized localization around the GV. Upon maturation mitochondria showed three different patterns: big clumps, smaller clumps and smooth distribution, which were prevalent in the 0, 10 and 25% pff groups respectively. Furthermore, approximately half of the oocytes matured in 25% pff displayed stronger mitochondria fluorescence, indicating a higher activity. The results indicated that also in pig mitochondria distribution is different between oocytes with different developmental competence (Table 1).

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Table 1  
Effect of follicular fluid on maturation, cleavage, blastocyst rates and mitochondria distribution

% pff in IVM (n)	MII (%)	Cleavage (%)	Blastocyst (%)	Prevalent mitochondria pattern at MII
0 (329)	83.33 <sup>a</sup> ± 7.64	80.1 <sup>a</sup> ± 3.60	6.78 <sup>a</sup> ± 4.84	Big clumps
10 (278)	87.37 <sup>a</sup> ± 2.7	83.9 <sup>a</sup> ± 5.21	5.99 <sup>a</sup> ± 2.3	Small clumps
25 (249)	91.25 <sup>a</sup> ± 6.29	87.6 <sup>a</sup> ± 2.9	28.63 <sup>b</sup> ± 11.67	Even distribution

Results are expressed as mean ± S.D. and analyzed with one-way ANOVA. <sup>a,b</sup>Different superscripts in the same column indicate statistical differences ( $P < 0.05$ ).

## A NOVEL INTEGRATED IN VITRO MATURATION AND IN VITRO FERTILIZATION SYSTEM FOR SWINE

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There have been a number of modifications to the protocols for in vitro production of porcine embryos. Many of these alterations have been to media components, timing of certain events, and atmospheric conditions. The method and degree of oocyte/embryo handling, however, have remained unchanged. Recently, the development of microscale culture devices (microchannels) has changed the manner in which embryologists perform the processes of in vitro maturation and in vitro fertilization. These microchannels are constructed using gas permeable poly-dimethylsiloxane (PDMS) fabrication techniques with channel dimensions (250  $\mu\text{m}$   $\times$  1000  $\mu\text{m}$ ; height  $\times$  width) on the same order as the cumulus-oocyte-complexes. These microchannels are designed to more closely mimic the function of the oviduct by allowing changing of media and their components without extensive manipulation of the oocytes. This study was designed to compare the integration of IVM-IVF of pig oocytes in PDMS-glass microchannels to conventional microdrop methods both in a static culture system. Oocytes were obtained by aspiration of ovarian antral follicles. Oocytes were placed in either traditional 500  $\mu\text{l}$  microdrops (50 oocytes per drop) or into a microchannel (50 oocytes per drop) filled with 500  $\mu\text{l}$  of medium for maturation. The maturation medium, TCM 199, was supplemented with LH, FSH, GF, cysteine, PVA, and antibiotics. All oocytes were allowed to mature in TCM 199 for 44 h humidified 5%  $\text{CO}_2$  in air atmosphere at 39  $^\circ\text{C}$ . The medium in the loading/unloading wells of the microchannel was removed by a pipette and then filled with 0.1 mg/ml hyaluronidase to remove cumulus cells. This medium was pulled through the microchannel and allowed to sit for 5 min. The procedure of changing medium was repeated and the microchannel was filled with 200  $\mu\text{l}$  modified Tris-buffered medium (mTBM) and pulled through the microchannel without disturbing the denuded oocytes. Similarly, 15 denuded oocytes were placed into a pre-equilibrated 50  $\mu\text{l}$  drop of mTBM covered with warm paraffin oil in a petri dish. Capacitated sperm cells in mTBM were added to the oocytes in both fertilization systems at a final concentration of  $3 \times 10^5$  sperms/ml and co-incubated in humidified 5%  $\text{CO}_2$  in air atmosphere at 39  $^\circ\text{C}$  for 6 h. Presumptive zygotes were washed and cultured in North Carolina State University-23 medium covered with mineral oil in humidified 5%  $\text{CO}_2$  in air atmosphere at 39  $^\circ\text{C}$  for 48 h. Cleavage rates were recorded on Day 2 of development, and data from five replicates were analyzed by an analysis of variance using generalized linear model in SAS. A total of 92/180 (51.1%) and 91/185 (49.2%) embryos cleaved after fertilization in the 50  $\mu\text{l}$  drops and microchannels, respectively. There was no significant difference ( $P > 0.05$ ) found between the two integrated IVM-IVF systems. These are the initial experiments involving media changes without disturbing the oocytes during the processes of IVM and IVF. Additional parameters, including flow rates, are being explored to optimize the microfluidic system for in vitro maturation and embryo production.

POTENTIAL FOR IN VITRO PRODUCTION OF EMBRYO FROM FOLLICULAR OOCYTE OF YELLOW AND YELLOW-RED SINDHI CROSSBRED CATTLE

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Local Yellow and Yellow × Red Sindhi (Laisind) bovine are the major resource of slaughtered cattle in Vietnam. The investigation was undertaken to study the potential of using the oocytes from these species for developing cattle embryo technology. In this paper we present the results of oocyte collection, in vitro maturation (IVM) and in vitro fertilization (IVF) realized during the dry season (from February to May). Cumulus–oocyte complexes (COCs) were aspirated from follicles of 2–5 mm of slaughterhouse-derived ovaries using 18-gauge needles. The COCs with more than four cumulus layers were matured in TCM199 medium supplemented with 10% FCS, estradiol-17β (1 µg/ml), FSH (0.5 µg/ml), LH (0.1 µg/ml) for 24 h in 5% CO<sub>2</sub> in air at 39 °C. After IVM, a group of COCs was removed from cumulus cells by pipetting in TC199-Hepes containing 1% hyaluronidase (Sigma). They were used for observation of maturation based on the presence of the first polarbody. The remaining COCs were rinsed in Fert-TALP medium and were inseminated with Holstein Friesian semen (Moncada Station-Viet Nam). After thawing in a 37 °C water bath for 1 min, motile spermatozoa were separated by the swim-up procedure [Parrish et al., *Theriogenology* 1986;25:591–600]. Final concentration of spermatozoa in fertilization droplets was adjusted to 1 × 10<sup>6</sup>/ml. The IVF medium consisted of Fert-TALP medium [Parrish et al., *Theriogenology* 1988;38:1171–80] supplemented with 1 µM epinephrine, 10 µM hypotaurine, 20 µM penicillamine and 0.5 µg/ml heparin. IVF was carried out at 39 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Following IVF, the presumptive zygotes were co-cultured in 50 µl droplets of Menezo B2 supplemented with 5% FCS and Vero cell monolayer at 39 °C, 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. The number of oocytes cleaved at 48 h after fertilization and the number of embryos developed to the morular-blastocyst stage on Days 6, 7 and 9 (Day 0 = day of IVF) were assessed. The results were presented in Table 1. The results show that the rate of maturation, fertilization and cleavage of oocytes from Yellow and Yellow–Red Sindhi cattle are in the range of 65–80% reported for other cattle breeds; however, the rate of embryo developed to blastocyst stage was lower. Compared to Yellow cattle, the efficiency of in vitro embryo production from the oocytes of Yellow–Red Sindhi crossbred was significantly higher because of the higher number of COC collected per ovary. In conclusion, the oocytes collected from both breeds during the dry season can be used as the material resource for embryo technology. Supported by AIRE-Development, French.

Table 1  
In vitro production of embryo from Yellow and Laisind cattle

Breed	No. COCs/ovary	No. of oocytes after IVM	% Oocytes with PB	No. of oocytes used for IVF	% Oocytes cleaved	% Morula	% Blastocyst (Day 7)
Yellow	4.34 ± 0.3 <sup>a</sup>	140	70 <sup>c</sup>	302	66.9 <sup>c</sup>	38.6 <sup>c</sup>	24.3 <sup>c</sup>
Laisind	16.8 ± 2.6 <sup>b</sup>	148	74.3 <sup>c</sup>	201	70.1 <sup>c</sup>	40.4 <sup>c</sup>	29.8 <sup>c</sup>

<sup>a,b</sup>Different superscripts within the same column differ significantly ( $P < 0.05$ , Student's *t*-test); <sup>c</sup>Values within the same column do not differ significantly ( $P > 0.05$ , chi-square test).

## ASSOCIATION OF FOOT AND MOUTH DISEASE VIRUS (FMDV) WITH BOVINE OOCYTES DURING IN VITRO MATURATION

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The risk of FMDV transmission by in vivo-produced embryos is extremely small. In vitro-produced (IVP) embryos carry a greater risk of transmitting FMDV. IVP day 7 embryos, exposed to FMDV, could not be freed of the virus by washing [Marquant Le Guienne et al., *Theriogenology* 1998;50:109–116]. No research has been done on the association of FMDV with bovine embryos during IVF and IVF. Exposing oocytes to FMDV during IVF and IVF would give a more realistic model to judge if oocytes collected from infected donor cattle pose a risk to transmit FMDV. This is of importance, since high virus concentrations were found in follicular fluid of cows in the viraemic stage of the disease [McVicar et al., *Theriogenology* 1986;26:595–603].

The aim of this study was to test if bovine oocytes, which are co-incubated with FMDV during IVF, can be washed free of the virus after maturation.

Five batches of 200 oocytes each, with an intact corona radiata and at least three layers of compact cumulus cells (COCs), were obtained from ovaries from a local abattoir. Each batch was matured for 22 h in TCM 199 (Earle's salts) supplemented with 25 µg/ml gentamicin, 100 µM 2-mercaptoethylamine, 25 mM HEPES, 5% steer serum and  $2 \times 10^6$  TCID<sub>50</sub> FMDV at 39 °C in 5% CO<sub>2</sub> in air with 100% humidity. Matured oocytes from each batch were split into two groups. Half was kept as entire COCs and the other half of the oocytes were denuded of cumulus cells by vortexing. Each group was further divided into two equal groups. Each of the resulting groups of 50 was treated as follows: COCs were washed 10 times in 300 µl IVF medium, i.e. modified TALP including 25 µg/ml gentamicin, 0.6% essentially fatty acid free BSA and PHE (2.0 mM penicillamine, 1.0 mM hypotaurine and 25 µM epinephrine). One group of 50 was put in 1 ml of PBS for virus detection on pig kidney monolayers (PKM). The other 50 COCs were washed and put in 200 µl PBS to be analyzed by PCR. The denuded oocytes from each batch were treated in the same way. The IVF fluid of each batch, containing the cumulus cells, was also analyzed for the presence of FMDV.

FMDV was detected in all samples on PKM; therefore, no PCR tests were run. After 24 h of incubation the cytopathic effect was, subjectively assessed, distinctly less severe for the denuded oocytes than for the other samples.

This trial shows that in vitro matured bovine COCs, cumulus cells and denuded oocytes, which are matured in the presence of FMDV, are not free of the virus after washing them 10 times. The results do not indicate whether the FMDV infects the cumulus cells and the oocytes, if it does only adhere to their surface or if 10 washes are not enough to remove all the IVF fluid containing the virus. Low pH is used to destroy viral particles in cell culture media without affecting internalized virus [Rieder et al., *J Virol* 1993;67 (9):5139–5145]. In a follow-up trial, COCs, cumulus cells and denuded oocytes should be exposed to an acidic environment for a few seconds. At pH 5 the FMDV is inactivated at a rate of 90% per second [Acharya et al., *Nature* 1989;337:709–716]. Assuming that FMDV does not penetrate the zona pellucida it is likely that exposing oocytes to an acidic environment would render denuded oocytes or embryos free of the virus without destroying them.

EFFECT OF SPERM NUCLEAR DAMAGE ON FERTILIZATION AND  
SUBSEQUENT EMBRYO DEVELOPMENT

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Male factor infertility correlates with anomalies in the composition of sperm nuclei, namely loosely packaged chromatin and damaged DNA. In this study, the fertilizing ability of DNA damaged sperm and the consequences of this damage for embryo development were evaluated. To induce DNA damage, bull sperm was exposed to 0, 0.60, 1.25, 2.50, 5.00 or 10.00 Gray (Gy) gamma-irradiation prior to insemination. The level of DNA damage was assessed using the sperm chromatin structure assay (SCSA), which applies acridine orange staining and flow cytometry to determine the structural stability of sperm nuclear chromatin (defined as the susceptibility of DNA to denaturation in situ: [Evenson et al., 1980]). The fertilizing ability of irradiated sperm and the rate of subsequent embryo development was tested by IVF. Bovine cumulus-oocyte complexes (COCs) were aspirated from antral follicles (2–8 mm). COCs were cultured in groups of 35 in 500 µl of IVM medium for 23 h. The IVM medium consisted of TCM199 supplemented with 10% FCS and 0.05 IU/ml recombinant human FSH. IVF took place in TALP medium supplemented with heparin and PHE (D-Penicillamin, Hypotaurine and pinephrine) using frozen-thawed spermatozoa irradiated with different dosages of gamma-irradiation and subsequently centrifuged over a percoll gradient. After 20 h of incubation, the oocytes were freed from cumulus cells and placed in a co-culture of BRL (Buffalo rat liver) cells. IVM, IVF and in vitro embryo culture (IVC) took place at 39 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The effect of irradiation versus control groups was statistically analyzed by one-way ANOVA and if there was a difference among groups then Dunnett's Multiple Comparison Test was performed. SCSA demonstrated that gamma-irradiation induced sperm DNA damage in a dose-dependent fashion. Fertilization up to cleavage on Day 4 was nearly unaffected even at the highest irradiation dose (Table). However, irradiation doses >2.5 Gy almost completely blocked blastocyst formation. It is concluded that even at relatively low irradiation levels, sperm DNA is extensively damaged. However, oocytes appear to be capable of repairing sperm DNA damage up to a certain threshold value. Finally, while cleavage does not seem to be critically regulated by the integrity of paternal DNA, blastocyst formation is.

	Number of oocytes	Cleavage rate Day 4 (%) S.D.	Blastocyst rate Day 7 (%) S.D.	Blastocyst rate Day 9 (%) S.D.	Blastocyst rate Day 11 (%) S.D.
Control	890	68	15	28	27
0.60 Gy	305	65	15	24	22
1.25 Gy	344	70	11	12*	12*
2.50 Gy	299	63	5*	5**	5**
5.00 Gy	375	58	1**	3**	3**
10.0 Gy	388	46*	1**	1**	<1**

\* $P < 0.05$ , \*\* $P < 0.001$ , number of replicates for control was 8 and for other groups was 3.

EFFECT OF GROWTH RATE VARIATION ON OPU-IVF EMBRYO PRODUCTION  
IN SUPEROVULATED OVERFED DAIRY HEIFERS

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The aim of this study was to investigate the effect of short-term variation of energy intake and associated growth rate on oocyte and in vitro embryo production after superovulation in previously overfed Holstein heifers. Sixteen Prim Holstein dairy heifers ( $14 \pm 1$  months old,  $340 \pm 25$  kg) were fed with a diet composed of hay, soybean, barley, minerals and vitamins. After induced heats synchronized with Norgestomet implants (Crestar<sup>®</sup>, Intervet, France), cumulus-oocyte complexes (COCs) were collected by ovum pick-up (OPU) every 2 weeks following superovulation. Heifers received a total dose of 250 µg FSH (Stimufol<sup>®</sup>, Merial, France) divided in five i.m. injections 12 h apart, at decreasing doses. OPU was performed 12 h after the last FSH injection and the quality of COC graded from 1 to 4 before in vitro maturation and fertilization. Embryos were cultured in SOF medium for 7 days. Their number, stage and quality were graded according to IETS criteria. After 15 days of adaptation, all heifers received individually for 6 weeks (Period 1 = P1 = OPU 1–4) a diet aiming at 1000 g per day live weight gain (LWG). Heifers were then allocated to two diets (overfeeding or dietary restriction) based on Period 1 LWG and oocyte production, for 8 weeks following OPU session 4 (Period 2 = P2 = OPU 5–8). Mean LWG achieved in P1 ( $n = 16$ ) was  $1040 \pm 330$  g per day. Three groups of growth rate were determined according to results observed during P2: group 1,  $1040 \pm 110$  g per day ( $n = 8$ ); group 2,  $620 \pm 140$  g per day ( $n = 4$ ) and group 3,  $1460 \pm 170$  g per day ( $n = 4$ ). Effects of Group, Period and interaction between Group and Period were analyzed using the mixed procedure of SAS (female effect as random) and lsmeans were compared with Scheffe's test. Overall, for the three groups, all oocyte and embryo production variables were increased in P2 when compared to P1 ( $P < 0.05$ , Table 1). However, Scheffe's test results show that the increase observed between P1 and P2 was only significant in group 2 (restricted group) for blastocysts and grade 1 embryos at Day 7 (respectively,  $P = 0.05$  and  $P = 0.01$ , Table 1). These data suggest that following an overfeeding period, dietary intake restriction, leading to a lower growth rate, may increase the number of Day 7 blastocysts and the quality of embryos.

Table 1  
Oocyte and embryo production (lsmeans  $\pm$  S.E.M.) in Periods 1 and 2, for the three groups of heifers

	Group 1 ( $n = 8$ )		Group 2 ( $n = 4$ )		Group 3 ( $n = 4$ )	
	Period 1 (1000 g per day)	Period 2 (1000 g per day)	Period 1 (1000 g per day)	Period 2 (600 g per day)	Period 1 (1000 g per day)	Period 2 (1400 g per day)
Collected oocytes	12.3 $\pm$ 2.6	12.9 $\pm$ 2.55	12.25 $\pm$ 3.6	13.25 $\pm$ 3.6	11.0 $\pm$ 3.6	15.1 $\pm$ 3.6
Inseminated oocytes	7.5 $\pm$ 1.9	11.4 $\pm$ 1.9	8.0 $\pm$ 2.7	11.5 $\pm$ 2.7	6.1 $\pm$ 2.7	10.1 $\pm$ 2.7
Day 7 embryos	1.9 $\pm$ 0.9	3.1 $\pm$ 0.9	2.4 $\pm$ 1.3	4.5 $\pm$ 1.3	1.1 $\pm$ 1.3	2.7 $\pm$ 1.3
Blastocysts	1.7 $\pm$ 0.8	2.5 $\pm$ 0.8	1.6 $\pm$ 1.1 <sup>a</sup>	4.4 $\pm$ 1.1 <sup>b</sup>	0.75 $\pm$ 1.1	2.2 $\pm$ 1.1
Grade 1 embryos	0.6 $\pm$ 0.4	1.4 $\pm$ 0.4	0.4 $\pm$ 0.6 <sup>a</sup>	2.4 $\pm$ 0.6 <sup>c</sup>	0.2 $\pm$ 0.6	0.9 $\pm$ 0.6

a vs. b,  $P = 0.05$  and a vs. c,  $P = 0.01$  (lsmeans compared with Scheffe's test).

PRODUCTION OF IVF EMBRYOS FROM ADULT AND JUVENILE CATTLE  
USING SEX-SORTED SPERM

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Two reproductive technologies currently being commercially developed for industry include the use of sex-sorted sperm for the predetermination of offspring sex and IVF of oocytes from juveniles for speed breeding. In this study we combined these technologies to produce embryos from oocytes collected from calves and fertilized with sex-sorted sperm.

COC's were collected from ovaries obtained from the abattoir (COW) or from stimulated Angus calves by TVR (CALVES). Angus calves, approximately 6 months of age and 150 kg live weight, received 2.0, 1.0, 0.5, 0.5 mg FSH (Ovagen) over 2 days prior to TVR. All viable COC's were matured for 24 h in 500- $\mu$ l wells containing TCM 199 + 10% FCS + hormones under oil. Fertilization was performed with either frozen/thawed sperm from a control bull (UNSORTED) or from a bull whose sperm had been sorted by DNA content using a MoFlow SX sperm sorter (Cytomation Inc.) to be predominately Y-bearing (SEX-SORTED). Fertilization was performed in 500- $\mu$ l wells containing SOFM and BSA (6 mg/ml) under oil at a concentration of approximately 3 million sperm per ml [Earl et al., *Theriogenology* 1997;47:255]. The zygotes were cultured in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> for 6 days after fertilization in SOFM containing BSA and amino acids. Cleavage rate and blastocyst development by Day 7 for each treatment were compared by Chi-square analysis. There was no difference in the cleavage rates between oocytes fertilized with UNSORTED (79%) or SEX-SORTED sperm (80%). However, cleavage rate derived from COW oocytes (89%) was significantly higher than those obtained from CALVES (55%,  $P \leq 0.05$ ). No difference in blastocyst production rate was found when COC's were in vitro fertilized with UNSORTED (79/191 = 41%) or SEX-SORTED (130/326 = 40%) sperm. Although significantly more ( $P \leq 0.05$ ) oocytes derived from COW developed to blastocysts by Day 7 (169/376 = 45%) than those from CALVES (40/141 = 28%), the percentage development from cleaved oocytes to blastocyst was similar (both 51%).

These results demonstrate that IVF with SEX-SORTED sperm can achieve similar fertilization and embryo development rates to UNSORTED sperm. Lower IVF rates of oocytes derived from CALVES compared to those for abattoir derived COW oocytes suggest differences in their intrinsic developmental capacity. SEX-SORTED sperm can be efficiently used to produce IVF embryos from oocytes collected from young calves, providing exciting opportunities in livestock breeding.

We thank XY Inc. for the supply of sex-sorted sperm.

## DEVELOPMENTAL COMPETENCY OF PREPUBERTAL AND ADULT PORCINE OOCYTES

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Studies in cattle, sheep and pigs indicate that the age of the donor animal affects oocyte developmental competence and, thus, the efficiency of in vitro embryo production [Armstrong et al., *Theriogenology* 2001;55:1303–1322]. In the pig, the incidence of polyspermic fertilization, which adversely affects developmental potential, is greater in prepubertal oocytes than in adult oocytes [Marchal et al., *Theriogenology* 2001;56:17–29]. A parthenogenetic study would enable an assessment of developmental potential to be made without the influence of polyspermic effects. The aim of this study was to determine the developmental competence of parthenogenetically activated prepubertal and adult porcine oocytes. Oocytes recovered from the ovaries of slaughtered prepubertal and adult pigs were matured, activated and cultured as described previously [Grupen et al., *Mol Reprod Dev* 2002;62:387–396]. Briefly, ovaries were transported in Dulbecco's PBS maintained at 37 °C, antral follicles 3–6 mm in diameter were aspirated, and oocytes with at least three compact cumulus cell layers were recovered from the collected fluid, washed and matured for 44 h. The maturation medium consisted of Medium 199 supplemented with antibiotics, 0.1 mg/ml Na-pyruvate, 10 µg/ml FSH, 5 µg/ml LH, 1 µg/ml estradiol, 0.5 mM cysteamine, 10 ng/ml EGF and 25% follicular fluid (with 1 mM dibutyryl cAMP for the first 20 h of maturation). Following maturation, oocytes were denuded of cumulus cells and those that had extruded a polar body were incubated sequentially with 5 µM ionomycin for 5 min and 2 mM 6-dimethylaminopurine for 3 h. Oocytes were then washed three times and cultured in NCSU-23 medium supplemented with 0.4% BSA for 7 days (10% FCS was added after 5 days of culture). Parthenote development was assessed and blastocyst cell numbers were counted following differential staining. Development data from three replicates were subjected to ANOVA and Chi-square analysis and cell number data were subjected to the *t*-test. Adult oocytes cleaved and formed blastocysts at greater rates than prepubertal oocytes (Table 1). Blastocysts derived from adult oocytes had greater numbers of trophoctoderm ( $42.6 \pm 2.0$  versus  $29.9 \pm 1.8$ ) and total ( $50.6 \pm 2.6$  versus  $36.1 \pm 2.5$ ) cells compared with those derived from prepubertal oocytes. The study demonstrated that the developmental competence of adult oocytes was superior to that of prepubertal oocytes. This finding is in agreement with those of IVF studies, and indicates that the reduced developmental potential of prepubertal porcine oocytes following fertilization cannot be attributed to polyspermic effects alone.

Table 1  
Development in vitro of parthenogenetically activated porcine oocytes

Oocyte source	<i>n</i>	% 2-cell	% 4-cell	% 7D blastocyst
Prepubertal	171	$73.2 \pm 6.2^a$	$52.3 \pm 4.2^a$	$37.9 \pm 2.4^a$
Adult	211	$92.2 \pm 1.9^b$	$76.3 \pm 6.7^b$	$57.0 \pm 6.7^b$

<sup>a,b</sup>*P* < 0.05.

DIFFERENTIAL STAINING OF TROPHECTODERM AND INNER CELL  
MASS CELLS OF CAT IN VITRO DERIVED BLASTOCYSTS

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Differential staining of inner cell mass (ICM) and trophoctoderm (TE) cells using bichromatic fluorochromes is a useful technique for assessing developmental potential of in vitro derived blastocysts. The aim of the present study was to determine if a chemically defined differential staining method could be used to quantite ICM and TE cell numbers in Day 7 and Day 8 cat in vitro derived blastocysts. Oocytes were recovered by mincing ovaries between 3 and 6 h after excision. Oocytes and embryos were cultured as described by Góomez et al. [Reprod Fertil Dev 2000;12:423–433]. Oocytes surrounded by compact cumulus cells were cultured in modified TCM 199 + 3 mg/ml BSA + 1 IU/ml hCG + 0.5 IU/ml eCGat 38 °C in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> for 24 h. For IVF, oocytes were inseminated with ejaculated spermatozoa (400,000 motile sperm/ml) in modified Tyrodes (mTy) + 6 mg/ml BSA. After 16 h, oocytes were rinsed and cultured in mTy + 3 mg/ml BSA + non-essential amino acids (NEAA). On Day 2 or 3, embryos were placed in mTy + NEAA + essential AA + 10% FBS. Embryos developing to the blastocyst stage on Days 7 and 8 were stained using a modification of the method described by K.D. Wells [Proceedings of Genetically Engineering and Cloning Animals: Science, Society and Industry, 2000, abstr. 12]. Blastocysts with intact zona pellucida were incubated in IVC-1 containing 1.0 mg/ml of the membrane permeant nuclear stain bisbenzimidide (Hoechst 33342; Ho) at 38 °C. After 30 min, embryos were exposed to 0.04% non-ionic detergent Triton-X 100 for 1 min to permeabilize the TE cells. Then, blastocysts were immediately transferred into IVC-1 containing 25 µg/ml of the membrane impermeant nuclear stain propidium iodide (PI) for 9 min, which stains all cells that have been permeabilized by Triton-X 100. After staining, blastocysts were pipetted onto a glass microslide into a drop of mounting medium (glycerol + 10 µg/ml Ho), gently flattened under a cover slip and ICM and T cells counted using epifluorescence microscopy. ICM cells were stained blue with Ho, and TE cells were stained red with PI. Comparison of blastocyst cell numbers between development stages were analyzed using *t*-test. Results are shown below:

Development stage	No. of blastocysts	Cell number			% ICM	Ratio ICM:TE
		Total cell#	ICM#	TE#		
Day 7	88	150 ± 50.8	42.7 ± 20.5	107.5 ± 42.6	28.4	1:2.5
Day 8	31	138 ± 40.5	39.0 ± 20.3	99.8 ± 37.4	28.2	1:2.5

There were no differences between Day 7 and Day 8 blastocysts in total number of cells, number of ICM cells or number of T cells. The percentage of ICM cells and the ratio of ICM:TE cells were similar to that reported for other species. These results demonstrate that a rapid, chemically defined differential staining technique can be used to determine ICM and T cell numbers in cat blastocysts. Such information will be useful for comparative evaluations of various methods used to generate embryos in vitro.

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A COMPARISON OF THE IN VITRO DEVELOPMENT OF COW  
AND CALF OOCYTES

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Calf oocytes appear to exhibit cytoplasmic deficiencies that may limit their in vitro developmental competence despite in vitro fertilization and early cleavage rates not differing significantly between calf and cow [Duby RT et al., *Theriogenology* 1996;45:121–130]. This study compares in vitro blastocysts production rates for adult and juvenile derived bovine oocytes. Four randomly selected Wagyu calves, aged 8–14 weeks, were stimulated on two occasions with four injections (40, 20, 20, 20 mg) FSH (Folltropin V, Vetrepharm, Canada) over 2 days. A Norgestomet ear implant (Crestar, Intervet, Australia) was inserted 3 days prior to stimulation. Cumulus–oocyte complexes (COCs) were aspirated from follicles  $\geq 2$  mm diameter 12 h after the last injection via midventral laparotomy under general anaesthesia. Unexpanded COC were recovered, matured, fertilized in SOF with BSA (6 mg/ml) and cultured in vitro [Kelly et al., *Theriogenology* 1997;47:291 (abstract)]. Oocytes from each collection were randomly assigned to be co-incubated with spermatozoa from either of two sires. Semen was prepared on a 3-layer percoll gradient and added at  $1 \times 10^6$ /ml concentration. Abattoir-sourced cow COC were used for comparison. Cleavage and embryo development rates were assessed. Variables were analyzed using procedure CATMOD in SAS. There was no significant ( $P > 0.05$ ) effect of either sire or oocyte source on cleavage rate. There was, however, a significant ( $P < 0.05$ ) sire effect and a significant ( $P < 0.05$ ) sire  $\times$  oocyte source interaction for blastocysts formation rate. These results indicate that under optimal in vitro conditions, calf oocytes are capable of developmental rates similar to those derived from cows. This conclusion is supported by the single transfer of 89 randomly selected calf blastocysts into programmed recipients resulting in 30 (34%) pregnancies. However, sire  $\times$  oocyte source interaction may play a greater role in determining developmental competence for oocytes derived from juveniles compared with adults.

Sire	Oocyte source	Oocytes in culture	No. of cleaved (%)	No. of blastocysts (%)
1	Cow	43	33/43 (77)	19 (58)
	Calf 1	14	11/14 (79)	9 (82)
	Calf 2	71	48/71 (68)	27 (56)
	Calf 3	12	7/12 (58)	5 (71)
	Calf 4	26	18/26 (69)	11 (61)
2	Cow	41	30/41 (73)	23 (77)
	Calf 1	34	29/34 (85)	13 (45)
	Calf 2	56	40/56 (71)	24 (60)
	Calf 3	12	7/12 (58)	1 (14)
	Calf 4	29	25/29 (86)	11 (44)

FERTILIZATION AND DEVELOPMENT IN VITRO OF PORCINE OOCYTES  
MATURED IN VITRO AND FREED FROM ZONA PELLUCIDA

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Zona pellucida (ZP) exists around mammalian ooplasm and plays an important role for fertilization and early embryonic development. One of the functions of ZP is prevention of polyspermy; this is known as zona block. However, the oocyte membrane is also considered to be the source of a block against excess sperm penetration (membrane block). The objectives of the present study were to evaluate the effect of ZP on sperm penetration and, if the fertilization of porcine oocytes without ZP is possible, to assess the developmental ability to the viable embryos. Porcine cumulus–oocyte complexes were matured in vitro for 42 h [Kikuchi et al., *Biol Reprod* 2002;66:1033–1041]. Oocytes with the first polar body were collected as matured oocytes after treatment with 150 IU/ml hyaluronidase and gentle pipetting. Then some of the matured oocytes were treated with 0.1% pronase for 30 s, and half-dissolved ZP were removed mechanically by pipetting in the medium without pronase. Both oocytes enclosed in ZP (ZP+ oocytes) and oocytes without ZP (ZP– oocytes) were subsequently cultured for 3 h in the maturation medium and fertilized in vitro by frozen–thawed epididymal spermatozoa from three Landrace boars (Boars A, B and C) in a modified Pig-FM [Suzuki et al., *Int Androl* 2002;25:84–93] for 3 h at  $1 \times 10^3$ – $10^5$  sperm/ml. Inseminated oocytes were further cultured for 7 h and fixed in whole mounted preparation. All culture was carried out at 39 °C under 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. Analysis of variance and Duncan's multiple range test revealed that the rate of sperm penetration was higher for ZP+ oocytes (ZP+, 48%; ZP–, 34%) and for oocytes inseminated at increased sperm concentrations ( $1 \times 10^3$ , 7%;  $1 \times 10^4$ , 27%;  $1 \times 10^5$ , 85%), but was not different significantly among sperm sources (Boar A, 48%; Boar B, 28%; Boar C, 47%). The rate of polyspermy increased significantly for ZP+ oocytes (ZP+, 33%; ZP–, 13%) and in the case of increased sperm concentrations ( $1 \times 10^3$ , 0%;  $1 \times 10^4$ , 6%;  $1 \times 10^5$ , 62%), but not among sperm sources (Boar A, 27%; Boar B, 13%; Boar C, 30%). The sperm number per oocyte also increased significantly in ZP+ oocytes (ZP+, 2.2; ZP–, 1.3) and in the case of increased sperm concentrations ( $1 \times 10^3$ , 0.7;  $1 \times 10^4$ , 1.2;  $1 \times 10^5$ , 3.3), but not among sperm sources (Boar A, 2.0; Boar B, 1.2; Boar C, 2.0). To assess the developmental ability, ZP– oocytes were fertilized by Boar A spermatozoa at  $1 \times 10^4$ /ml, enclosed in 3% alginate acid, and cultured for 6 days (first 2 days in IVC-PyuLac and other 4 days in IVC-Glu [Kikuchi et al., 2002]) at 38.5 °C and fixed. The rate of IVM/IVF oocytes that developed to the blastocyst stage was 20% and average cell number was 60.6. Although the effects of pronase on the oocyte membrane during liberation from ZP have not been of concern yet, the results suggest that the existence of ZP accelerates sperm penetration. ZP may promote the final capacitation and/or acrosome reaction of spermatozoa, and the membrane blocks against excess number of sperm penetrating the porcine oocytes may be possible. It is concluded that porcine oocytes without ZP can be fertilized and developed in vitro to the blastocyst stage.

## TIMING OF DEVELOPMENT AND SEX RATIO OF IN VITRO PRODUCED GOAT EMBRYOS

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The domestic goat is an important species for agriculture and embryo-based biomedical technology; however, the in vitro production and development of its embryos are poorly characterized. The aim of this study was to examine the chronology of development and sex ratio of in vitro produced goat embryos. To accomplish this, ovaries from culled goats were obtained from a local slaughterhouse, oocytes were aspirated from follicles, matured in M199 plus EGF (10 ng/ml) and 100  $\mu$ M cysteamine for 24 h, co-incubated with frozen-thawed sperm ( $10^6$  sperms/ml) from bucks of proven fertility for 17 h in SOF (-BSA) and 10% estrus sheep serum. Presumptive zygotes were cultured ( $D = 0$ ) in microdrops of SOF (+BSA) for 6–8 days at 38.5 °C in 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> atmosphere. On Day 1 of culture, 10% fetal calf serum was added to microdrops and the cleavage rate was assessed. Development was monitored on Days 5, 6, 7 and 8. Hatched blastocysts were removed from culture as they appeared and were stored at 20 °C until sex determination was performed. All remaining blastocysts were removed at Day 8. Sex determination was carried out by PCR amplification of SOX-2 and BRY.1 [Bernardi and Delouis, Hum Reprod 1996;11:621–626] known to be located on a caprine autosome and Y-chromosome, respectively.

In all, 392 oocytes distributed over 10 replicates were matured and co-incubated with sperm. The maturation rate in parallel studies was greater than 90%. The overall percentage of oocytes that cleaved, developed to the blastocyst stage and hatched by Day 8 was 70, 45 and 30, respectively. Among those embryos that hatched, 23, 35, and 42% hatched on Days 6, 7 and 8, respectively. Embryos from 7 replicate experiments were processed for sex determination. The sex of 80% of embryos could be successfully determined of which 52.9% were males and 47.1% were females. Among blastocysts that hatched on Days 6, 7 and 8 or remained within their zona pelucida on Day 8, the percentage of males was 52.3, 57.5, 56.9, and 59.5, respectively. The percentage of males did not differ ( $P \leq 0.05$ ) from the expected 50%.

It was concluded that in vitro produced goat embryos developed to the blastocyst stage after 5 days of in vitro culture with the majority hatching between Days 7 and 8. The overall sex ratio did not differ from the expected 1:1 ratio.

## IN VITRO OOCYTE FERTILIZATION, POLYSPERMY AND EMBRYO DEVELOPMENT IN PIGS IN THREE DIFFERENT MEDIA

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The aim of this study was to evaluate the effect of using North Carolina State University-23 (NCSU23), Whitten and TCM 199 as maturation media upon polyspermy, pronuclear formation and embryo development after IVF. In the first experiment, oocytes were matured for 42–44 h in NCSU23, Whitten and TCM199 media supplemented with hormones during the first 24 h of maturation then without hormones. Following maturation, oocytes were fertilized in Tris Buffered Medium (TBM) for 6 h with capacitated spermatozoa ( $3 \times 10^5$  spermatozoa/ml). After fertilization, cumulus cells were removed and oocytes were transferred to NCSU23 supplemented with 0.4% BSA for 18 h. After this period embryos were fixed in 4% paraformaldehyde and stained with Hoechst 33342 to evaluate rates of penetration and polyspermy. There was no significant difference, using the Chi-square test, in penetration rates (85, 79.69 and 80.26%) and polyspermic rates (78.43, 70.58 and 68.65%), respectively, for oocytes matured in NCSU23, Whitten and TCM199. In the second experiment oocytes were matured, fertilized and cultured in NCSU23 with 0.4% BSA for 5 days. At the morula and early blastocyst transition, NCSU23 was supplemented with 20% calf fetal serum (CFS) for 4 days. Blastocyst formation rates were not statistically different for oocytes matured in NCSU23 (18.9%), Whitten (21.85%) and TCM199 (20%). In addition, there was no difference in hatching rates on Day 7 post insemination (p.i.) and for oocytes matured in NCSU23 (7.14%), Whitten (18.18%) and TCM199 (17.64%), respectively. However, there was a difference in hatching rates 16.12, 34.28 and 32.3% on Day 9 p.i. for NCSU23, Whitten and TCM199, respectively. In conclusion, the three media supported oocyte maturation with subsequent fertilization and embryo development; however, in all three media there was a high penetration rate as well as a high incidence of polyspermy.

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## EFFECT OF VASCULAR ENDOTHELIAL GROWTH FACTOR ON THE DEVELOPMENT OF BOVINE EMBRYOS CULTURED IN SERUM-FREE MEDIA

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In cattle, Vascular Endothelial Growth Factor (VEGF) increases in follicular fluid following the development of the follicle [Einspanier et al., 1999, J Reprod Fertil Abstr Series;23:7). We accordingly presumed that VEGF affects oocyte maturation, and have shown that VEGF has beneficial effects on bovine oocyte maturation and early embryonic development in serum-supplemented media [Luo et al., J Vet Med Sci 2002;64(9) and 64(11), in press]. The objectives of this study were to determine the effect of VEGF on the in vitro developmental competence of bovine oocytes and embryos in serum-free condition and the synergistic effect of VEGF and serum component. To examine the effect of VEGF on the early development in serum-free media during IVM and IVC, recombinant human VEGF 165, obtained from R&D Systems (Minneapolis, MN), was employed at 5 ng/ml. SOF was supplemented with 1% BME-EAA (essential amino acid), 1% MEM-NEAA (non-essential amino acid), 1.5 mM Glucose, 2 mg/ml porcine FSH, 2 mg/ml estradiol-17 $\beta$  and 1% polyvinyl-alcohol (PVA) or 10% fetal bovine serum (FBS) for IVM, and 1% BME-EAA, 1% MEM-NEAA and 1% PVA or 1% FBS for IVC. Bovine COC were matured for 22 h in m-SOF supplemented and fertilized for 6 h in BO solution at 39 °C in an atmosphere of 5% CO<sub>2</sub> in air. Presumptive embryos surrounded by cumulus cells were cultured for 42 h (IVC<sub>1</sub>) in m-SOF at 39 °C in an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. Then, 4- to 8-cell embryos were cultured until Day 8 (IVC<sub>2</sub>) following the removal of surrounding cumulus cells. Each group contained 20  $\pm$  2 presumptive zygotes in 100-ml drop covered by paraffin oil. In treatment groups, VEGF was supplemented during both IVM and IVC<sub>1</sub>, but it was not used during both IVF and IVC<sub>2</sub>. A total of 451 COC was examined in the experiment with six replicates. The data were analyzed by ANOVA. The cleavage and development rates to 4-to 8-cell stage or to blastocysts were significantly high in VEGF groups (Table 1). The increased developmental potency of the oocyte to blastocyst resulted from the improvement of the development from oocyte to 4- to 8-cell embryo. These results indicate that the supplementation with VEGF both in serum-supplemented and serum-free media during IVM and for the initial 42 h during IVC improved the development of bovine embryos in vitro. It is suggested that the beneficial effect of VEGF on the developmental potency of bovine COC is regulated via the cumulus cells, and VEGF acts synergistically with serum component on oocyte maturation and/or embryonic development in vitro. In conclusion, the results of the present study demonstrated that VEGF improves the developmental competence of bovine oocyte and embryo both in serum-supplemented and serum-free media.

Table 1

Effect of VEGF on the development of bovine embryos cultured in serum-free media

Treatment	No. of replicates	No. of oocytes examined	Number (% $\pm$ S.E.) of oocytes developed to		
			$\geq 2$ cell	$\geq 4$ -to 8-cell	Day 8 blastocyst
P	6	111	49 (44.1 $\pm$ 4.3) <sup>a</sup>	35 (31.5 $\pm$ 5.5) <sup>a</sup>	19 (17.1 $\pm$ 6.2) <sup>a</sup>
P + V	6	114	62 (54.5 $\pm$ 1.8) <sup>b</sup>	51 (44.7 $\pm$ 2.5) <sup>b</sup>	26 (22.8 $\pm$ 3.9) <sup>a</sup>
F	6	112	66 (58.8 $\pm$ 3.2) <sup>b</sup>	56 (50.0 $\pm$ 3.7) <sup>b</sup>	36 (32.1 $\pm$ 1.9) <sup>b</sup>
F + V	6	114	79 (69.3 $\pm$ 4.0) <sup>c</sup>	67 (58.8 $\pm$ 4.0) <sup>c</sup>	48 (42.1 $\pm$ 1.8) <sup>b,c</sup>

P: PVA; V: VEGF; P + V: PVA + VEGF; F: FBS; F + V: FBS + VEGF. VEGF was supplemented during both IVM (for 22 h) and IVC<sub>1</sub> (for 42 h, at 48 h Pi). Values in the same column with different superscripts (a, b, c) differ significantly ( $P < 0.05$ ).

EFFECT OF ACTIVATION TREATMENT FOR EQUINE OOCYTES AFTER ICSI  
AND SUBSEQUENT EMBRYO'S FREEZABILITY

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Slow progress in equine IVF/IVP research is due in part to the difficulties in obtaining enough experimental oocytes as the supply of mare ovaries is limited. Recently, intracytoplasmic sperm injection (ICSI) has been applied to equine embryo production, due to low rate of IVF. In this study, we obtained oocytes from 1 day preserved ovaries and examined the effect of oocyte activation treatment after ICSI on fertilization and developmental ability. Furthermore, we tested freezability of ICSI-derived embryos. Mare ovaries were transported to the laboratory within 24 h of slaughter. Oocytes with expanded cumulus cells layers were matured in IVMD101 medium (chemically defined medium; Research Institute for the Functional Peptides Co., Ltd.). After 28 h of in vitro maturation, the oocytes with a first polar body were selected for ICSI. Frozen-thawed spermatozoa were treated with ionophore A23187 (2 min, 0.1  $\mu$ M) and were incubated in HHBSA [Palmer et al., *J Reprod Fertil Suppl* 1991; 44:375–384] containing 1 mM caffeine for 4–6 h at 37 °C. One sperm was injected in the oocyte using piezo micromanipulator. Injected oocytes were activated with A23187 (10 min, 10  $\mu$ M) and incubated in CR1aa containing cycloheximide (6 h, 10  $\mu$ g/ml). After ICSI, oocytes were cultured in CR1aa. At 20 h after ICSI, the oocytes were fixed and stained to evaluate their pronuclear formation. Furthermore, an additional in vitro culture was continued until Day 9 to evaluate developmental ability. Equine embryos were cryopreserved by slow cooling protocol in 10% ethylene glycol (EG) and 0.1 M sucrose [Hochi et al., *Theriogenology* 1996;46:1217–1224]. The effect of oocyte activation treatment after ICSI is shown in Table 1. There were no significant differences on pronuclear formation, cleavage and morula/blastocyst formation rates between activation and non-activation groups. Five morulae and blastocysts (on Days 6–9) were cryopreserved and all of them were survived and developed to the expanded blastocysts after thawing. These results suggest that equine oocytes derived from 1 day preserved ovaries can develop into blastocysts after ICSI and activation treatment after ICSI is not essential for equine embryo production. And ICSI-derived blastocyst stage has a high freezability with 10% EG and 0.1 M sucrose. This study was supported by the Japan Racing Association.

Table 1  
Effect of equine oocyte activation treatment after ICSI

Groups	Pronuclear formation (%)	Cleavage (%)	Morula/blastocyst (%)
Activation	20/26 (76.9)	24/37 (64.9)	5/37 (13.5)
Non-activation	12/20 (60.0)	19/39 (48.7)	9/39 (23.1)

CAPRINE IN VITRO FERTILIZATION UTILIZING CRYOPRESERVED EPIDIDYMAL SPERM FROM A 4-MONTH-OLD TRANSGENIC BUCK

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Transgenic dairy goats offer an economical alternative method for producing large volumes of recombinant molecules for human therapeutic use. Since the generation of transgenic dairy goats by pronuclear microinjection or somatic cell nuclear transfer is a labor-intensive procedure, the ability to quickly and efficiently generate F1 progeny from a founder buck is important. The ability to successfully collect and cryopreserve epididymal sperm from a young buck, prior to collection of ejaculated semen, ensures the ability to propagate F1 offspring. In this study, epididymal sperm was collected and cryopreserved, as previously described [Blash et al., *Theriogenology* 2000;54:899–905] from a 4-month-old F1 buck transgenic for the light immunoglobulin chain of a proprietary therapeutic monoclonal antibody. Ovulated oocytes and aspirated mature COCs were recovered from superovulated does. A single IVF experiment was performed in BO medium supplemented with 20% heat-inactivated estrus goat serum, 7.7 mM calcium lactate plus 6.25 µg/ml heparin. Following IVF, presumptive zygotes were transferred to equilibrated SOF plus 0.8% BSA and allowed to continue development in vitro for 24 h. On Day 2 cleavage was determined, then five 2- to 8-cell embryos were surgically transferred to the oviduct of each synchronized recipient. The remaining embryos were allowed to continue development in vitro to Day 7; then two or three blastocysts were surgically transferred to the uterine horn of each synchronized recipient. Pregnancies were determined by ultrasound on Day 50. In summary, IVF performed utilizing cryopreserved epididymal sperm from a 4-month-old transgenic buck resulted in successful cleavage and blastocyst development. Only surgical oviductal transfer of developing IVF embryos to recipient does on Day 2 established pregnancies. These results suggest a method exists for progeny development from immature transgenic founder male dairy goats (Table 1).

Table 1  
Summary of caprine IVF

Oocyte	<i>n</i>	#Cleaved (%)	#Pregnant/#recipients Day 2 transfers (%)	#Blastocysts (%)*	#Pregnant/#recipients Day 7 transfers (%)
Ovulated	57	53 (93)	7/7 (100)	5 (23)	0/2
Aspirated	18	9 (50)	0/1	0	ND
Totals	75	62 (83)	7/8 (88)	5 (14)	0/2

\*: *n*-Day 2 transfers.

DEVELOPMENT TO BLASTOCYSTS STAGE OF PIG OOCYTES MATURED,  
FERTILIZED AND ELECTROACTIVATED IN VITRO

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Livestock biotechnology uses an arsenal of in vitro reproductive procedures, such as gene transfer, cryopreservation of oocytes and embryos, in vitro production of embryos, embryo sexing, and nuclear transfer. There is tremendous interest in producing large quantities of matured pig oocytes and embryos through in vitro maturation, fertilization and culture (IVM/IVF/IVC) techniques. Because of their physiological similarities to humans, pigs have become increasingly important as potential xenograft donors and as transgenic animals that produce specific proteins. The significant progress achieved in animal cloning by using nuclear transfer has aroused great interest in the artificial activation of eggs of large domestic animals. This study was designed (1) to determine the effectiveness of two in vitro maturation (IVM) media (tissue culture medium TCM 199 (Gibco, Gran Island, NY, USA) and synthetic oviduct fluid supplemented with amino acids [SOFaa]), (2) to compare the effects of two in vitro fertilization (IVF) media (Tris-buffered medium [TBM] and SOFaa) on the developmental competence of pig oocytes, and (3) to test the activation ability of IVM pig oocytes matured in TCM 199 or SOFaa, electro-activated and cultured in SOFaa. Ovaries were collected from prepubertal landrace gilts in a local slaughterhouse. In Experiment 1, SOFaa and TCM 199 media were supplemented with 10 ng/ml epidermal growth factor (EGF; Sigma), 100 IU/ml penicillin G potassium, 0.1 mg/ml streptomycin sulfate, 10% (v/v) heat-treated Fetal cow serum, 10 IU/ml PMSG (Denka Pharmaceutical Co., Kawasaki, Japan), and 10 IU/ml hCG (Denka). In Experiment 2, matured oocytes were fertilized in either TBM or SOFaa medium using frozen-thawed spermatozoa. In Experiment 3, oocytes matured in TCM 199 and SOFaa were electro-activated by a single DC pulse treatment of 1.65 kV/cm for 100 ms and cultured in SOFaa. The percentage data were assessed by ANOVA. Means were compared by a least significant difference test. All percentage data were converted by using arcsine transformation before ANOVA. A probability level of  $P < 0.05$  was considered statistically significant. In Experiment 1, the nuclear maturation rates were similar between IVM media (91.0% versus 89.0%). In Experiment 2, there was no significant difference between SOFaa and TBM in the percentage of embryos with two pronuclei 33.2% versus 13.8% or polypronuclei 5.3% versus 13.4%. The cleavage rate was the same in both media. SOFaa had a significantly higher ( $P < 0.05$ ) blastocyst percentage than TBM (12.7% versus 3.9%). A similar result was obtained when the activation rates were 54.2 in TCM 199 and 56.0% in SOFaa, and the blastocyst rates were 7.9% and 6.1%, respectively. We conclude that SOFaa can enhance in vitro maturation, fertilization and culture of pig oocytes.

## EFFECTS OF DIFFERENT CONCENTRATIONS OF POTASSIUM PHOSPHATE ON THE DEVELOPMENT OF IN VITRO PRODUCED MOUSE EMBRYOS

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It is well known that oviduct cells and potassium in high proportions in the oviduct play a significant role in embryo development. The aim of this study was to investigate the effects of different  $\text{KH}_2\text{PO}_4$  proportions (0.59 mM low, 1.19 mM control, 2.38 mM high) on overcoming the 2-cell block occurring in culture media after in vitro fertilization of oocytes belonging to inbred mice. For this purpose, the present work has evaluated the development of oocytes/embryos from inbred BALB/C mice as a result of culture and co-culture with mouse oviduct epithelium cells (MOEC) in Whitten's media. Experiments replicated eight times, in each study 96, 91, 169, 138, 101, 231, 223, 142 oocytes were collected. In each  $\text{KH}_2\text{PO}_4$  and with co-culture groups, totally 1072 oocytes were used (175 in low, 179 low + co-culture, 182 control, 181 control + co-culture, 177 high, 178 high + co-culture). Oocytes collected from superovulated mice were fertilized for 4–6 h with epididymal sperm and capacitated in TYH (Toyoda, Yokojoma and Hoshi) media for 1.5–2 h. Following fertilization, the presumptive zygotes were divided into six groups; three of the groups were placed in co-culture with MOEC, and three were without co-culture, and all groups were examined every 24 h for a total 96-h culture time. Results are presented in Table 1. After 24 h, no statistically significant differences were found between the groups with respect to cleavage rates. After 48-h cleavage rates increased in all groups, and this increase was higher in the co-culture groups. Also, while the highest rate of reaching the 4–6 and 8-cell stage was seen in the control potassium and control potassium + co-culture groups ( $P < 0.05$ ), no significant difference was observed between the other groups. After 72 h the rate of development to the morula stage was highest in the control potassium and control  $\text{KH}_2\text{PO}_4$  + co-culture groups ( $P < 0.05$ ). The difference between rates of development to the morula stage in the low- and high-potassium groups was found to be significant ( $P < 0.05$ ). At 96 h the highest rate of development of embryos to the early blastocyst and blastocyst stages occurred in the medium containing control  $\text{KH}_2\text{PO}_4$ , followed by the control  $\text{KH}_2\text{PO}_4$  + co-culture groups ( $P < 0.05$ ). It was concluded that different proportions of potassium did not influence the 2-cell block and did not affect cleavage rates. Co-culture with MOEC affected embryo development positively. Low potassium has a more positive effect than high potassium in producing blastocysts.

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Table 1  
Development stages of in vitro fertilized mouse embryos cultured in Whitten's medium with different concentrations of  $\text{KH}_2\text{PO}_4$

	Culture without MOEC				Culture with MOEC			
	Cleavage (%)		Morulae (%)	Blastocyst (%)	Cleavage (%)		Morulae (%)	Blastocyst (%)
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Low	48.0 <sup>a</sup>	60.0 <sup>a</sup>	11.5 <sup>c</sup>	12.4 <sup>c</sup>	57.4 <sup>a</sup>	68.7 <sup>a</sup>	31.6 <sup>c</sup>	16.3 <sup>bc</sup>
Control	55.5 <sup>a</sup>	65.9 <sup>a</sup>	55.3 <sup>a</sup>	30.0 <sup>a</sup>	56.9 <sup>a</sup>	67.9 <sup>a</sup>	44.8 <sup>b</sup>	26.0 <sup>ab</sup>
High	50.8 <sup>a</sup>	61.6 <sup>a</sup>	16.9 <sup>d</sup>	11.0 <sup>cd</sup>	53.4 <sup>a</sup>	62.4 <sup>a</sup>	28.6 <sup>d</sup>	7.20 <sup>d</sup>

Values with different superscripts (a, b, c, d, e) differ significantly in the same column ( $P < 0.05$ ) (Chi-square and Student's *t*-test).

USE OF IN VITRO FERTILIZATION AS A PREDICTOR OF SEMEN QUALITY  
PRIOR TO CERVICAL INSEMINATION IN SHEEPS. Papadopoulos<sup>1</sup>, J.P. Hanrahan<sup>2</sup>, A. Donovan<sup>2</sup>, P. Duffy<sup>1</sup>, M. Wade<sup>1</sup>, M.P. Boland<sup>1</sup>,  
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Artificial insemination (AI) is probably the most important single technique devised to facilitate the genetic improvement of farm animals. The availability of an efficient sheep AI service would yield similar benefits to those achieved in cattle and would greatly enhance the scope for pedigree and commercial breeders to respond to consumer demands. An in vitro assay for determining ram fertility, which could replace field fertility trials, would be of great benefit to AI programs. The objective of this study was to determine if the quality of frozen–thawed ram semen could be effectively evaluated through IVF procedures prior to insemination as a means of improving pregnancy rate. There is little field data available from rams whose frozen semen has been used for cervical insemination in Ireland. However, such data are available from large-scale inseminations in Norway. Experiment 1 was designed to determine if the differences detected between rams at field level in Norway could be accurately identified via IVF evaluation. Frozen semen from Norwegian rams that differed in fertility was imported for IVF. A minimum of 100 inseminations per ram was the criterion for selection for IVF testing. Semen from eight rams was selected for IVF evaluation based on non-return rates achieved at farm level (high non-return rate:  $n = 4$ ; low non-return rate:  $n = 4$ ). Field non-return rates ranged 45.7–73.8%. The parameter examined (IVF score) was the proportion of oocytes cleaved at 48 h post-insemination (hpi). In Experiment 2, in order to eliminate factors such as inseminator effect, farm effect, year of insemination, nutritional effect, and ewe breed, the objective was to determine if differences observed in the IVF scores of rams were reflected in differences in pregnancy rates following cervical inseminations under controlled experimental conditions. Semen from six of the eight Norwegian rams was selected for use in inseminations based on IVF scores (high IVF score,  $n = 3$ ; low IVF score,  $n = 3$ ). Ewes of various breeds ( $n = 90$ ) were synchronized using an intravaginal progestagen pessary (FGA), followed by 500 IU eCG at pessary removal as described above. Cervical inseminations with frozen–thawed semen were carried out at 56–58 h post-pessary removal. Semen from individual rams was balanced across ewe breed. Ewes were slaughtered 28 days later and pregnancy rate determined. Data on non-return rate and pregnancy rate were analyzed using GENMOD procedure of SAS. Data on IVF rate were analyzed using generalized linear model (GLM) procedure of SAS. In Experiment 1, despite significant differences in fertility found at field level, there were no significant differences between rams in IVF values (65.9–82.1% cleavage at 48 hpi). In Experiment 2, in which several factors, such as inseminator effect, farm effect, year of insemination, nutritional effect, and ewe breed were controlled for, significant differences in pregnancy rates were found between individual rams ( $P < 0.02$ , range: 12.9–65.8% pregnancy rate). However, there was no relationship between rams rated as “high” or “low” based on IVF and pregnancy rate. In conclusion, there was no evidence from this study that the pregnancy rate achieved following cervical insemination with frozen–thawed ram semen could be predicted by prior evaluation using IVF.

## HETEROLOGOUS CO-CULTURE OF BOVINE EMBRYOS WITH MOUSE EMBRYOS STIMULATES BLASTOCYST DEVELOPMENT AND DIFFERENTIATION

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The culture of embryos in groups has been found to stimulate in vitro development in a number of mammalian species. However, for most exotic and endangered species, groups of embryos are not common and each embryo is very valuable. This study was, therefore, designed to evaluate the effect of heterologous embryo co-culture in which a single bovine embryo was cultured together with a group of mouse embryos. The effects on blastocyst development and differentiation were evaluated. The use of the bovine embryo was intended as a model for endangered mammals. Bovine ovaries were obtained from a slaughterhouse and antral follicles 2–10 mm were aspirated. The COCs were matured and fertilized in standard conditions. Resultant embryos were cultured in medium G1 for 72 h at which time, 8-cell bovine embryos were randomly assigned into three treatment groups. Treatment I: embryos cultured individually in 30- $\mu$ l drops of medium G2. Treatment II: embryos cultured in groups of four in 30- $\mu$ l drops of G2 (standard bovine incubation ratio). Treatment III: single embryos cultured together with 15 8-cell CF1 mouse embryos in 30- $\mu$ l drops of G2 (standard murine incubation ratio). A total of four bovine 8-cell embryos was used for each treatment in each replicate. Embryos in all three treatments were cultured a further 72 h at 38.5 °C in an atmosphere of 6% CO<sub>2</sub>, 5% O<sub>2</sub>, 89% N<sub>2</sub>, after which blastocyst development, total cell number, and inner cell mass (ICM) cell number were determined. The experiment was replicated 17 times ( $n = 68$  bovine 8-cell embryos/treatment). Data for blastocyst development was analyzed using generalized linear modeling, while data for cell counts were analyzed using one-way ANOVA and Bonferroni's post-test for multiple comparisons. Total blastocyst development was 30% for Treatment I, 41% for Treatment II, and 56% for Treatment III. Development in Treatment I was significantly lower than the other groups ( $P < 0.05$ ). Total blastocyst development between Treatments II and III was not significantly different. The percentage of expanded blastocysts was significantly greater in Treatments II (29/4%;  $P < 0.05$ ) and III (40%;  $P < 0.05$ ) compared with Treatment I (13%), while the percentage of expanded blastocysts in Treatments II and III was not significantly different. Blastocysts in Treatment I had a mean total cell number of  $79.7 \pm 5.6$  and a mean ICM cell number of  $22.2 \pm 2.5$  yielding a mean %ICM of  $27.1 \pm 2.0\%$ . Each of these parameters was significantly lower when compared with Treatment II ( $113.8 \pm 6.8$ ,  $38.0 \pm 2.5$ , and  $34.8 \pm 2.2\%$ ;  $P < 0.01$ ,  $P < 0.001$ , and  $P < 0.05$ , respectively) and Treatment III ( $139.5 \pm 5.6$ ,  $51.9 \pm 2.6$ , and  $36.2 \pm 1.7\%$ ;  $P < 0.001$ ,  $P < 0.001$ , and  $P < 0.01$ , respectively). Total cell number and number of ICM cells were both significantly higher in Treatment III compared with Treatment II, ( $P < 0.01$  and  $P < 0.001$ ) while the %ICM was not different. These data show that culturing bovine 8-cell embryos in the presence of a group of 8-cell mouse embryos produced significantly more blastocysts, and blastocysts of higher quality than those embryos cultured individually. This, therefore, has potential for the culture of small numbers of valuable embryos such as those derived from exotic and endangered species.

# Male Physiology

## THE INFLUENCE OF DIRECT SOLAR RADIATION ON SKIN, SCROTAL AND DEEP BODY TEMPERATURES IN HOLSTEIN BULLS

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Elevated environmental conditions can decrease fertility and milk production in dairy cows, and decrease semen quality and libido in bulls. Cattle gain most environmental heat input in day-light hours through direct or indirect solar radiation [Fuquay JW, J Anim Sci 1981;52(1):164–174]. Under sunny conditions dairy cows with predominantly black skin have higher rectal and skin surface temperatures than cows with mainly white skin [Hansen PJ, Vet Record 1990;127:333–334]. The aim of this study was to assess the influence of direct sunlight and coat color markings on the respiratory rates, skin, scrotal and rectal temperatures of bulls at two locations. Bulls were located at one of two properties (P1 and P2). P1 was located in a valley (130-m altitude; temperature range 2.9–36.6 °C), while P2 is situated on a relatively barren plain above P1 (160 m; 2.5–36.6 °C). Air temperature and relative humidity (RH) were measured using six data loggers ( $\pm 0.1$  °C). Temperatures were measured in 17 mature Holstein sires (5 at P1, 12 at P2; aged 5–9 years) on 17 days when the temperature was  $>30$  °C. Hide temperatures were measured (once the hair was brushed of excess dirt) at the shoulder, flank, rump and leg from a distance of 0.5–1 m with an infrared thermometer ( $\pm 0.5$  °C). This thermometer also measured proximal, mid and distal scrotal temperatures. Core body temperatures were measured with a rectal probe ( $\pm 0.2$  °C). Coat color markings, respiration rates and macro environmental conditions (direct sunlight or cloud) were recorded. During periods of  $>30$  °C, the average temperature at P2 ( $32.3 \pm 0.1$  °C S.E.) was greater ( $P < 0.05$ ) than at P1 ( $31.9$  °C). Conversely, the average RH at P1 ( $26.7 \pm 0.3\%$ ) was greater ( $P < 0.05$ ) than at P2 ( $24.7 \pm 0.5\%$ ). Bulls located at P1 had lower ( $P < 0.001$ ) respiratory rates ( $48.8 \pm 0.6$  breaths/min versus  $53.3 \pm 0.4$  breaths/min) and rectal temperatures ( $38.7 \pm 0.3$  °C versus  $38.9 \pm 0.0$  °C), while having higher ( $P < 0.001$ ) shoulder ( $39.1 \pm 0.3$  °C versus  $36.6 \pm 0.2$  °C), flank ( $38.9 \pm 0.3$  °C versus  $37.9 \pm 0.1$  °C), rump ( $39.2 \pm 0.3$  °C versus  $33.4 \pm 0.1$  °C) and leg ( $33.5 \pm 0.1$  °C versus  $32.2 \pm 0.1$  °C) temperatures than those at P2. The proximal scrotum was hotter ( $P < 0.05$ ) in bulls located at P1 ( $33.1 \pm 0.1$  °C versus  $32.8 \pm 0.1$  °C). There were no differences in mid or distal scrotal temperatures between locations. Respiratory rates increased ( $P < 0.001$ ) when bulls were standing in direct sunlight ( $52.1 \pm 0.2$  breaths/min versus  $50.4 \pm 0.4$  breaths/min). Temperatures at each of the four skin locations were also between 0.3 and 2.8 °C higher ( $P < 0.05$  to  $P < 0.001$ ) in direct sunlight, while rectal temperatures were higher ( $38.9 \pm 0.0$  °C versus  $38.8 \pm 0.0$  °C;  $P < 0.001$ ). In contrast, bulls standing in direct sunlight had lower proximal ( $32.8 \pm 0.0$  °C versus  $33.1 \pm 0.0$  °C;  $P < 0.05$ ), middle ( $32.0 \pm 0.0$  °C versus  $32.3 \pm 0.0$  °C;  $P < 0.05$ ) and distal ( $30.9 \pm 0.2$  °C versus  $31.3 \pm 0.1$  °C;  $P < 0.001$ ) scrotal temperatures than bulls not in direct sunlight. Skin color alone did not influence respiratory rates, scrotal or rectal temperatures ( $P > 0.05$ ). Bulls with black markings at the shoulder, flank, rump and leg displayed higher ( $P < 0.001$ ) skin temperatures at these sites than bulls with white markings in direct sunlight (skin color  $\times$  sunlight). The Holstein bulls exposed to environmental conditions associated with this study appeared able to control scrotal temperatures in response to both elevated air temperatures and direct sunlight that were sufficient to increase respiratory rates, skin and rectal temperatures.

## GLYCOSIDASE ACTIVITY AND FERTILIZING ABILITY IN VITRO BY INCUBATION OF FROZEN–THAWED SPERMATOOZOA IN THE PIG

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This study has evaluated effect of the spermatozoa incubation on the glycosidase activity and fertilizing ability in vitro in the pig. Use of chlortetracycline fluorescence analysis, as well as various glycosidase analyses and the oocyte penetration test showed that glycosidases can affect the fertilizing ability and glycosidase activity of frozen–thawed spermatozoa in vitro. To identify sperm glycosidases specific for sugar residues found in the zona pellucida of pig oocytes, the spermatozoa were treated experimentally and assayed for activities of L-fucosidase, D-mannosidase, D-galactosidase and N-acetyl-D-glucosaminidase (GlcNAc'ase). The glycosidase's activity was higher in spermatozoa incubated for 2 h than without incubation. The GlcNAc'ase activity was at least two-fold higher than other glycosidase regardless of spermatozoa incubation. In the same glycosidases, the activity had a tendency to increase as time of spermatozoa incubation was prolonged, but there were no differences in spermatozoa incubated during the various periods (4, 8, 12, 16, 20 and 24 h). The percentages of spermatozoa that reached acrosome reaction were affected by glycosidases ( $P < 0.05$  for D-mannosidase), and were higher in spermatozoa with that than without incubation. On the other hand, the sperm motility was decreased with incubation periods, but no effects by different glycosidases on the change of sperm motility during the various periods of incubation. In another experiment, the zona binding and penetration of spermatozoa were tested with oocytes matured in vitro. The penetration rates were decreased with incubation of spermatozoa when oocytes were inseminated in fertilization medium with different glycosidases. These rates were higher in spermatozoa non-incubated than with incubation for 2 h ( $P < 0.05$  for GlcNAc'ase). The sperm-zona binding rate in control group was higher than in medium with glycosidases. In addition, the highest binding rate was obtained in medium with GlcNAc'ase among the glycosidases. In all glycosidases, the sperm-zona binding rate in spermatozoa without incubation was higher than incubation for 2 h, and the significant differences were obtained in spermatozoa treated with D-mannosidase ( $P < 0.05$ ). These results suggest that GlcNAc'ase is present mainly in the plasma membrane of pig spermatozoa. It was also shown that the glycosidase activity was increased in spite of low sperm-zona binding and penetration rates by spermatozoa incubation.

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EFFECT OF LACTOFERRIN ON MOTILITY AND CAPACITATION  
OF BOVINE SPERMATOZOA

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Lactoferrin (Lf) that is present in the milk and in several other external secretions has possible roles involved in antimicrobial activity, immunomodulatory anti-inflammatory properties and antioxidant activity. Although reproductive organs such as the seminal vesicles and the prostate secrete Lf, its role on physiology remains unclear. The present study was conducted to demonstrate effect of Lf on motility and capacitation of bovine spermatozoa. The epididymal sperm ( $n = 3$ ) and ejaculated sperm ( $n = 4$ ) suspended in an assay buffer [Accot et al., 1983] containing 33 mM the ophylline and adjusted to 2.5–3.0  $\times 10^7$  cells/ml. They were incubated for 30 min supplemented with Lf (100  $\mu\text{g/ml}$ ) or transferrin (100  $\mu\text{g/ml}$ ), and then sperm motility analysis was carried out by a CASA system (Hamilton–Thorne Research). Second, to examine effect of Lf on sperm capacitation, the suspensions of ejaculated sperm ( $n = 3$ ) were diluted with equal volume of BO medium [Brackett and Oliphant, 1975] containing 20 mg/ml BSA, 5 U/ml heparin and 0–1000  $\mu\text{g/ml}$  Lf, and then incubated for 120 min. Sperm capacitation was monitored by a CTC staining [Fraser et al., 1995]. Comparisons of data were performed with the two-way analysis of variance with no replication and Student's *t*-test. The motility percentage (mean  $\pm$  S.D.) in the specimen of epididymal sperm was quite low in absence of Lf, and after incubation with Lf increased slightly (without versus with Lf,  $12.1 \pm 1.3$  versus  $19.3 \pm 4.1$ ;  $P < 0.05$ ), whereas the percentage of straightness (STR) and linearity (LIN) increased (in STR  $\pm$  S.D.,  $44.3 \pm 8.6$  versus  $71.3 \pm 5.9$ ;  $P < 0.05$ ; in LIN  $\pm$  S.D.,  $23.0 \pm 5.6$  versus  $39.7 \pm 4.0$ ;  $P < 0.05$ ). In the specimen of ejaculated sperm, the motility increased significantly after incubation with Lf ( $81.9 \pm 6.0\%$  versus  $90.2 \pm 6.5\%$ ;  $P < 0.001$ ). Neither of the percentage of STR or LIN was not affected by supplement of Lf. The motility and the characteristic velocity were not increased by addition of transferrin. Although the motility percentage in the specimen of ejaculated sperm was decreased after treated with BSA and heparine but without Lf, the supplementation of Lf improved the motility percentage. By analysis of CTC staining patterns, the ratio of F (uncapacitated state)-typed sperm decreased time-dependently, whereas the ratio of B (capacitated state)-typed sperm increased by supplement with Lf. Our results suggest that Lf may have accelerating effect on motility and capacitation of bovine spermatozoa.

## STUDY OF PRESENCE OF CUMULUS–OOCYTE COMPLEXES ON FUNCTIONAL CHANGES ON ARGENTINE CREOLE PATAGONIC BOVINE SPERMATOZOA

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Detailed studies about gamete physiology are a prerequisite for developing effective assisted-breeding programs for autochthon species. The Argentine Creole Patagonic is descendant of the original cattle brought with the Spanish conquest of the New World. These animals have been adapted to the local environments for around 400 years. At the moment, little information is available about reproduction characteristics and among others, the sperm functionality has not been studied yet. The aim of these experiments was to investigate the effect of presence of cumulus–oocyte complexes on functional changes of Creole spermatozoa using Holstein spermatozoa as control. In six replicates, Creole and Holstein semen was thawed at 37 °C for 30 s and selected by swim-up [Parrish et al. *Theriogenology* 1986;25:591–600] in TCM199 medium supplemented with caffeine-sodiumbenzoate and heparin sodium. The sperm from two breeds was incubated in TCM199 and TCM199 with in vitro mature cumulus–oocyte complexes [First and Parrish, *Reprod Fertil* 1987;34:151–165] for 150 min at 38.5 °C and 5% CO<sub>2</sub>. Every 30 min, tail membrane integrity and the acrosome status were evaluated by hypo-osmotic swelling test (HOS) and by double stain (Trypan blue-Giemsa), respectively. Data were analyzed by analysis of variance. There were significant effects ( $P < 0.01$ ) of presence of cumulus–oocyte complexes on HOS results only in the Holstein spermatozoa, where the values were highest in spermatozoa incubated with cumulus–oocyte complex. However, the percentage of spermatozoa with acrosome reacted was affected by culture time ( $P < 0.01$ ) in both media the same manner in the two breeds studied. Contrary to expected results, no effect of cumulus–ovocyte complexes on acrosome reaction was detected in both breeds. The possible reason for these results could be that (1) in vitro conditions would be not adequate enough to study this process or (2) the staining system is not precise enough if compared with computerized systems as flow cytometry or (3) more probably the effect of heparine supplemented to medium is so high that the effect of glycosaminoglycans produced by the cumulus–oocyte complexes is masked. Anyway, more studies are necessary to determine the semen characteristics of Argentine Creole Patagonic in order to use them under in vitro conditions.

## SEPARATION OF PRESUMPTIVE ROUND SPERMATIDS FROM BULL TESTIS

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Round spermatids can be used for treating severe human male infertility and for developing an approach for transgenic animal production. However, little has been reported on the isolation of the cells from bull testis. The objective of this research was to isolate the cells by Percoll density gradient and to test the effect of the isolation method on survivability and apoptosis of round spermatids from four bull testis. After being minced into small pieces, testicular tissues were incubated in 0.2 mg/ml pronase solution for 2 min and filtered through a 20- $\mu$ m nylon mesh. The cells were then placed onto the top of the 20% Percoll solution and then centrifuged at  $650 \times g$  at 4 °C for 25 min through the gradients (20, 35, 40, 45 and 90% Percoll solution). In Experiment 1, both microscopic observation and DNA analysis by flow cytometry after DAPI (4, 6-diamino-2-phenylindole) staining showed that 34% of cells collected from 35% Percoll gradient were presumptive round spermatids, whereas in 40% Percoll gradient, mostly primary spermatocytes were observed. Experiment 2 compared the effect of the Percoll density isolation on the incidences of cell apoptosis and necrosis to those of untreated cells with the gradient. To evaluate the rates of apoptosis, necrosis and membrane intact, cells were stained with YO-PRO-1 dye and propidium iodide and analyzed by flow cytometry. The rates (mean  $\pm$  S.D.) of apoptosis and early necrosis in Percoll-treated group were significantly lower ( $P < 0.05$ ) than in untreated group ( $1.2 \pm 0.9$  and  $14.2 \pm 7.5\%$  versus  $2.3 \pm 1.6$  and  $25.4 \pm 4.0\%$ , respectively). The proportional incidences (mean  $\pm$  S.D.) of membrane intact in Percoll-treated group were significantly higher ( $P < 0.05$ ) than in untreated group ( $82.9 \pm 9.4\%$  versus  $71.8 \pm 5.3\%$ , respectively). In combination, these results suggest that isolation method of presumptive round spermatid by Percoll density gradient is effective to eliminate apoptotic and early necrotic cells.

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## THE EFFECT OF ALBUMIN AND SODIUM CITRAT ON THE DEVELOPMENT OF IN VITRO PRODUCED BOVINE EMBRYOS

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Beneficial effects of adding of sodium citrate (Na<sub>3</sub>)/bovine serum albumin (BSA) to SOF defined medium to enhance blastocyst development has been reported previously [Theriogenology 2001;55:593–606, and 1999;52:683–700]. The objective was to evaluate the source of BSA and the effect of sodium citrate on CR1 defined culture system that supports repeatable high rate of embryo development. Bovine oocytes with at least 4 layers of cumulus cells were collected from slaughterhouse ovaries, and matured for 20–22 h at 39 °C, 5% CO<sub>2</sub> in humidified air in 7.5% FBS M199 medium supplemented with, 0.5 µg/ml FSH 5.0 µg/ml LH, and 2 µg/ml estradiol. Oocytes were subsequently subjected to in vitro fertilization (IVF) using standard BO fertilization procedure. Six hours after IVF, cumulus cells were removed from oocytes by vortexing. Presumptive zygotes were cultured in 75 µl defined CR1 medium supplemented with MEM and NEA amino acid (CR1aa), and subjected to a 2 × 2 factorial treatment. Two sources of BSA are used at the concentration of BSA of 6 mg/ml, (1) Sigma BSA (Sigma, A-6003, St. Louis, USA), with essentially fatty acid free initially fractionated by cold alcohol precipitation, and (2) ICP BSA (ICP Bio, ABRZ-010, Auckland, New Zealand), a freeze-dried powder irradiated to 2.5 MRad. Embryos were cultured under 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> at 39 °C with high humidity for 2 days, and then cleaved embryos at 4–8-cell stage were transferred to 75 µl CR1aa medium plus 10% FBS under 5% CO<sub>2</sub> in air for subsequent culture of 4.5 days. The number of expanded blastocysts with tighter compaction and the integrity of the inner cell mass (C1) and large blastocysts (C1–2) on day 6.5 was recorded. The data were compared by General Linear Model (GLM, Univariate, SPSS 9.0, 1999, SPSS Inc., Chicago, IL 60606). The results (Table 1) showed that there were no significant differences among four treatments in the rates of cleavage, 8-cell and morula development. However, CR1aa medium with addition of Sigma–BSA and Na<sub>3</sub> significantly increased total blastocyst development compared with ICP–BSA groups (37% versus 19–21%, *P* < 0.05). There was no difference on total blastocyst rate within Sigma–BSA groups (28% versus 37%, *P* > 0.05) and within ICP groups (19% versus 21%, *P* > 0.05), respectively. Likewise, the best development of high-quality embryos (C1 BL) was generated from CR1aa Sigma–BSA medium supplemented with Na<sub>3</sub> (26% versus 11–17%, *P* < 0.05) among other treatments. It demonstrated a synergistic effect of sodium citrate and the system of BSA extraction on in vitro development of bovine IVP embryos. The necessity of factor(s) for promoting competent blastocyst development in cattle is retained in Sigma extraction system, while that of promoting agent(s) appears depleted in ICP irradiated BSA.

Table 1  
Effects of BSA and Na<sub>3</sub> on the development of in vitro produced embryos

Treatment		No.	Cleavage (%)	8-cell (%)	Morula (%)	D6.5 blastocyst (%)	
BSA	Na <sub>3</sub> (mM)					Total BL	C1 BL
Sigma	0.34	818	565 (69)	473 (58)	424 (52)	299 (37) <sup>a</sup>	212 (26) <sup>a</sup>
Sigma	0	790	586 (74)	480 (61)	271 (34)	223 (28) <sup>a,b</sup>	132 (17) <sup>b</sup>
ICP	0.34	406	278 (68)	237 (58)	144 (35)	84 (21) <sup>b</sup>	61 (15) <sup>b</sup>
ICP	0	619	452 (73)	362 (58)	158 (26)	116 (19) <sup>b</sup>	65 (11) <sup>b</sup>

Values with different superscripts within columns are significantly different (*P* < 0.05).

## MITOCHONDRIAL ACTIVITY AND FERTILIZATION POTENTIAL OF FRESH AND CRYOPRESERVED BUFFALO SPERM

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Although in vitro embryo production efficiency has greatly improved in buffalo species, cleavage rate is still poor. Aim of this work was to investigate if the low fertilization rate is related to a poor quality of frozen semen. Fresh and frozen-thawed buffalo sperm was prepared by swim-up procedure and separated into portions, one of which was used to measure mitochondrial activity and a second retained for in vitro fertilization (IVF). Fresh and frozen-thawed semen from a Fresian bull was used as a control. Fertilization ability of both buffalo and bovine sperm was assessed utilizing bovine oocytes as a model. Measurement of mitochondrial activity was achieved by incubating samples for 30 min in culture medium containing 0.5 mM JC-1. Motile sperm samples were immobilized under a fibrin clot made by adding a solution of Thrombin (100 IU/ml) to culture medium containing 20 mg/ml fibrinogen. An Olympus Fluoview confocal microscope, based on an Olympus IX-70 inverted microscope, was used to image single sperm mitochondria. A Kr/Ar laser was used to produce the excitation laser line at 488 nm and emission wavelengths were separated by a 530 nm dichroic mirror followed by analysis in a photo multiplier after further filtering through a 515–530 nm band pass filter (green emission) or a 585 nm long pass filter (red emission). Mitochondrial activity is presented as a ratio (not calibrated). IVF was performed with both fresh and cryopreserved bovine and buffalo sperm. Bovine cumulus–oocyte complexes ( $N = 457$ ) from slaughtered animals, were in vitro matured, fertilized and cultured with our standard procedure [Gasparri et al., *Theriogenology* 2000;54:1537–1542]. All sources of sperm were treated by swim-up and used at a final concentration of  $10^6$  and  $20^6$  ml<sup>-1</sup>, respectively, for bovine and buffalo semen. After 24 h of culture presumptive zygotes were fixed in 4% paraformaldehyde and stained with diamidino-2-phenylindole (DAPI) to assess penetration rate (PR) and cleavage rate (CR) under UV. Ratiometric analysis of mitochondrial activity was performed with fresh, refrigerated for 24 h and frozen semen. Neither bovine semen (ratio fresh:  $1.29 \pm 0.64$ ,  $n = 34$ ; 24 h:  $1.27 \pm 0.79$ ,  $n = 22$ ; frozen:  $1.04 \pm 0.13$ ,  $n = 11$ ) nor buffalo sperm (ratio fresh:  $1.22 \pm 0.78$ ,  $n = 31$ ; 24 h:  $0.85 \pm 0.51$ ,  $n = 21$ ; frozen:  $1.16 \pm 0.14$ ,  $n = 14$ ) was characterized by significant differences between groups (ANOVA). When bovine oocytes were fertilized with bovine sperm no differences in penetration and cleavage rate were observed among fresh and frozen-thawed sperm (PR:  $87.4 \pm 7.5$  versus  $88.9 \pm 7.8$ , respectively; CR:  $82.8 \pm 5.5$  versus  $81.4 \pm 11.2$ , respectively). No differences were observed also between frozen and fresh buffalo semen in penetration and cleavage rate (PR:  $69.4 \pm 9.4$  versus  $79.6 \pm 4.7$ ; CR:  $60.3 \pm 4.2$  versus  $70.5 \pm 7.3$ , respectively). The results suggest that neither the mitochondrial activity nor the fertilization capability of buffalo or bovine sperm is affected by cryopreservation and hence that other factors may contribute to the low success rate in buffalo IVF.

# Microstructural Analysis

## DISTRIBUTION OF HYALURONAN IN THE BOVINE OVIDUCT

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Glycosaminoglycans (GAGs) are present in predictable amounts in the fluid of the oviducts of several species, including the bovine, where they are ascribed influences on sperm function. For instance, the sulfated GAG heparin triggers sperm capacitation while the non-sulfated hyaluronan (HA, hyaluronic acid) is believed to have a protective role against attacks from the female immune response. Bull spermatozoa presents receptors for HA and exogenous HA appears to stimulate sperm motility in vitro. Although HA had previously been localized by immunohistochemistry in the sperm reservoir during the preovulatory stage by our group [Rodriguez-Martinez et al., 1998, *Gametes: Development and Function*, Serono Symposia, Italy, p. 239–274], a detailed screening of its localization along the oviduct during other phases of the estrous cycle is yet missing. The aim of this study was to elucidate the distribution of HA in the bovine oviduct during the estrous cycle. Oviductal tissue was obtained at surgery or immediately post-mortem from Swedish dairy heifers and cows, either during standing estrous ( $n = 14$ ), metaestrous or diestrous ( $n = 13$ ). Immunohistochemistry using biotinylated hyaluronan-binding protein (HABP) was performed on paraformaldehyde-fixed, paraffin-embedded, transversal tissue slides from selected tubal segments: Uterotubal junction (UTJ), isthmus and ampulla. Control slides were either pre-incubated with 50 units/ml of *Streptomyces* hyaluronidase, to specifically degrade HA and indicate the degree of specificity of the labelling, or replacements of the biotinylated HABP by TRIS. Positive HA labeling was localized to the lamina propria of the ampullar (strong immunostaining) and isthmus (weaker immunostaining) segments of the bovine oviduct irrespectively of the stage of the estrous cycle. As for the lining epithelium, positive immunostained cells were seen in the sperm reservoir (UTJ-adjacent isthmus) during standing estrous (pre-ovulation), a staining that disappeared during the luteal phase, to be restricted to the basal lamina. The epithelium of the other segments explored appeared negative for HA, during all stages of the estrous cycle hereby studied. Hyaluronan immunolocalization in the bovine oviduct appears restricted to the epithelium of the preovulatory sperm reservoir, and to the basal lamina and the underlying lamina propria during the estrous cycle.

**ATOMIC FORCE MICROSCOPY OF BOVINE ACROSOME-INTACT AND REACTED SPERMATOZOA; THEIR FINE STRUCTURE AND NUMERICAL ANALYSIS**K. Saeki<sup>1,2</sup>, N. Sumitomo<sup>2</sup>, Y. Nagata<sup>2</sup>, Y. Hosoi<sup>1,2</sup>, K. Matsumoto<sup>1,2</sup>, and A. Iritani<sup>1,2</sup><sup>1</sup>Institute of Advanced Technology, Kinki University, Kainan, Wakayama 642-0017, Japan, <sup>2</sup>Department of Genetic Engineering, Kinki University, Uchita, Wakayama 649-6493

Atomic force microscopy (AFM) provides nanometer-resolved, topographic data images of the natural surface structure of the samples. AFM also indicates a novel three-dimensional (3-D) image-contrast mechanism. In this study, we have used AFM to study topology of bovine frozen-thawed, capacitated and acrosome-reacted sperm heads. Also, numerical analysis of the volumes of sperm heads was carried out using 3-D images stored by AFM scanning. Bovine frozen-thawed semen from a bull was washed with Percoll solution [Saeki et al., *Theriogenology*, 1990]. Washed spermatozoa were then incubated in a medium [m-DM, Saeki et al., *Theriogenology*, 1994] containing 10 µg/ml heparin for 4 h to induce sperm capacitation. They were further incubated with 100 µg/ml lysophosphatidylcholine (LPC) for 15 min to induce acrosome reaction [Parrish et al., *Biol Reprod* 1988]. A portion of the spermatozoa was stained with naphthol yellow S and erythrosin B for light microscopic examination of the acrosomal status [Lenz et al., *Biol Reprod* 1983]. Washed, capacitated and acrosome-reacted spermatozoa were smeared and air-dried. The samples were subjected to observation with AFM (SPI 3800, Seiko Instruments Inc., Chiba, Japan) using dynamic force mode. AFM images of spermatozoa after washing showed their clear shape of acrosomal caps, equatorial segments, post-acrosomal regions and necks. By analysis of 3-D images, the heads measured about 9, 4 and 0.4 µm in length, width and thickness, respectively. Images of capacitated spermatozoa treated with heparin also indicated their complete acrosomal caps. After LPC treatment, most spermatozoa had no acrosomal caps by light microscopy after staining. AFM images of stained acrosome-reacted sperm heads indicated the loss of acrosomal caps, but rough surfaces were detected probably due to dyes attached to sperm surfaces. Areas of median plane of sperm heads were measured by counting pixels of stored image. Areas of acrosome-reacted sperm heads ( $2679 \pm 616$ ) indicated 40% decrease compared with those of intact heads ( $4535 \pm 174$ ,  $P < 0.05$ ). These results indicate that 3-D AFM enables numerical volume analysis of bovine sperm heads as well as observation of fine surface structure without fixation, coating and extensive de-hydration. Shrinkage of sperm heads even after air-drying has been reported [Allen et al., *Mol Reprod Dev* 1996]. Thus, further studies are needed to examine the intact shape and volume of bovine sperm heads by AFM under fully hydrated status.

## KINETICS OF H<sub>2</sub>O<sub>2</sub> AND MITOCHONDRIAL ACTIVITY DURING EARLY EMBRYO DEVELOPMENT IN IN VITRO PRODUCED BOVINE EMBRYOS

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The aim of this research was to determine the kinetics of H<sub>2</sub>O<sub>2</sub> production and mitochondrial activity during early development of in vitro produced bovine embryos with high and low developmental potential. *Methodology*: According to the potential to reach the blastocyst stage, the embryos were grouped as follows: Those that reached the two-cell stage at 32 h post-insemination (hpi) were considered competent, while those that did not, were considered non-competent. Twenty embryos were taken from each group 32, 40, 50, 72 and 100 hpi to be evaluated for H<sub>2</sub>O<sub>2</sub> and mitochondrial function by epifluorescence. Half of the embryos in each pool were incubated with di-hydrorhodamine 123 (DHR) (1 µM) for 15 min, while the other half were stained with JC-1 (5 µM) for 30 min; then the embryos were washed three times with PBS before direct observation through epifluorescent microscopy. Green fluorescent embryos after DHR staining were assumed to be positive for H<sub>2</sub>O<sub>2</sub>. Functional mitochondria as revealed by JC-1 staining were determined by red fluorescent dots. The experiment was repeated three times. Altogether a total of 362 competent embryos and 345 non-competent embryos was evaluated with DHR, while 290 competent embryos and 310 non-competent embryos were evaluated with JC-1. The data were analyzed (mean ± S.D.) between pools (32, 40, 50, 72 and 100 hpi) and between groups (competent versus non-competent), by test of hypothesis comparing *Z* values calculated from the data with *Z* values in the Table. A progressive increase on H<sub>2</sub>O<sub>2</sub> positive embryos was observed with a peak at 50 hpi. Although this tendency was maintained among both groups, there was a significant difference ( $P < 0.05$ ) between the number of positive events in each group: the number of H<sub>2</sub>O<sub>2</sub> positive embryos was greater in the non-competent group at each observation time. There was not significant difference between groups nor between pools on mitochondrial function, but this function was very low in both groups of early embryos. The large number of non-competent embryos showing high H<sub>2</sub>O<sub>2</sub> levels may be related to the cell cycle arrest observed under culture conditions, and it is consistent with poor blastocyst production in delayed embryos. Increased production of H<sub>2</sub>O<sub>2</sub> does not seem to be related to mitochondrial function, and additionally low mitochondrial function does not seem to be a factor involved in the cell cycle arrest. Other investigators have previously reported metabolic rearrangements which may be compatible with the low mitochondrial function observed by us.

## Oocyte Activation

### EFFECTS OF CALCIUM IN THE MEDIUM ON THE ACTIVATION OF PORCINE OOCYTES AFTER THE PUNCTURE USING PIEZO-ELECTRIC ELEMENTS

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Direct exposure of a somatic cell nucleus into the cytoplasm of matured oocytes is an elegant way of nuclear transfer, in that injected donor cells will take some time to undergo nuclear reorganization. "Premature" activation of oocytes at manipulation and reduction of the activity of maturation promoting factor (MPF), however, might abolish this advantage. Calcium, a regular component of medium as inorganic salts, must be a cause of this failure, though calcium itself is important for the health of cells. Thus, in the present study, we tested whether oocytes can maintain metaphase II arrest even after the penetration by an injection pipette for up to 6 h in a calcium-free medium. Cumulus–oocyte complexes (COCs) derived from slaughterhouse ovaries were cultured for 44 h in a modified NCSU 23 (mNCSU 23) medium. The oocytes that had matured to metaphase II were selected after removal of cumulus cells and transferred to a calcium-free TALP-hepes medium supplemented with cytochalasin B. Then the zona pellucida and cytoplasmic membrane were penetrated by the injection pipette using piezo-electric elements. The tip of the injection pipette stayed in the cytoplasm for 5 s. After the manipulation, oocytes were cultured for 2 or 6 h in mNCSU 23 medium either with (2.27 mM) or without calcium. In general, nuclear transfer consists of two manipulations of oocytes, enucleation and nuclear-injection or electro-fusion. Thus, in the next experiments, oocytes received the second penetration 2 h after the first penetration, and were cultured for 4 h. The rates of oocytes at metaphase II after the single penetration were 86.8% versus 94.8% (2 h); 71.7% versus 90.0% (6 h) in the medium with or without calcium, respectively. The differences were significant ( $P < 0.05$ , ANOVA with Fisher's test). The rates at metaphase II at 4 h after the second injection were 40% versus 72.4%, in the medium with or without calcium, respectively. Thus, the difference became greater after the second injection. These results indicate that extracellular calcium in the culture medium augments the chance of oocyte activation after the penetration by an injection pipette, but not in the absence of calcium.

INCREASED PARTHENOGENETIC DEVELOPMENT OF BOVINE OOCYTES  
ACTIVATED WITH A COMBINED PHOSPHORYLATION AND PROTEIN  
SYNTHESIS INHIBITOR

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Oocyte activation is imperative for successful nuclear transfer. Common activation protocols use either protein synthesis or a protein phosphorylation inhibitor in combination with a  $\text{Ca}^{2+}$ -elevating agent. However, these treatments are relatively inefficient at stimulating embryo development. The objective of this study was to assess parthenogenetic development of bovine oocytes activated with a combination of a protein synthesis and protein phosphorylation inhibitor. Oocytes were matured in TCM-199 supplemented with 10% FCS,  $\text{Na}^{2+}$  pyruvate, rEGF, bLH, and bFSH for 22 h at 39 °C in 5%  $\text{CO}_2$  and air. Denuded oocytes were placed in Zimmerman's fusion medium and electrically pulsed (EP, 1.2 kV/cm for 30  $\mu\text{s}$ ). Oocytes were randomly assigned to one of the following activation treatments: (1) TCM-199 plus 10% FCS, (2) 1.9 mM 6-DMAP for 3.5 h, (3) 1.9 mM 6-DMAP +10  $\mu\text{g}/\text{ml}$  cycloheximide (CHX) for 3.5 h, (4) 10  $\mu\text{g}/\text{ml}$  CHX +5  $\mu\text{g}/\text{ml}$  cytochalasin B (CB) for 6 h, or (5) 2.5  $\mu\text{g}/\text{ml}$  cytochalasin D (CD) for 6 h. Following activation, presumptive parthenotes were cultured in CR1aa supplemented with 0.3% BSA in 5%  $\text{CO}_2$ , 5%  $\text{O}_2$ , and 90%  $\text{N}_2$  for 48 h and transferred to CR1aa supplemented with 10% FCS in 5%  $\text{CO}_2$ , for an additional 5 days. Cleavage and blastocyst development were assessed on Day 7. Data were analyzed using GLM ANOVA with Fisher's LSD Multiple-Comparison Test and are summarized in the table below. Data indicate treatment with a combination of 6-DMAP and CHX in addition to EP resulted in significantly higher cleavage, morula and blastocyst development compared to all other treatments examined. Treatment of oocytes with EP combined with either a phosphorylation or protein synthesis inhibitor may not be adequate for optimal parthenote development. The combined treatment to inhibit multiple cell cycle regulators together with EP may be more efficient at improving nuclear transfer development.

Treatment	<i>n</i>	Cleaved (%)	Morula + blastocyst (%)	Blastocyst/cleaved (%)	Total blastocyst (%)
EP	258	6.2 ± 2 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
EP + 6-DMAP	212	51 ± 2 <sup>b</sup>	11.3 ± 0.8 <sup>b</sup>	18.8 ± 1.7 <sup>b</sup>	9.3 ± 0.9 <sup>b</sup>
EP + 6-DMAP + CHX	316	70 ± 2 <sup>c</sup>	32.3 ± 0.8 <sup>c</sup>	31.5 ± 1.7 <sup>c</sup>	22.0 ± 0.9 <sup>c</sup>
EP + CHX + CD	276	58 ± 2 <sup>d</sup>	12.3 ± 0.8 <sup>b,d</sup>	20.0 ± 1.7 <sup>b</sup>	11.5 ± 0.9 <sup>b</sup>
EP + CHX + CB	282	41.3 ± 2 <sup>d</sup>	14.0 ± 0.8 <sup>b</sup>	26.8 ± 1.7 <sup>a</sup>	11.0 ± 0.9 <sup>b</sup>

INFLUENCE OF MULTIPLE ELECTRICAL PULSES ON PARTHENOGENETIC  
ACTIVATION AND SUBSEQUENT IN VITRO DEVELOPMENT OF  
MOUSE OOCYTES

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In mammalian oocytes, the incoming sperm causes an increase in  $\text{Ca}^{2+}$  excitability that provokes a sustained calcium oscillation that is responsible for oocyte activation [Swann K et al., *Int Rev Cytol* 1994;152–183]. However, an electrical pulse can evoke a single intracellular  $\text{Ca}^{2+}$  transient in mature oocytes that induces a transient decline in maturation promoting factor (MPF) activity [Collas P et al., *Mol Reprod Dev* 1995;40:253–258]. The objective of this study is to investigate the influence of multiple pulses on oocyte activation and subsequent parthenogenetic development in mouse oocytes. Oocytes were recovered 14–16 h post-hCG from 6- to 8-week-old C57BL/6  $\times$  CBA F1 mice. The COC were denuded with hyaluronidase. These oocytes were then placed between two 1-mm diameter stainless steel round wire electrodes approximately 1 mm apart and overlaid with HEPES-buffered Chatot, Ziomek and Bavister (CZB) medium. Parthenogenetic activation was accomplished by square electrical direct current pulses delivered by electro-cell manipulator (ECM 2001, BTX, USA). Subsequently, the oocytes were cultured in CZB medium supplemented with 5 mg BSA/ml and 5  $\mu\text{g}$  cytochalasin B/ml for 5 h and then further cultured in CZB supplemented with 5 mg BSA/ml. The number of 2-cell parthenogenetic embryos and blastocysts at Day 1 and Day 5 post-activation was recorded. Four experiments were performed and Pearson chi-square test was used to analyze the data. In the first experiment, 4 different voltage field strengths, i.e. 1.0, 1.5, 2.0, 2.5 kV/cm, were applied as a single pulse of 50  $\mu\text{s}$  duration to the oocytes. However, electro-activation rate was low with 4.1, 19.0, 52.9, and 47.8% developing to 2-cell embryos, respectively. In the second experiment, field strength of 500 V/cm, 30  $\mu\text{s}$  duration was used in combination with 3, 6, 9, 12, 15 pulses. The percentage of oocytes dividing into 2-cell was 30, 75.7, 87.5, 64.8 and 21.7%, respectively and correspondingly 33.3, 39.5, 23.8, 23.6 and 20% of these 2-cell embryos developed to blastocysts. In the third experiment, 2 sets of 6 consecutive pulses of 500 V/cm, 30  $\mu\text{s}$  duration with rest intervals 1, 2 or 3 h between them were performed. The percentage of 2-cell parthenogenetic embryos was 91.3, 89.2, and 88.1%, respectively and correspondingly, the percentage of blastocysts that developed from these parthenogenetic embryos was 30.6, 32.8, and 36.5%. The length of rest interval between the 2 sets of similar electro-activation parameters did not affect the outcome of the experiment. However, 2 sets instead of 1 set of 6 consecutive pulses of 500 V/cm, 30  $\mu\text{s}$  duration significantly increased the activation rate (88.1% versus 75.5%,  $P < 0.05$ ) and blastocyst formation rate was not significantly reduced. In the fourth experiment, 3 sets of 6 consecutive pulses of 500 V/cm, 30  $\mu\text{s}$  duration with rest interval 1 h between them were performed. Compared to the third experiment with 1-h rest interval, there was a significant decrease in the number of parthenogenetic 2-cell embryos (91.3% versus 73.7%,  $P < 0.05$ ) and blastocyst formation rate (30.6 versus 21.4%,  $P < 0.05$ ). The data suggest the combination of multiple pulses of lower field strength would be beneficial to the parthenogenetic activation and subsequent development.

INTRACELLULAR CALCIUM ( $[Ca^{2+}]_i$ ) TRANSIENT INDUCED BY  
MICROINJECTION OF BOVINE SPERM EXTRACT INTO  
HOMOLOGS IVM-OOCYTES

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Oscillating calcium peaks appear to be necessary for oocyte activation during mammalian fertilization. In order to induce embryo development in the absence of a fertilizing spermatozoa, treatments have to be applied that mimic this sperm-mediated event. Recently, soluble sperm factors, extracted from sperm of several mammals, have shown to support parthenogenetic activation and early embryonic development when microinjected into heterolog or homolog oocytes. The ability of unfractionated bovine sperm extract (SE<sub>b</sub>) to induce Ca<sup>2+</sup>-oscillation in a homolog in vitro system has not yet been investigated. The objective of this study was to figure out if intracytoplasmatic injection of SE<sub>b</sub> would support such  $[Ca^{2+}]_i$  transients in bovine oocytes matured in vitro. Ca<sup>2+</sup>-oscillation was evaluated by fluorescence ratios using Fluo-4AM (Molecular Probes, Inc.) loaded oocytes (observation period 20 min). Oscillation pattern was described by amplitude (nM), interval (s) and frequency (n/20 min) of Ca<sup>2+</sup> peaks. Experimental sperm source was prepared by pooling 4 native ejaculates of 4 German Holstein bulls. Following separation of spermatozoa from seminal plasma, sperm concentration was estimated and aliquots were stored frozen (−196 °C) until further use. Prior to injection, SE<sub>b</sub> was prepared by consecutive freeze-thawing and centrifugation of aliquots from the stored sperm pool (final protein concentration: 2 mg/ml; 1.5–2.285 sperm equivalents/injection (25.5 pl)). Bovine cumulus–oocyte complexes collected from abattoir ovaries were matured in vitro (22 h); thereafter cumulus cells were removed (hyaluronidase: 4 min, 3 mg/ml). Preloading (60 min) denuded oocytes (*n* = 84) with Fluo-4AM prior to microinjection (SE<sub>b</sub> resp. sham (Ca<sup>2+</sup>/Mg<sup>2+</sup>-free D-PBS)) enabled individual monitoring of the succeeding Ca<sup>2+</sup>-response. Beside its appearance, we estimated fluorescence brightness of the first polar body (PB, bright–/non fluorescence) before further manipulations were performed. Injection of SE<sub>b</sub> (*n* = 64) resulted in 50% (32/64) activation rate as assessed by measurable  $[Ca^{2+}]_i$ -transients expressing oscillation pattern (versus 0% (0/20) Ca<sup>2+</sup>-oscillation for sham injection (*n* = 20)). Oocytes showing Ca<sup>2+</sup> oscillation in response to SE<sub>b</sub> injection triggered this event in strongly individual patterns (total range: peak amplitude (17.39–849.14 nM); peak interval (71.53–248.08 s), peak frequency (1–16/20 min). Remaining (50%) SE<sub>b</sub> injected oocytes (32/60) did not show any response. While first polar-body appearance had no influence on the occurrence of oscillating  $[Ca^{2+}]_i$ -patterns, PB's brightness under fluorescence optic before SE<sub>b</sub> injection and oscillating  $[Ca^{2+}]_i$  transient thereafter was significantly related (*P* < 0.05). In conclusion, our results demonstrate that SE<sub>b</sub> injection causes  $[Ca^{2+}]_i$ -transients in an oscillating manner in bovine in vitro matured oocytes. Considering that the SE<sub>b</sub> was prepared from a uniform sperm source, we conclude that further studies have to be done to clarify whether differences of oocyte quality and maturational stage are the reason for effects in appearance and individual performance of the  $[Ca^{2+}]_i$ -transients. The role of the fluorescence behavior of the first polar body as a potential indicator for Ca<sup>2+</sup> oscillation induceability by SE<sub>b</sub> also needs further research.

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## LOCALIZATION OF LAP2 $\beta$ IN OOCYTES AND EMBRYOS DURING EARLY DEVELOPMENT IN CATTLE

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Nuclear functions, such as DNA replication, transcription and RNA processing, are highly dependent on nuclear architecture, which is largely determined by the various components of the nuclear membrane. The inner nuclear membrane and nuclear lamina contain unique proteins, including nuclear membrane proteins p58/lamin B receptor, lamin associated polypeptide (LAP)1, 2 etc. The peripheral nuclear membrane proteins include type A and type B lamins. LAP2 $\beta$  belongs to the LAP2 family of nuclear proteins. LAP2 $\beta$  has been isolated and characterized as an inner nuclear membrane protein that binds to lamin B and chromosomes in a phosphorylation-dependent manner; however, its function has been not clarified. In this experiment, we focused on the LAP2 $\beta$  protein and examined the localization of LAP2 $\beta$  in activated or fertilized bovine oocytes. Oocytes were obtained by aspiration of antral follicles. After maturation in SOF for 22 h, the oocytes were denuded and activated by exposure to calcium ionophore (CaA; 5  $\mu$ M for 5 min) or CaA followed by incubation with 6-dimethylaminopurine (2.5 mM for 4 h). Cumulus-enclosed and not denuded oocytes were subjected to IVF. Activated and fertilized oocytes were cultured in SOF for 7 days. They were fixed at various culture periods by 3.5% paraformaldehyde in PBS containing 1% polyvinylalcohol (PVA) and 0.5% Triton X-100. Fixed oocytes or embryos were incubated with anti-LAP2 $\beta$  mouse monoclonal antibody (1:100, overnight), washed and then incubated with FITC-conjugated anti-mouse IgG (1:200, for 1 h) after washing with PBS containing 1% BSA and PVA (PBS-BSA). They were stained with 2.0  $\mu$ g/ml Hoechst 33342 for detection of nuclear DNA by using an epifluorescence microscope. Oocytes at the germinal vesicle stage exhibited staining with LAP2 $\beta$ . As the nuclear envelope was dissolved, LAP2 $\beta$  nuclear epitope became non-detectable. After activation or fertilization, these epitopes reappeared on the interphase nuclear envelope. Both the male and female pronuclei exhibited reactivity with anti-LAP2 $\beta$  antibodies. This expression pattern persisted until the blastocyst stage. As the embryos proceeded through development, LAP2 $\beta$  staining became progressively stronger than that in pronuclear stage oocytes. There were no significant differences between the parthenogenetic and fertilized embryos in LAP2 $\beta$  expression and localization. For the first time we demonstrated that LAP2 $\beta$  was present in mammalian oocytes as well as embryos.

PARTHENOGENETIC DEVELOPMENT OF BUFFALO OOCYTES  
AFTER ELECTRICAL AND CHEMICAL ACTIVATION

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Effective activation protocols that can be used during nuclear transfer experiments in buffalo need to be developed. In this study, effects of various activation treatments on the *in vitro* development of buffalo oocytes were investigated. Slaughterhouse-derived oocytes were matured *in vitro* by routine methods for 22 h. Denuded oocytes with the presence of the first polar body were selected for activation. Oocytes were activated by 2 DC pulses of 2.1 kV/cm for 30  $\mu$ s (1  $\mu$ s apart), 7% ethanol and 5  $\mu$ M calcium ionophore (A23187) with or without subsequent administration of 6-dimethylaminopurine (6-DMAP) for 4 h. Activated oocytes were cultured for 7 days in TCM199 supplemented with 10% FBS. Cleavage and blastocyst development were determined on Day 2 and Day 7 of culture. Day 7 blastocysts were stained by Hoechst 33342 and number of nuclei were recorded (Table 1).

The results indicate that 6-DMAP treatment of electrically or chemically activated buffalo oocytes is the critical step to increase parthenogenetic activation and subsequent development *in vitro*. Blastocyst development of buffalo oocytes activated by ethanol or calcium ionophore combined with 6-DMAP was higher than those activated by electrical pulses.

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Table 1  
Parthenogenetic development of buffalo oocytes after various activation treatments

Treatments	<i>n</i>	Cleavage (%)	Blastocyst (%)	Cell number (mean $\pm$ S.D.)
Electrical pulses	25	11 (44) <sup>a</sup>	0 (0) <sup>a</sup>	0 <sup>d</sup>
Ethanol	54	36 (67) <sup>a,b</sup>	1 (2) <sup>a</sup>	36.0 $\pm$ 0.0 <sup>e</sup>
A23187	56	24 (43) <sup>a</sup>	1 (2) <sup>a</sup>	26.0 $\pm$ 0.0 <sup>e</sup>
DMAP	35	0 (0) <sup>c</sup>	0 (0) <sup>a</sup>	0 <sup>d</sup>
Electrical pulses + DMAP	51	27 (53) <sup>a</sup>	19 (37) <sup>b</sup>	79.3 $\pm$ 4.2 <sup>f</sup>
Ethanol + DMAP	61	50 (82) <sup>b</sup>	34 (56) <sup>b,c</sup>	72.6 $\pm$ 9.6 <sup>f</sup>
A23187 + DMAP	44	34 (77) <sup>b</sup>	26 (59) <sup>c</sup>	81.3 $\pm$ 7.8 <sup>f</sup>

<sup>a,b,c</sup>*P* < 0.05 (Chi-square test); <sup>d,e,f</sup>*P* < 0.05 (one-way ANOVA).

CORTICAL GRANULES DYNAMICS IN BOVINE OOCYTES ACTIVATED  
WITH STRONTIUM, IONOMYCIN AND 6-DMAP

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Artificial activation mimics the events promoted by spermatozoa in oocytes during fertilization, such as cortical granules (CG) exocytosis and cortical reaction. In this way, we sought to evaluate CG dynamics in bovine oocytes treated with different activation protocols. The oocytes were IVM in TCM 199 supplemented with FCS, estradiol, FSH, hCG, pyruvate and kanamicin for 26 h at 38.5–39 °C and 5% CO<sub>2</sub> in air. After that, they were denuded with hyaluronidase, selected for first polar body presence (oocyte in metaphase II–MII) and assigned to one of the following activation treatments: control group (C); 20 mM SrCl<sub>2</sub> for 6 h (S); 5 μM ionomycin for 5 min (I); 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h (D); treatment I followed by S (IS); treatment I followed by D (ID); treatment I followed by S alone for 2 h, then S combined with D for 4 h (which totaled 6 h for S and 4 h for D) (ISD); treatment S followed by I (SI); and treatment S for 2 h and then S combined with D for 4 h (SD). The oocytes were stained with 10 μg/ml *Lens culinaris* FITC conjugated for CG examination and with 10 μg/ml Hoechst 33342 for nuclear evaluation just before (in MII) and 2–6 h after activation. In oocytes, CG distribution was classified in: clusters of CG (cytoplasmic immaturity); CG in transition from the center to the periphery (partial maturity); peripheral CG, located above viteline membrane (maturity); and CG reduction (CG exocytosis). The results in the table below are expressed in oocyte number (percentage) and they were analyzed by chi-square test using SAS ( $P = 0.05$ ). In conclusion, CG exocytosis is dependent upon activation treatment and the treatments S, IS, ID, ISD and SD were more efficient since they showed less peripheral CG and more CG reduction. Furthermore, nuclear maturation is not always followed by cytoplasmic maturation, since in all treatments oocytes with characteristics of immaturity (clusters of CG and CG in transition) were seen. This fact may compromise activation and further embryonic development. Considering that CG can be used as indicative of cytoplasmic maturation, CG staining could be allied to nuclear maturation observation in order to ensure the efficiency of IVM protocols in bovine oocytes.

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Treatment	Clusters of CG	CG in transition	Peripheral CG	CG reduction
MII	2 (8%) <sup>a</sup>	5 (21%) <sup>a</sup>	12 (50%) <sup>b,c</sup>	5 (21%) <sup>c,d</sup>
C	2 (6%) <sup>a</sup>	3 (9%) <sup>a,b</sup>	28 (80%) <sup>a</sup>	2 (5%) <sup>d</sup>
I	4 (9%) <sup>a</sup>	2 (4%) <sup>b</sup>	27 (60%) <sup>a,b</sup>	12 (27%) <sup>c</sup>
S	3 (8%) <sup>a</sup>	6 (15%) <sup>a,b</sup>	9 (23%) <sup>d</sup>	21 (54%) <sup>a,b</sup>
D	5 (14%) <sup>a</sup>	2 (6%) <sup>a,b</sup>	13 (36%) <sup>c,d</sup>	16 (44%) <sup>b,c</sup>
IS	3 (8%) <sup>a</sup>	1 (3%) <sup>b</sup>	8 (21%) <sup>d</sup>	26 (68%) <sup>a</sup>
ID	3 (9%) <sup>a</sup>	3 (9%) <sup>a,b</sup>	7 (21%) <sup>d</sup>	20 (61%) <sup>a,b</sup>
ISD	4 (11%) <sup>a</sup>	4 (11%) <sup>a,b</sup>	6 (16%) <sup>d</sup>	23 (62%) <sup>a,b</sup>
SI	4 (10%) <sup>a</sup>	5 (12.5%) <sup>a,b</sup>	13 (32.5%) <sup>c,d</sup>	18 (45%) <sup>b,c</sup>
SD	2 (6%) <sup>a</sup>	6 (18%) <sup>a</sup>	9 (27%) <sup>c,d</sup>	16 (49%) <sup>a,b</sup>

Values with the same letter (a, b, c, d) are not significantly different ( $P = 0.05$ ).

## STRONTIUM AND BARIUM ACTIVATE IN VITRO-MATURED PIG OOCYTES

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Mouse oocytes are parthenogenetically activated by the exposure to culture medium containing strontium or barium ions or intra-cytoplasmic injection of the divalent cations. The injection of strontium increases in intracellular free calcium concentration ( $[Ca^{2+}]_i$ ) of mouse oocytes. It is known that pig oocytes are parthenogenetically activated by the intra-cytoplasmic injection of calcium ( $Ca^{2+}$ ) chloride, which induces a series of events of oocyte activation, such as exocytosis of cortical granules, meiotic resumption, MPF inactivation, pronuclear formation and embryonic development to blastocysts. Here, we examined the activation events after intra-cytoplasmic injection of strontium ( $Sr^{2+}$ ) or barium ( $Ba^{2+}$ ) chloride into in vitro-matured pig oocytes. Oocyte-cumulus-granulosa cell complexes were dissected from pig ovarian follicles 4–6 mm in diameter. They were cultured in TCM199 containing 10% fetal calf serum and 0.1 IU/ml hMG under an atmosphere of 5%  $CO_2$  in humidified air at 38.5 °C for 45–48 h. After the maturation culture, oocytes were denuded, and those emitting the first polar body (MII oocytes) were microinjected with 8.2–14.1 pl (0.9–1.6% of the oocyte volume) of buffer (20 mM HEPES, pH 7.4) supplemented with  $Sr^{2+}$ ,  $Ba^{2+}$  or  $Ca^{2+}$  to make a final concentration of 0.5–1 mM in the oocytes. These oocytes were further cultured in TALP medium for 6 h and then they were fixed and stained to evaluate the meiotic resumption and pronuclear formation. In order to measure an initial  $Ca^{2+}$  transient by confocal microscopy, oocytes were injected with  $Ca^{2+}$  indicator dye fluo-4 dextran before injection of the divalent cations. Exocytosis of cortical granules was also examined by the staining with FITC-labeled peanut agglutinin after 6 h. To test the developmental ability, injected oocytes were treated with cytochalasin B for 4 h and then cultured up to 168 h in NCSU23 medium. In all experiments, oocytes injected with the buffer were used as the controls. Data were analyzed by ANOVA and Scheffe's mean separation test. Microinjection of  $Sr^{2+}$ ,  $Ba^{2+}$  or  $Ca^{2+}$  induced a rapid elevation of  $[Ca^{2+}]_i$ . The duration of the spike induced by  $Ba^{2+}$  injection was longer than that by  $Sr^{2+}$  or  $Ca^{2+}$  that lasted approximately 1 min. In both MII and control oocytes, cortical granules were located just under the plasma membrane as a bright continuous ring. In the oocytes injected with divalent cations the ring became discontinuous, and patches of cortical granule materials were observed on the oocyte surface. After 6 h of the injection with  $Sr^{2+}$ ,  $Ba^{2+}$  and  $Ca^{2+}$ , 64% (30/47), 71% (36/51) and 86% (42/49) of the oocytes were released from MII-arrest, and 51, 66 and 86% formed female pronuclei, respectively (Table 1). Control oocytes resumed meiosis at a significantly lower rate ( $P < 0.05$ ). After 168 h, 29% ( $Sr^{2+}$ ; 15/51), 29% ( $Ba^{2+}$ ; 15/51) and 51% ( $Ca^{2+}$ ; 26/49) of the injected oocytes developed to the blastocyst stage, while none of the 47 control oocytes developed to blastocysts. These results indicate that intra-cytoplasmic injection of  $Sr^{2+}$  or  $Ba^{2+}$ , like  $Ca^{2+}$ , induces in vitro-matured pig oocytes to release from MII-arrest and leads them to a series of events related with oocyte activation.

Table 1  
Activation of pig oocytes after microinjection of divalent cations

Compound injected	Number of oocytes injected	Number of oocytes resuming meiosis (%)	Number of oocytes having pronucleus (%)
$Sr^{2+}$	47	30 (64) <sup>a</sup>	24 (51) <sup>a</sup>
$Ba^{2+}$	51	36 (71) <sup>a</sup>	34 (67) <sup>a</sup>
$Ca^{2+}$	49	42 (86) <sup>b</sup>	41 (84) <sup>b</sup>
Control <sup>1</sup>	47	2 (4) <sup>c</sup>	2 (4) <sup>c</sup>

Values within a column having different superscripts (a–c) are significantly different ( $P < 0.05$ ).

<sup>1</sup> HEPES buffer.

## OVINE OOCYTES METABOLISM DEPENDING ON FOLLICLE SIZE

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In vitro embryo production requires a previous selection of those gametes which will presumably be able to develop to blastocysts. Follicle size is a visible characteristic that can permit a first selection of the oocytes prior to aspiration, as it seems that follicle size is related to developmental capacity of the oocyte [Otoi et al., *Theriogenology* 1997;48:769–774 in bovine]. The study of oocyte and embryo metabolism can lead us to a better understanding of the phenomena which take place in embryonic development. There are some studies [Rieger et al., *J Reprod Fertil* 1998;112:123–130] which demonstrate a relationship between cleavage rate and oxidative metabolism, indicating that mitochondrial function is related to developmental potential. The aim of the present study was to compare oocytes from different follicle sizes in terms of their metabolism through the pentose phosphate pathway and glycolytic metabolism. Oocytes were obtained from ovaries of adult ewes slaughtered at the local abattoir by aspirating visible follicles, which were classified in three sizes: small follicles (1–3 mm diameter), medium follicles (3–5 mm diameter) and large follicles (>5 mm diameter). A total of 197 good-quality oocytes (complete and compact cumulus, homogeneous cytoplasm) was cultured with radiolabelled substrates for 3 h in a substrate free medium (H-SF) with 5.56 mM [<sup>1-14</sup>C]Glucose (specific activity 55 mCi mmol<sup>-1</sup>; Amersham) or with 5.56 mM [<sup>5-3</sup>H]Glucose (specific activity 17.5 mCi mmol<sup>-1</sup>; Amersham). Groups of two oocytes were cultured in a 4 µl drop placed in the cap of a sterile cryotube with 1.5 ml NaOH 0.1 M, at 37 °C in 5% CO<sub>2</sub> [Rieger D et al., *Reprod Fertil Dev* 1992;4:547–557]. After culture, we added 20 ml of scintillation fluid to the NaOH and disintegrations per minute (dpm) were counted in a liquid scintillation counter (Beckman, USA). The same procedure was made with the same reagents without oocytes to control non-specific counts. Data were analyzed by means of a variance analysis with the General Linear Model (GLM) procedure of SAS<sup>TM</sup> (Table 1). Metabolism of oocytes depending on follicle size (pmol isotope oocyte<sup>-1</sup> h<sup>-1</sup> ± S.E.M.). Results show that the use of glucose through the pentose phosphate pathway (measured with the [<sup>1-14</sup>C]Glucose metabolism) does not change significantly as follicle size increases, though the values tend to decrease with increasing follicle sizes. On the other hand, and even if there are not significant differences in [<sup>5-3</sup>H]Glucose metabolism, the values of glycolytic metabolism tend to increase with increasing follicle size. Our results agree with the changes in the enzyme activities in rat oocytes observed by Tsutsumi et al. [*Mol Reprod Dev* 1992;33:333–337]. They detected that enzymes involved in glycolysis metabolism increased during maturation while pentose phosphate pathway enzymes decreased. As follicles grow, the oocytes inside also grow [Arlotto et al., *Theriogenology* 1996;45:943:956] and acquire developmental capacity. These results show that oocytes from larger follicles may start the maturation metabolism phenomena while oocytes from smaller follicles remain with the metabolic routes of immature oocytes.

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Table 1

Metabolism of oocytes depending on follicle size (pmol isotope oocyte<sup>-1</sup> h<sup>-1</sup> ± S.E.M.)

Radiolabelled substrate	Small follicles	Medium follicles	Large follicles
[ <sup>1-14</sup> C]Glucose	1.54 ± 0.32 <sup>a</sup>	1.35 ± 0.30 <sup>a</sup>	1.06 ± 0.42 <sup>a</sup>
[ <sup>5-3</sup> H]Glucose	0.44 ± 0.07 <sup>a</sup>	0.53 ± 0.08 <sup>a</sup>	0.59 ± 0.17 <sup>a</sup>

# Oocyte Maturation

## MEIOSIS INHIBITION AND REVERSIBILITY WITH DIFFERENT CYCLIN DEPENDENT KINASE INHIBITORS IN BOVINE OOCYTES

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In vivo, bovine oocytes complete their growth period and remain blocked in prophase I, within the still-growing follicle, until they are stimulated to resume meiosis (maturation) after the LH surge. Oocytes for in vitro maturation (IVM) are usually obtained from 2 to 6 mm follicles and immediately resume meiosis when placed in suitable medium. Cyclin-dependent kinase inhibitors (CDKIs), such as butyrolactone I (BL I) and roscovitine (ROS), are able to maintain bovine oocytes blocked in the germinal vesicle stage (GV). Bohemine (BOH), another CDKI, has been used only to activate bovine oocytes. The aim of the present study was to determine whether BOH blocks meiosis and to compare its effectiveness to the other CDKIs, ROS and BL I, regarding meiosis inhibition and subsequent nuclear in vitro maturation in bovine oocytes. Oocytes were aspirated from 2 to 6 mm follicles of slaughterhouse ovaries, and evenly granulated oocytes with more than three layers of compact cumulus cells were selected for use. All cultures were in 100 µl droplets of the appropriate medium under oil, at 38.5 °C and 5% CO<sub>2</sub> in air. Meiosis inhibition medium was composed of TCM 199 + 3 mg/ml BSA and antibiotics added with one of the inhibitors. IVM medium was TCM 199 + 10% FCS, 0.5 µg/ml FSH, 5.0 µg/ml LH and antibiotics. To assess the stage of meiosis, oocytes were fixed (ethanol:acetic acid, 3:1, v/v, for 24 h) and stained with 1% (w/v) orcein. Data from three replicates were analyzed by ANOVA and Tukey's test. In Experiment 1, a 24-h prematuration culture with increasing concentrations of BOH (0, 50, 100 and 150 µM) was evaluated to determine the most effective concentration for blocking meiosis. Meiosis inhibition rates (% of oocytes in GV) were 76.6% (98/128), 81.4% (105/129) and 92.5% (111/120) for 50, 100 and 150 µM BOH, respectively. There was no significant difference between these concentrations and the group of oocytes evaluated immediately after aspiration (100% GV, 105/105). These groups were all superior to BOH-free medium (10.5%, 13/124,  $P < 0.05$ ). In the next experiment, for the comparison of meiosis inhibition, oocytes were treated 24 h with the different inhibitors BOH (100 µM), ROS (25 µM) and BL I (100 µM) remained in GV in 85.3% (99/116), 79.8% (95/119) and 97.4% (112/115) of the cases, respectively. In the control group (no inhibitor) only 19.1% of the oocytes remained in GV stage ( $P < 0.05$ ), the majority (78.2%) of them having reached nuclear maturation (metaphase II # MII). To evaluate reversibility of meiosis inhibition, the oocytes were submitted to IVM for 18 h after the 24-h inhibiting treatments. Control oocytes were only IVM for 18 (C18) or 24 h (C24). The percentage of oocytes reaching MII was greater than 82.5% in all treatments, including controls. The results show that BOH can effectively inhibit meiosis resumption and that the inhibition is equally effective and reversible for the inhibitors tested.

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## EFFECTS OF DIFFERENT SPECIFIC INHIBITORS OF MATURATION PROMOTING FACTOR (MPF) ON BOVINE EMBRYO DEVELOPMENT IN VITRO

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Bovine oocytes used for in vitro maturation (IVM) are usually obtained from 2 to 6 mm follicles, and a large number of them is considered incompetent for supporting embryonic development after in vitro fertilization (IVF) and culture (IVC). Several studies have been performed using meiosis inhibition aiming to provide an additional preparation time for the oocytes before they are submitted to the IVM procedure in an effort to improve the results obtained for in vitro embryo production (IVP). The present study aimed to compare the effects of different cyclin-dependent kinase inhibitors (CDKIs), bohemin (BOH), rocovitine (ROS) and butyrolactone I (BL I), which inhibit MPF activity, on embryo development following IVM, IVF and IVC. Bovine ovaries were obtained from local slaughterhouses and oocytes were aspirated from 2 to 6 mm follicles. Oocytes which were evenly granulated and with more than three layers of compact cumulus cells were selected for use. All cultures were performed in 100 µl droplets of the appropriate medium under oil, at 38.5 °C and 5% CO<sub>2</sub> in air. Prematuration (inhibition) culture was in TCM 199 + 3 mg/ml BSA and antibiotics added with one of the inhibitors for 24 h and IVM was in TCM 199 + 10% FCS, 0.5 µg/ml FSH, 5.0 µg/ml LH and antibiotics for 18 or 24 h. For the experiments, oocytes were placed in prematuration culture with the different inhibitors BOH (100 µM), ROS (25 µM) and BL I (100 µM) to induce meiosis inhibition, which was then reversed by submitting treated oocytes to IVM for 18 h. Control oocytes were only IVM for 18 (C18) or 24 h (C24). After IVM, all groups of oocytes were submitted to IVF (frozen-thawed Percoll gradient selected spermatozoa, co-culture with  $2 \times 10^6$  sperm cells/ml for 18 h in TALP medium supplemented with 2 mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine and 20 µg/ml heparin) and IVC (TCM 199 + 10% FCS and antibiotics) up to the blastocyst stage (Day 8). Data from three replicates were analyzed by ANOVA and Tukey's test. Cleavage rates after 48 h IVC were 77–80% in all groups. Regarding blastocyst development, 20.3% (27/133), 24.2% (33/136), 32.1 (44/137), 43.1% (56/130) and 34.8% (47/135) of oocytes reached the blastocyst stage in BOH, ROS, BL I, C18 and C24 groups, respectively. C18 and C24 were superior to BOH and ROS ( $P < 0.05$ ) and similar to BL I, which was also superior to BOH ( $P < 0.05$ ). The results show that prematuration of oocytes with meiosis inhibitors may either decrease (BOH and ROS) or maintain (BL I) embryo development rates being, therefore, not indicated to be used in the concentrations studied when the aim is to improve developmental rates of IVP embryos.

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## EFFECT OF PROTEIN SUPPLEMENTATION AND INTRACELLULAR CAMP LEVELS ON THE DEVELOPMENTAL COMPETENCE OF IN VITRO MATURED BUFFALO OOCYTES

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Improving the efficiency of buffalo embryos in vitro production (IVP) would help the programs of genetic selection in this species. Previous studies in cattle demonstrated that modulating intracellular cAMP levels during IVM significantly improved oocyte developmental competence [Luciano et al., 1999 MRD 54,86]. The present study was conducted to evaluate the effect of modulating the intracellular level of cAMP during oocyte in vitro maturation (IVM) in the presence of two protein sources (BSA and FCS) on the ability of buffalo oocytes to cleave and develop to the blastocyst stage in vitro. In this experiment cAMP levels were regulated by the combined addition of dibutyl-*c*-AMP (db-*c*-AMP), a cAMP analog that can penetrate intact cell membranes, and of 3-isobutyl-1-methyl-xantine (IBMX), a phosphodiesterase inhibitor. Egyptian buffalo ovaries were collected at the local slaughterhouse and cumulus-oocyte complexes (COCs) were aspirated from 2 to 8 mm follicles in TCM 199 supplemented with 0.5 mM IBMX and 0.1 mM db-*c*-AMP. Only COCs with  $\geq 3$  cumulus cell layers were used for IVM. Basic maturation medium (BMM) consisted of TCM 199 supplemented with 0.1 IU FSH, 0.1 IU LH (Pergovet, Serono) and 50  $\mu$ g/ml gentamycin. COCs were randomly allocated into four experimental groups: A = BMM + 4 mg BSA/ml; B = BMM + 4 mg BSA/ml + 0.01 mM db-*c*-AMP; C = BMM + 10% FCS; D = BMM, 10% FCS + 0.01 mM db-*c*-AMP. Maturation was performed for 24 h at 38.5 °C in 5% CO<sub>2</sub> at high humidity. Matured oocytes were fertilized using frozen thawed buffalo spermatozoa ( $1.5 \times 10^6$  sperm/ml) in CR1aa medium supplemented with 10  $\mu$ g/ml heparin and 10 mM caffeine. After 18 h, oocytes were cultured in TCM 199 medium + 10% FCS + 50  $\mu$ g/ml gentamycin, at 38.5 °C under 5% CO<sub>2</sub> at high humidity. Cleavage, morula, blastocyst, and hatched blastocyst rates were checked on Days 2, 5, 7 and 9 of culture, respectively. Results are shown in Table 1. Addition of db-*c*-AMP and IBMX to aspiration and maturation medium increased cleavage and morula rates irrespective of the kind of protein supplementation. However, blastocyst rate was higher only in the presence of FCS. No effect of cAMP levels, nor of protein supplementation, was observed on the hatching rate. In conclusion, addition of db-*c*-AMP and IBMX to in vitro maturation medium had a beneficial effect on the first stages of embryonic development (cleavage and morula formation) in the presence of either BSA or FCS. Blastocyst development was stimulated only in the presence of FCS while hatching was not affected. This suggests that cAMP modulation with an appropriate protein supplementation during oocyte IVM may lead to an improved efficiency of buffalo IVP but further experiments are required in order to find the optimal combination that can ensure positive results also for the later part of in vitro embryo development.

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Table 1  
Effect of maturation medium of buffalo oocyte developmental competence

IVM group	Matured oocytes (n)	Fertilized oocytes (n)	Cleaved embryos; embryos on Day 2	Morulae on Day 5 (%)	Blastocyst on Day 7 (%)	Hatched blastocysts on Day 9 (%)
A	263	244	62.1 $\pm$ 1.5	55.0 $\pm$ 1.2	19.2 $\pm$ 1.3	4.2 $\pm$ 1.4
B	242	222	77.4 $\pm$ 2.6**	62.6 $\pm$ 2.4*	22.7 $\pm$ 2.4	4.1 $\pm$ 1.5
C	159	149	64.4 $\pm$ 3.7	58.2 $\pm$ 1.1	19.2 $\pm$ 0.2	4.6 $\pm$ 1.6
D	233	226	82.1 $\pm$ 2.6**	66.4 $\pm$ 1.9**	26.0 $\pm$ 0.7**	3.1 $\pm$ 1.8

Results are expressed as mean  $\pm$  S.E.M. Cleavage rate is relative to fertilized oocytes; the other rates are relatively to cleaved embryos. Each experiment was replicated three times and data were analyzed with Student's *t*-test.

\*  $P > 0.05$ .

\*\*  $P > 0.01$ .

THE POTENTIAL ROLES OF COMMUNICATIONS THROUGH GAP JUNCTION  
BETWEEN CUMULUS CELLS AND OOCYTES DURING IN VITRO  
MATURATION OF BOVINE OOCYTES

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Preliminary studies in our laboratory have indicated that modulating cumulus expansion early or late during culture had a profound influence on the subsequent development of enclosed oocytes. One of the routes by which the factors are transmitted from cumulus cells to the oocyte is gap junctional communication (GJC). The function of cumulus cells in in vitro maturation of bovine oocytes was investigated by using gap junction inhibitors, 1-heptanol and 1-octanol. Also, to manipulate the level of cumulus expansion, we evaluated the effect of short-term treatment (first 2 or 6 h) during in vitro maturation (IVM) with recombinant human follicle-stimulating hormone (r-hFSH). The effect of long-term treatment (last 18 h) with r-hFSH during IVM was also studied. cumulus-oocyte complexes (COCs) from ovaries of slaughtered cows were matured for 24 h ( $38.5 < \text{BC}$ , 5%  $\text{CO}_2$  in air with 100% humidity) in modified synthetic oviduct fluid (mSOF) supplemented with MEM nonessential amino acids, MEM essential amino acids, 1.5 mM glucose and 1 mM glutamine. Then, in vitro fertilization took place on droplets composed of modified Tyrode lactate medium for 15–18 h. After fertilization, 1-cell embryos were cultured in mSOF for 8 days under mineral oil in a humidified atmosphere of 5%  $\text{CO}_2$ , 7%  $\text{O}_2$  at  $38.5 < \text{BC}$ . Data from three replicates were analyzed by ANOVA, and multiple comparisons were made with Fisher's LSD test. No cumulus expansion was observed at 24 h when COCs were cultured with r-hFSH for the first 2 or 6 h compared with those cultured for 24 h. A highly significant improvement in blastocyst development rate as a proportion of cleaved oocytes was always noted after IVM of bovine oocytes in the presence of r-hFSH for the first 6 h (48 and 2.3%,  $n = 60$ ) compared to those matured with r-hFSH for the first 2 h (27 and 1.9%,  $n = 67$ ) or 24 h (29 and 2.8%,  $n = 65$ ). Addition of r-hFSH to the maturation medium during the last 18 h only also improved the developmental competence of oocyte as reflected by the blastocysts yield obtained after in vitro fertilization (33 and 4.7%,  $n = 65$ ). When COCs were matured with r-hFSH for the first 6 h followed by 18 h with 1-heptanol or 1-octanol, developmental competence of oocyte was significantly decreased as reflected by the blastocyst yield (17 and 1.0%,  $n = 63$ ; 13 and 2.1%,  $n = 66$ , for 1-heptanol and 1-octanol, respectively). However, when oocytes were incubated initially for 18 h in the presence of these uncoupling agents, then incubated for a further 24 h in maturation medium control (mSOF BSA, modified synthetic oviduct fluid medium containing bovine serum albumin, (8 mg/ml) + r-hFSH), the ability of these oocytes to develop the blastocyst stage was not affected, indicating that treatment with 1-heptanol and 1-octanol was neither cytotoxic nor irreversible (31 and 0.9%,  $n = 58$ ; 27 and 2.5%,  $n = 60$ ; 28 and 1.7%,  $n = 55$ , for Control, 1-octanol and 1-heptanol, respectively). A brief treatment with the weak base, methylamine (15 mM for 30 min before maturation), significantly reversed the inhibitory action of these agents. In conclusion, high development rates of bovine oocytes can be achieved with short exposure to r-hFSH. This effect is believed to be mediated through gap junctions as developmental competence of oocytes is compromised by inhibition of their function.

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## USE OF ANTIOXIDANTS IN THE IN VITRO MATURATION OF BOVINE OOCYTES

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The aim of this investigation was to determine the effect of  $\beta$ -mercaptoethanol (B-ME) or cysteamine (CYS), low-molecular weight thiol compounds, on the development and viability of embryos obtained by in vitro fertilization of in vitro matured. Maturation media were prepared with the following concentrations of B-ME or CYS: 0 (Control), 10, 50, 100, 200, and 400  $\mu$ M, two additives were tested separately. Oocytes were obtained from slaughterhouse ovaries, and those showing compact cumulus and uniform ooplasm were selected. In vitro maturation was performed in 100  $\mu$ l droplets of TCM-199 with BSA (5  $\mu$ g/ml) HCG (100 IU/ml), FSH (0.5  $\mu$ g/ml), estradiol (1.0  $\mu$ g/ml), pyruvate s(0.2 mM), penicillin (70  $\mu$ g/ml) and streptomycin (50  $\mu$ g/ml) and supplemented with specific concentration of antioxidant, in 5% CO<sub>2</sub> in air at 39.0 °C for 24 h. For fertilization, semen was thawed and separated using a Percoll gradient, capacitated with heparin and hyperactivated with PHE. Final semen concentration was adjusted to  $25 \times 10^6$  cells/ml. Oocytes were placed in 100  $\mu$ l droplets of TALP medium with 5  $\mu$ l of semen solution and cultured at 39. °C in a 5% CO<sub>2</sub> atmosphere for 20 h. After fertilization, zygotes were cultured in 100 ml droplets of SOF medium with FCS (2%) and BSA (5 mg/ml) under mineral oil. Cleavage was assessed 40 h after fertilization and expanded blastocyst development on the 7th day of culture. The quality of obtained embryos was assessed by counting total cell number. Statistical analysis was performed using the Chi-square test. There are no significant effect of different B-ME concentrations in cleavage and blastocyst rates ( $P > 0.05$ ) (0  $\mu$ M = 89.9 and 51.1%, 10  $\mu$ M = 88.1 and 40.3%, 50  $\mu$ M = 88.2 and 44.4%, 100  $\mu$ M = 84.3 and 46.6%, 200  $\mu$ M = 88.2 and 38.4%, 400  $\mu$ M = 90.7 and 28.2%); however, the proportion of embryos that developed to the blastocyst stage decreases when the B-ME concentration was increased from 10 to 400  $\mu$ M. No differences were detected ( $P > 0.05$ ) among CIS concentrations in cleavage rate and blastocyst rate (0  $\mu$ M = 81.1 and 47.2%, 10  $\mu$ M = 81.4 and 45.1%, 50  $\mu$ M = 78.2 and 38.6%, 100  $\mu$ M = 80.7 and 47.3%, 200  $\mu$ M = 78.7 and 49.0%, 400  $\mu$ M = 75.1 and 41.9%). The number of cells present in blastocysts obtained was affected by B-ME or CIS concentration. A higher number of cells was found in embryos matured in the 100  $\mu$ M B-ME ( $P < 0.05$ , 0  $\mu$ M =  $89 \pm 30.7$ ; 10  $\mu$ M =  $98 \pm 27.8$ ; 50  $\mu$ M =  $09 \pm 32.1$ ; 100  $\mu$ M =  $110 \pm 31.6$ ; 200  $\mu$ M =  $69 \pm 26.9$ ; 400  $\mu$ M =  $59 \pm 22.6$ ) and in the 100  $\mu$ M CYS media (0  $\mu$ M =  $99 \pm 21.0$ ; 10  $\mu$ M =  $119 \pm 23.9$ ; 50  $\mu$ M =  $125 \pm 25.1$ ; 100  $\mu$ M =  $129 \pm 36.8$ ; 200  $\mu$ M =  $103 \pm 31.9$ ; 400  $\mu$ M =  $103 \pm 29.3$ ). In conclusion, the use of B-ME and CYS at 100  $\mu$ M during oocyte maturation does not raise the blastocyst rate, though the number of blastocyst cells is higher, therefore showing a greater embryo viability.

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## OOCYTE QUALITY IN REPEAT-BREEDER DAIRY HEIFERS AND EFFECTS OF OPU ON REPRODUCTIVE PERFORMANCE

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Repeat-breeder heifers (RBH) deviate from normal considering hormonal interplay during estrus, with effects on estrus and ovulation [Båge et al., *Theriogenology* 2002;57:2257–2269]. This study evaluated the effect of repeated ovum pick-up (OPU) on RBH characteristics. The aims were to study (1) effects of OPU on estrous cyclicity, follicle development and oocyte recovery rate, (2) IVM and IVF competence and oocyte morphology after IVM, and (3) carry-over effects on fertility. Ten Swedish red and white-breed heifers were used: defined RBH, ( $n = 5$ , average age 3.5 years,  $\geq 3$  AIs with returns to estrus within normal inter-estrous intervals) and virgin heifers (VH, Controls,  $n = 5$ , average age 2.7 years). Oocytes were retrieved by OPU performed twice weekly during 4–6 consecutive estrous cycles. OPU was restricted to the first 12 cycle days, starting after spontaneous ovulation of the pre-ovulatory follicle had occurred. A total of 269 RBH oocytes and 174 VH oocytes was collected. Oocytes were quality scored prior to conventional IVM and IVF. Ten immature and 10n IVM oocytes from either category were fixed for morphology. Nuclear and mitochondrial changes after IVM were studied by laser confocal microscopy (LSM 510, Zeiss, Oberkuchen, Germany) after staining with propidium iodide and Mitotracker Green (Molecular Probes, Leiden, The Netherlands). Ultrastructure was studied by TEM (JEM-1230, JEOL Ltd., Akishima, Japan). At first estrus after OPU, the heifers were artificially inseminated. Plasma progesterone concentrations were analyzed by RIA (Coat-A-Count<sup>®</sup>, Diagnostic Products Corporation, Los Angeles, CA) on cycle D0 (onset of standing estrus), 6, 12 and 16, plus D21 and 23 after AI. Pregnancy was confirmed by ultrasonography 30 days after AI. Frequency distributions were compared by Chi-square test, and a Mann-Whitney test or Wilcoxon signed rank test was used for group-wise comparisons (Minitab software, Minitab<sup>®</sup> Release 10.2, Minitab Inc., PA). During OPU, RBH progesterone concentrations were higher ( $P < 0.05$ ) on D0, but lower ( $P < 0.01$ ) on D6, 12 and 16 compared to VH. Pre-ovulatory follicle diameter was greater ( $P < 0.05$ ) at ovulation in RBH. Estrus intensity and inter-estrous intervals were equal in RBH and VH, and unaffected by OPU (NS). There was no depletion of follicles over time ( $P < 0.01$ ) in RBH or VH. At quality scoring, more ( $P < 0.05$ ) oocytes were classified as good (Grades 1 and 2) for VH (55%) than for RBH (42%). Cleavage rates after IVF were equal (NS) in RBH (33%) and VH (34%). Degree of chromatin condensation in immature oocytes (before GVBD) and after IVM (MI or MII) was similar in RBH and VH (NS), but spatial distribution of mitochondria and cortical granules after IVM was less advanced in RBH. Pregnancy rates after AI were comparable to field fertility (RBH 2/5, VH 4/5). In conclusion, an OPU schedule with physiological ovulation, CL formation and a third, unpunctured follicular wave does not change RBH characteristics concerning progesterone concentrations and pre-ovulatory follicle development. Correspondingly, control heifers were unaffected by this OPU regime. While nuclear maturation after IVM and cleavage rates after IVF were similar in RBH and VH, RBH showed a sub-optimal cytoplasmic maturation in vitro and the expected fertility after controlled AI.

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## TEMPORAL EFFECT OF IGF-I ON NUCLEAR AND CYTOPLASMIC MATURATIONS IN EQUINE OOCYTES

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The ability of fertilized oocytes to develop depends upon normal nuclear and cytoplasmic maturations. Both are essential for an oocyte to develop the capacity for fertilization and normal embryonic development [Eppig, *Reprod Fertil Dev* 1996;8:485–489]. Insulin-like growth factor-I (IGF-I) plays an important role in *in vivo* maturation [Carneiro et al., *Theriogenology* 2002;58:685–688] and has a positive effect on nuclear and cytoplasmic maturation of equine oocytes [Carneiro et al., *Biol Reprod* 2001;65:899–905]. For an oocyte to achieve developmental competence, nuclear and cytoplasmic maturation must proceed in a highly integrated fashion [Albertini et al., *Serono-Symposia USA series* 1993;3–21]. The temporal effects of IGF-I on the progression of meiosis were examined in equine oocytes. Nuclear maturation, by the assessment of metaphase-II formation and polar body extrusion, and cytoplasmic maturation, based on the cortical granule (CG) density measured by quantifying the presence in the center versus the periphery of the oocyte [Carneiro et al., *Theriogenology* 2002;57:363], were determined at 24, 30, and 48 h after the onset of maturation. In mammalian oocytes, the migration of CG is an important step in cytoplasmic maturation and has been used as a criterion in the assessment of maturity and organelle organization [Damiani et al., *Mol Reprod Dev* 1996;45:521–534]. Equine cumulus–oocyte complexes (COCs) were recovered by aspiration and subsequent scraping of the follicles from ovaries excised from euthanized adult cycling mares at the Veterinary Medical Teaching Hospital (UC Davis, California). Compacted COC were *in vitro* matured in M-199 + 0.4% BSA (maturation medium), supplemented (treatment group) or not (Control group) with 200 ng/ml IGF-I, according to Carneiro et al. [*Biol Reprod* 2001;65:899–905]. Nuclear maturation data were analyzed by the Chi-square test and cytoplasmic maturation based on cortical granule migration by ANOVA. Exposure to IGF-I in serum-free maturation medium significantly ( $P < 0.05$ ) increased the frequency of oocytes with extruded first polar body (PB) and metaphase-II formation at 24 h (8/32 versus 15/30;  $P = 0.04$ ) and 30 h (9/30 versus 22/36;  $P = 0.01$ ), but not at 48 h (18/31 versus 20/34;  $P = 0.09$ ) of maturation in the treatment group compared to the Control, respectively, whereas no significant effects were observed in cytoplasmic maturation between groups. These results indicate that the progression of meiosis in equine COC is temporally affected by the exposure to IGF-I in serum-free maturation medium without apparent effects on cytoplasmic maturation based on cortical granule migration kinetics. Equine COC matured in the presence of IGF-I achieved nuclear maturation earlier than Controls. This apparent unbalance of synchronicity between nuclear and cytoplasmic maturation might have an important implication on fertilization and embryonic development. Further studies are necessary to understand the mechanisms associated with such effect.

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BOVINE OOCYTES MATURED IN VITRO WITH RETINOIC ACID AND ETHANOL  
BECOME BLASTOCYSTS WITH ALTERED DIFFERENTIAL CELL COUNTS

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Retinoids, especially retinoic acid (RA), lead to cell differentiation and are important factors in the oocyte development [Duque et al., Hum Reprod 2002, in press; Mohan et al., Biol Reprod 2002;66:692–700]. Ethanol decreases RA synthesis by inhibiting alcohol-dehydrogenase, and therefore outlines the effects of exogenous RA when used as a solvent. The present work analyzes the effect of 9-*cis*-RA and ethanol during in vitro maturation (IVM) on the trophectoderm (TE) differentiation in Day 7 and Day 8 embryos. Cumulus–oocyte complexes (COCs) from slaughterhouse ovaries matured for 24 h in TCM199 with FSH, LH, E<sub>2</sub> and PVA at 39 °C and 5% CO<sub>2</sub> in air. Treatments during IVM were: (1) 9-*cis*-RA 5 nM; (2) 1% ethanol (vehicle); (3) no additives; and (4) 10% FCS. Zygotes were cultured in Synthetic Oviduct Fluid, and nuclei from TE and inner cell mass (ICM) were counted in expanded and hatched blastocysts [Van Soom et al., Mol Reprod Dev 1996;45:171–182]. Embryos were incubated in trinitrobenzenesulfonic acid and in rabbit antiserum, and then in guinea-pig complement. After staining and ethanol fixation fluorescence micrographs revealed groups of red (stained with propidiumiodide and bisbenzimidide, Hoechst 33342) and blue (stained with bisbenzimidide) spots representing TE and ICM nuclei, respectively. Data were analyzed by ANOVA and Duncan's multiple range test. Ethanol in IVM gave blastocysts with high ICM and low TE counts and, as a consequence, increased proportions of cells in the ICM. Besides ethanol, and although ICM and TE counts were unaffected, increased ICM proportions were observed with 9-*cis*-RA. Late expanding embryos (Day 8) were responsible for most of differences observed, while Day 7 embryos only differed between 9-*cis*-RA and ethanol for ICM counts ( $P < 0.05$ ; data not shown). The controlled manipulation of the retinoid pathway could lead to blastocysts with appropriate ICM/TE ratio, and contribute to gain knowledge of bovine stem cells, which are relevant goals in reproductive biotechnology (Table 1).

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Table 1

Single (Day 8) and cumulative (Day 7 + 8) cell counts in blastocysts (means  $\pm$  S.E.M.)

	Group	N	Number of cells		Percentage	
			ICM	TE	Total	ICM/total
Day 8	9- <i>cis</i> -RA	21	40.0 $\pm$ 2.7 <sup>x</sup>	53.5 $\pm$ 3.9 <sup>a</sup>	93.5 $\pm$ 5.2 <sup>a</sup>	43.1 $\pm$ 2.3 <sup>x</sup>
	Ethanol	12	62.1 $\pm$ 8.1 <sup>y</sup>	40.7 $\pm$ 4.8 <sup>b</sup>	111.9 $\pm$ 7.0	63.5 $\pm$ 4.3 <sup>y</sup>
	Control (–)	22	30.8 $\pm$ 4.5 <sup>z</sup>	63.8 $\pm$ 5.0 <sup>c</sup>	94.6 $\pm$ 7.3 <sup>a</sup>	31.5 $\pm$ 3.1 <sup>z</sup>
	FCS	9	49.7 $\pm$ 5.1 <sup>xy</sup>	69.3 $\pm$ 3.6 <sup>c</sup>	119.0 $\pm$ 7.2 <sup>b</sup>	41.1 $\pm$ 2.3 <sup>xz</sup>
Day 7 + 8	9- <i>cis</i> -RA	33	38.1 $\pm$ 2.3 <sup>a</sup>	60.9 $\pm$ 3.6	99.0 $\pm$ 4.5 <sup>a</sup>	38.9 $\pm$ 1.9 <sup>x</sup>
	Ethanol	28	5.1 $\pm$ 5.0 <sup>b</sup>	57.5 $\pm$ 4.3 <sup>a</sup>	116.3 $\pm$ 5.8 <sup>b</sup>	50.0 $\pm$ 3.3 <sup>y</sup>
	Control (–)	38	34.2 $\pm$ 3.5 <sup>a</sup>	68.0 $\pm$ 3.4 <sup>b</sup>	105.0 $\pm$ 5.3	33.6 $\pm$ 2.5 <sup>z</sup>
	FCS	22	49.4 $\pm$ 2.8 b	67.8 $\pm$ 4.1 b	117.2 $\pm$ 1 <sup>b</sup>	42.4 $\pm$ 2.1 <sup>xz</sup>

Superscripts in the same column within Day 8 or Day 7 + 8 embryos express significant differences: (a, b, c):  $P < 0.05$ ; (x, y, z):  $P < 0.01$ .

## EFFECTS OF MEIOSIS-ACTIVATING STEROL ON PORCINE CUMULUS-OOCYTE COMPLEX MATURATION, FERTILIZATION AND PRONUCLEUS FORMATION

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Follicular-fluid-meiosis-activating sterol (FF-MAS) has been isolated from the follicular fluid of several species including man. FF-MAS affects the quality of in vitro oocyte maturation, since the developmental potential of oocytes exposed to FF-MAS during in vitro maturation is improved. The aim of the present study was to investigate how FF-MAS affects porcine in vitro maturation and pronucleus formation. Porcine cumulus-oocyte complexes (COCs) were isolated from abattoir gilt ovaries and in vitro matured 48 h in NCSU 37 medium supplemented with 1 mg/l cysteine, 10 ng/ml epidermal growth factor and 50 µM 2-mercaptoethanol with or without 10% porcine follicular fluid (pFF). For the first 22 h 1 mM db-cAMP and 10 IE PMSG/hCG were added. Both media were supplemented with 0.3, 10.0, 30.0 or 100.0 µM FF-MAS dissolved in ethanol. After maturation the COCs were denuded and in vitro fertilized with fresh semen ( $5 \times 10^5$  spermatozoa/ml) for 3.5 h [Laurincik et al., 1994b]. The presumptive zygotes were evaluated 18 h after fertilization for pronucleus formation. Data from five replicates were analyzed by Chi-square with GraphPad Prism 3 (Table 1).

FF-MAS did not affect maturation, fertilization or polyspermy rates in the presence of pFF, whereas polyspermy rate and pronucleus formation decreased with increasing FF-MAS concentrations. In the absence of pFF, FF-MAS decreased dose dependently the M-II rate and decreased the polyspermic rate. In the absence of pFF, FF-MAS reduced the polyspermy rate and accelerated the pronucleus formation. The block in meiotic progression observed in the absence of pFF at high FF-MAS concentrations is difficult to explain; some yet unknown components of the pFF may interact with FF-MAS to overcome this block. The effect of FF-MAS was most significant in the absence of pFF, where the polyspermy rate was reduced from approximately 19–5% and the pronucleus formation increased from 6 to 17%. In conclusion, it appears that FF-MAS benefits porcine in vitro maturation in the absence of pFF. The FF-MAS concentration should be selected carefully, since at concentrations higher than 30 M, FF-MAS has a negative influence on maturation rate as well as pronucleus formation. Future studies should address in more details the effects of FF-MAS on porcine COCs at the lower micromolar range in the absence of pFF.

Table 1

Effects of FF-MAS on porcine COC in vitro maturation, fertilization, polyspermy and pronucleus formation

Treatment	Concentration of FF-MAS (µM)	Total no. of COCs	Number (% of total) matured	Number (% of total) fertilized	Number (% of total) polyspermic	Number (% of total) pronuclei
pFF + FF-MAS	0	188	188 (100)	120 (64)	41 (22) <sup>a</sup>	81 (43) <sup>a</sup>
pFF + FF-MAS	3	110	110 (100)	73 (66)	22 (20) <sup>a</sup>	39 (35) <sup>ac</sup>
pFF + FF-MAS	10	96	95 (99)	61 (64)	32 (33) <sup>b</sup>	25 (26) <sup>bc</sup>
pFF + FF-MAS	30	104	101 (97)	70 (67)	36 (35) <sup>b</sup>	30 (29) <sup>bc</sup>
pFF + FF-MAS	100	50	48 (96)	27 (54)	14 (28) <sup>ab</sup>	12 (24) <sup>bc</sup>
pFF + FF-MAS	0	48	46 (96) <sup>a</sup>	14 (29) <sup>a</sup>	9 (19) <sup>a</sup>	3 (6) <sup>ab</sup>
pFF + FF-MAS	3	67	64 (96) <sup>a</sup>	19 (39) <sup>a</sup>	3 (5) <sup>b</sup>	11 (17) <sup>a</sup>
pFF + FF-MAS	10	71	61 (86) <sup>a</sup>	17 (24) <sup>a</sup>	3 (4) <sup>b</sup>	11 (15) <sup>a</sup>
pFF + FF-MAS	30	59	41 (69) <sup>b</sup>	5 (9) <sup>b</sup>	4 (7) <sup>ab</sup>	0 (0) <sup>b</sup>
pFF + FF-MAS	100	40	18 (45) <sup>c</sup>	4 (10) <sup>b</sup>	2 (5) <sup>ab</sup>	0 (0) <sup>b</sup>

Different letters within each column indicate statistical difference ( $P < 0.05$ ) by chi-square.

**OOCYTE PRODUCTION IN NON-HUMAN PRIMATE: EFFECT OF THE COMBINATION OF PRIOR IN VIVO HORMONAL TREATMENT AND IN VITRO CULTURE ON THE MATURATIONAL COMPETENCE**

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The production of a large number of mature oocyte from non-human primate species has been reported difficult due to the low efficiency of in vitro maturation and the effect of nonbreeding season [Lefevre et al., *Pathol Biol* 1990;38:166–169; Smith et al., *J Reprod Fertil* 1978;54:91–95]. The study to control the oocyte production has been the essential question in the developing of the research using in these species [Schramm and Bavister, *Human Reprod* 1996;11:1698–1702]. We present in this study the attempt to combine the prior in vivo hormonal treatment and the in vitro culture for long duration of 48 h to maximize the number of mature oocytes produced in cynomolgus monkeys (*Macaca fascicularis*). Adult female monkeys were distributed into three treatment groups: (1) injection of human recombinant follicle stimulating hormone (r-hFSH, Serono, Aubonne, Switzerland, 35 IU per day, intramuscularly) for 10 days and an injection of human chorionic gonadotropin (hCG, 1000 IU, intramuscularly) 34 h before oocyte collection (G.I); (2) injection of r-hFSH (35 IU per day) for 4 days and an injection of hCG (1000 IU) 34 h before oocyte collection (G.II); (3) injection of r-hFSH (6 IU per day) for 4 days and an injection of hCG (1000 IU) 34 h before oocyte collection (Grade III). Cumulus–oocyte complexes (COCs) were collected by follicular aspiration using 18-gauge needles and then cultured at 39 °C in a water-saturated CO<sub>2</sub> incubator with 5% CO<sub>2</sub> in TCM-199 medium (Gibco) supplemented with, estradiol-17β (1 µg/ml), FSH (0.5 µg/ml), LH (0.1 µg/ml) and 5% heat-treated monkey serum collected from animal in estrus. The number of oocytes with the presence of the first polar body was recorded at the moment of collection, 24 and 48 h after collection. For observation, the COCs with expanding cumulus were denuded immediately after collection, COCs with no expanding cumulus cells were denuded after culture for 24 h. The results are presented in Table 1. From these data, it can be concluded that the FSH treatment at low dose and short duration of 4 days can be applied to produce mature oocytes under conditions of IVM for 48 h. However, the rate of oocytes matured in this case was significantly lower than that obtained in monkeys receiving treatment of a high dose of FSH for 10 days (31–52% versus 81%).

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Table 1  
Effect of prior hormonal treatment and in vitro culture on the production of mature oocyte

Group	No. of animals treated	No. of animals responded (%)	No. of oocytes collected/animal (mean ± S.E.M.)	No. of oocytes observed	% (No.) of oocytes with PB [0 h]	% (No.) of oocytes with PB [24 h]	% (No.) of oocytes with PB [48 h]
I	25	24 (96)	18.8 ± 3.1 <sup>a</sup>	451	53.5 (241)	68.9 (311)	81.3 (367)
II	6	6 (100)	13.3 ± 3.0 <sup>a</sup>	80	0 (0)	21.1 (17)	52.0 (42)
III	14	14 (100)	5.6 ± 3.2 <sup>b</sup>	78	0 (0)	9.0 (7)	31.8 (25)

Different superscripts with the same column differ significantly ( $P < 0.05$ ).

## EFFECTS OF MIDKINE ON THE GENE EXPRESSION IN BOVINE CUMULUS CELLS

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We previously reported that midkine (MK) added to in vitro maturation (IVM) medium of bovine cumulus-enclosed oocytes (CEOs) enhances the developmental competence of the oocytes to the blastocyst stage after in vitro fertilization via its direct action to the surrounding cumulus-granulosa cells [Ikeda et al., *Biol Reprod* 2000;63:1067–1074]. The present study was conducted to identify mRNAs which are more abundant in MK treated cumulus cells than in untreated control cells. Cumulus cells were obtained by mechanical stripping from CEOs collected from antral follicles (2–5 mm in diameter) in slaughterhouse-derived bovine ovaries. Groups of  $8 \times 10^4$  cumulus cells were cultured in 50  $\mu$ l drops of IVM medium (synthetic oviduct fluid containing 2% (v/v) BME amino acid solution, 1% (v/v) MEM non-essential amino acid solution, 0.5 mg/ml polyvinyl alcohol, 1  $\mu$ g/ml estradiol-17 $\beta$ , and 100 IU/ml human chorionic gonadotropin) with or without (control) 200 ng/ml recombinant bovine MK for 24 h in 5% CO<sub>2</sub> in air at 39 °C. After the culturing, cumulus cells were frozen and stored until mRNA extraction. mRNA were extracted from pooled cumulus cells ( $3 \times 10^6$ ) in both groups and subjected to PCR-based suppression subtractive hybridization followed by differential screening with four kinds of probes, i.e. subtracted cDNA of MK treated cumulus cells, subtracted cDNA of control cumulus cells, unsubtracted cDNA of MK treated cumulus cells, and unsubtracted cDNA of control cumulus cells. Ninety putative positive clones were obtained from subtracted cDNA of MK treated cumulus cells. Differential screening on the clones revealed that only 24 clones among them were true positive. Preliminary DNasequence analysis identified genes that shared high homology with sequences of proteins with unknown functions (ovarian cancer related protein; OVN6-2, a hypothetical protein; FLJ10652) and that seemed to encode novel proteins. We are currently studying the characterization of these clones and their potential association with the enhancing effect of MK upon the oocyte competency via surrounding cumulus-granulosa cells.

## ULTRASTRUCTURAL AND DEVELOPMENTAL COMPARISON OF PORCINE OOCYTES MATURED IN TCM-199 AND MSOF

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The objective of this study was to examine the effects of in vitro maturation (IVM) media on the distribution of cellular organelles in the cytoplasm and development of porcine oocytes. Ovaries of prepubertal gilts were collected in a local slaughterhouse and immature porcine cumulus–oocytes complexes (COCs) aspirated from follicles of diameter ranging from 2 to 6 mm were cultured in BSA free both media supplemented with 10% pFF, 10 ng/ml GF, 10 IU/ml PMSG and 10 IU/ml hCG for 22 h and cultured without hormone for another 24 h. After 46 h IVM, cumulus cells and corona cells were denuded by pipetting. By Hoechst staining, maturation rate was examined and a total of 40 oocytes with polar body in each group was examined after 48 h IVM for transmissible electron microscopy (TEM). The equatorial section of each oocyte ( $N = 15$ ) was examined for lipid droplets, mitochondria, smooth endoplasmic reticulum (SER) complexes, and cortical granules. After IVF & IVC [Funahashi et al., Biol Reprod 1996;54:1412–1419], the developmental competence was examined (four replicates). The electron micrographs were analyzed by morphometry [Cran, J Reprod Fertil 1985;74:237–245] and data were analyzed by PROC-GLM in SAS program statistically. All percentage data were subjected to arc sine transformation before statistical analysis. Results are expressed as least squares mean  $\pm$  S.E.M. (mSOF versus TCM-199). There was not a significant difference in  $M \pm$  maturation rate (%) between the two groups ( $84.6 \pm 1.8$  versus  $80.5 \pm 2.0$ ,  $P > 0.05$ ). The porcine oocytes matured in TCM-199 have higher electron dense and significantly larger lipid droplets (volume fraction (%):  $0.02 \pm 0.2$  versus  $0.09 \pm 0.1$ ,  $P < 0.05$ ) than those matured in mSOF. However, the number per unit volume of lipid droplets was not different significantly ( $0.15 \pm 0.2$  versus  $0.10 \pm 0.2$ ,  $P > 0.05$ ). There was no significant differences in the number per unit volume ( $0.12 \pm 0.2$  versus  $0.12 \pm 0.2$ ,  $P > 0.05$ ) and the volume fraction ( $0.05\% \pm 0.3$  versus  $0.07\% \pm 0.3$ ,  $P > 0.05$ ) of mitochondria. In addition, SER-mitochondria-lipid droplets complexes (SER complexes) were well developed in oocytes matured in TCM-199, and the distribution of cortical granules was not different significantly between two groups. There was no significant differences in the cleavage rate (%) ( $77.1 \pm 3.5$  versus  $60.1 \pm 3.9$ ,  $P > 0.05$ ). The 7-day expanding blastocyst rate was higher ( $16.4 \pm 6.1$  versus  $8.1 \pm 3.1$ ,  $P < 0.05$ ) in oocytes matured in mSOF group. In this study, we report that compared to mSOF, porcine oocytes matured in TCM199 contained larger lipid droplets and well-developed SER complex in the cytoplasm, and mSOF could be employed for porcine IVM medium to study lipid droplets of porcine oocytes.

## EFFECT OF SPHINGOSINE-1-PHOSPHAT ON IN VITRO MATURATION OF PORCINE OOCYTES

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Sphingosine-1-phosphate (S1P, Sigma) is one of the sphingolipid metabolites that affects a variety of cellular processes including the proliferation, differentiation, growth, survival, migration and gene expression. The present study was performed to investigate the effect of S1P on nuclear maturation of porcine oocytes. In vitro maturation frequency of porcine oocytes was compared in three different medium groups: group I: NCSU23 + 0.1% PVA, group II: NCSU23 + 10% porcine follicular fluid (PFF), and group III: NCSU23 + 10% PFF + 10 ng/ml EGF + 2.5 mM 2-mercaptoethanol. Each group containing 0.1 mg/ml cysteine was divided into four sub-groups of S1P concentration (0, 50, 500 and 5000 nM). Porcine COC were incubated in each maturation medium supplemented with hormones (10 IU/ml PMSG and 10 IU/ml hCG) for 22 h and then further cultured in the same medium without the hormones for 22 h. After completion of in vitro maturation, the oocytes were examined for nuclear maturation by using a basic fuchsin (Pararosaniline, Sigma) staining method. Maturation rates of embryos among experimental groups were analyzed by Student's *t*-test and Duncan's multiple range test using General Linear Models procedure in SAS. In group I, when porcine oocytes were incubated in 0 nM (control), 50, 500 and 5000 nM S1P concentration, frequency of metaphase II chromosome was 45.4 (30/66), 66.7 (54/81), 56.6 (26/46) and 48.7% (37/76), respectively. Thus, 50 nM S1P group showed a higher maturation rate than control group ( $P < 0.05$ ). In group II, maturation rate of porcine oocytes was also higher in 50 nM S1P concentration (83.5%, 116/139) as compared to control group (62.7%, 42/67;  $P < 0.05$ ). No difference was observed in the nuclear maturation of porcine oocytes in group III. Thus, S1P is not effective on the nuclear maturation of pig oocytes under the maturation medium containing EGF because growth factors such as GF stimulate sphingosine kinase and then intracellular S1P levels increase. When sphingosine kinase inhibitor (*N,N*-dimethylsphingosine, Sigma), which blocks generation of intracellular S1P, treated at a concentration of 0, 10, 50 and 100 nM, maturation rate of the oocytes was 47.0 (14/60), 19.4 (20/103), 24.5 (23/94) and 23.3% (14/60), respectively. These results indicate that maturation of porcine oocytes is partially arrested by sphingosine kinase inhibitor. It has been generally known that S1P acts as a mitogenic mediator in the cell by activating MAP kinase and cyclin-dependent kinase. Thus, our findings also suggest that S1P plays an important role in the nuclear maturation of porcine oocytes.

ACTIVITY OF MATURATION PROMOTING FACTOR (MPF) AND MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) DURING IN VITRO MATURATION OF EXPANDED OR COMPACT HORSE CUMULUS-OOCYTE COMPLEXES

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The aim of this work was to investigate maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPk) activity levels during in vitro maturation of horse oocytes derived from expanded or compact COCs. Cumulus-oocyte complexes (COCs) recovered from the horse follicles can be classified into two categories according to their cumulus expansion. Different studies comparing meiotic competence of horse oocytes show that a higher rate of maturation in vitro was achieved by those derived from expanded COCs, although cumulus expansion in the horse, as in other species, is strongly associated with follicle atresia. The progression of meiosis is regulated by the fluctuation of kinase activities of two factors: MPF and MAPk. The kinetic of MPF and MAPk activities during meiosis has been documented for a variety of mammalian species and it has been shown that the MPF activity is related to oocyte quality. The COCs recovered from ovaries of slaughtered mares were selected on the basis of their cumulus morphology and divided into two groups: expanded and compact. Both groups were matured in TCM 199 supplemented with FCS (10%), FSH (0.1 IU/ml), LH (0.1 IU/ml) at 38.5 °C, 5% CO<sub>2</sub> in air. COCs were collected from the culture system, stripped from the cumulus cells, evaluated for nuclear configuration by Hoechst staining and stored (1 oocyte/vial) at -70 °C according to the maturation stage, pending MPF and MAPk assays. Only oocytes that reach the maturation stage specific to the analyzed times (GV, MI, A-T, MII collected at 0, 6, 18 and 24 h, respectively for expanded cumulus group and at 0, 6, 24 and 30 h, respectively for compact cumulus group) were used in this experiment. MPF and MAPk activity (15 oocytes/meiotic stage in three replicates) was measured respectively by histone H1 kinase and myelin basic protein kinase assays according to the method described by Naito et al. (1992). The kinase activities of both MPF and MAPk were quantified by measuring the density of the relative bands in the autoradiographic film with a densitometer. Data were analyzed using GLM (Minitab). Results showed that the kinetics of both MPF and MAPk activities were similar during meiotic progression between oocytes from expanded and compact COCs. MPF level was low at GV stage, rose during GVBD and chromosome condensation until MI, dropped to basal levels during A/T transition and increased again at MII. MAPk level was low at GV stage, increased during GVBD and remained high through progression to MII. At MI and MII stages, oocytes from the expanded COCs group showed higher kinase activity levels of MPF compared to the compact group. If the activity of the MPF is arbitrarily assumed to be 100 in each meiotic stage of expanded COCs derived oocytes, those from the compact group showed 70% of this activity ( $P < 0.01$ ). Using the same relative quantification, MAPk activity was lower (65%,  $P < 0.01$ ) in MI, A-T and MII oocytes from compact COCs compared to those derived from the expanded ones. This study evidenced that oocytes collected from expanded COCs showed higher activity of the factors regulating the cell cycle compared to those derived from compacted ones that could be an index of a more adequate cytoplasmic maturation and meiotic competence of expanded COCs.

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RELATIVE MESSENGER RNA ABUNDANCE IN BOVINE OOCYTES COLLECTED  
AT THE LH SURGE AND MATURED IN VITRO OR THEIR  
COUNTERPARTS MATURED IN VIVO

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In the cyclic cow, final maturation of the ovulatory follicle is initiated by the preovulatory LH surge. During the subsequent 24-h period the oocyte nucleus undergoes meiotic progression to metaphase II, and several changes in cytoplasmic organization take place. We have previously shown that oocytes recovered at the time of the LH peak and matured in vitro are less competent to reach the blastocyst stage than their counterparts recovered 20 h later following in vivo maturation, despite both groups undergoing IVF and culture in parallel [Rizos et al., *Mol Reprod Dev* 2002;61:234–248]. The objective of this study was to compare, using real-time quantitative RT-PCR, the relative abundance of various developmentally important gene transcripts in these oocytes. The groups used were mature bovine oocytes originating from (1) preovulatory follicles punctured by ovum pick-up just before the LH surge (40 h after prostaglandin administration, i.e. immature, [Vos et al., *Theriogenology* 1996;45:329]) and matured in vitro, or (2) preovulatory follicles punctured 20 h later, just prior to ovulation (following GnRH administration at 40 h, i.e. in vivo matured). We examined the relative mRNA expression of five enzymes involved in protection against free oxygen radicals (mitochondrial Mn-superoxide dismutase, MnSOD, cytosolic Cu/Zn superoxide dismutase, Cu/ZnSOD,  $\gamma$ -glutamyl-cysteine transferase, GCS, glutathione peroxidase, GPX, sarcosine oxidase, SOX), a transcript involved in follicular development (growth differentiation factor-9, GDF-9), transcripts involved in glucose metabolism (glucose-6-phosphate dehydrogenase, G6PDH, glucose transporter type 1 and 8, Glut-1, Glut-8) and genes involved in cell cycle events, Cyclin A and B and poly(A) polymerase (PAP). Data on mRNA expression were analyzed using one-way repeated measures ANOVA. Differences of  $P < 0.05$  were considered significant. Transcripts for all genes were detected, irrespective of oocyte origin. Transcripts for GDF-9 were expressed at significantly higher levels in oocytes recovered at the LH peak and matured in vitro than in those matured in vivo. For all other genes, while differences existed between the groups they did not reach significance. The observations with GDF-9 are interesting as this gene is believed to be essential for normal folliculogenesis and may be important in the regulation of early follicle and oocyte growth. The results of this study demonstrate differences in the relative mRNA abundance of several developmentally important gene transcripts in bovine oocytes which may be related to developmental competence. In particular, there is a relationship between GDF-9 transcript abundance and oocyte developmental competence. It would seem that, while GDF-9 is important during folliculogenesis, low levels of GDF-9 transcripts in the mature oocyte may be indicative of high developmental competence.

## EVALUATION BY ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY (ESEM) OF PIG OOCYTES MATURED AND FERTILIZED IN VITRO

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In this study our objectives were to get to know more about the pig oocyte in vitro maturation and fertilization. Oocytes and eggs were observed under scanning electron microscopy. Environmental scanning electron microscope technology (ESEM) examination of truly wet specimens at room temperature using low-beam accelerating voltage and current was made. An installed reservoir takes care of the steam injection to the chamber. In this technique a Gaseous Secondary Electron Detector is used. The ability to image materials without coating or other preparation also allows much faster sample throughput. The microscope's flexibility and nondestructive operation makes it ideal for living cells examination.

Cumulus–oocyte complexes were harvested from slaughterhouse ovaries, evaluated and matured in modified TCM199. Forty five to fifty oocytes were transferred into 4-well plates, containing 500  $\mu$ l of culture medium. After the completion of the maturation oocytes was denuded by vortexing, 25–30 oocytes were placed in 50  $\mu$ l drops of mTBM medium covered with oil. Preparation of spermatozoa was carried out by thawing of two 0.25 ml straw washed in 8 ml TCM199 by centrifugation for 2 min, 2000 rpm. The pellet resuspended in 40  $\mu$ l mTBM, and later on 50  $\mu$ l of semen was added to drops of oocytes to give the final concentration of  $2 \times 10^5$  cells/ml. Fertilization was performed at 38.5 °C for 4 h. Oocytes and presumptive zygotes were examined by ESEM. No preparation is necessary of the samples. No beam damage was detected during the examination. The dynamical examination would be realized by changing the parameters of the sample chamber (pressure, temperature). Magnification up to 50,000 times—with resolution guaranted to 100 Å—is possible in such environments. Microphotographs of oocytes and eggs were taken under a Philips XL 30 ESEM microscope. The samples were examined between 1 and 30 kV voltage and between 1 and 26.6 mbar pressure. The first step is to find the circumstances, where oocyte membranes, polar bodies or spermatozoa look appropriate. The best results would be to get oocytes 20–30 kV 6 mbar at low spot size (3) and sperm cells 5 kV and 13 mbar at high spot size (6). In zygotes it was a big problem to see the oocytes and spermatozoa together. TCM 199 medium that we use the maturation is good for examination but the mTBM, the fertilization medium, is not suitable to examination because this medium evaporates too quickly. It was necessary to put the zygotes from mTBM to TCM 199 medium. Mineral oil was extremely disagreeing to ESEM evaluation.

The results suggest that ESEM represent one of the most exciting breakthroughs in electron microscopy since invention of the electron microscope. This instrument has opened doors to an unexplored world of microscopic phenomena previously unattainable with the traditional S.E.M. In conclusion we described methods allowing us to know more about the oocyte and sperm cell final morphology. Our aim in the near future is to try to get to know more about the morphological difference of in vitro and in vivo developed embryos.

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## CDC2 KINASE AND MAP KINASE ACTIVITY IN NUCLEOLATED AND ENUCLEOLATED PORCINE OOCYTES

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During oocytes growth, rRNAs are synthesized in the nucleolus and stored in the cytoplasm. When oocytes attain their full size, rRNA synthesis ceases although prominent nucleoli are present in germinal vesicles (GV). The nucleoli disappear before germinal vesicle breakdown. The role of nucleoli in the process of oocyte maturation has not been elucidated yet. In this study, enucleolated and enucleated porcine oocytes at GV stage were cultured in vitro and changes in Cdc2 and MAP kinase activities, which regulate oocyte maturation, were examined. In Experiment 1, porcine oocytes at GV stage were collected from 4 to 6 mm ovarian follicles, and denuded from cumulus cells. They were treated with 5.0 µg/ml cytochalasin B for 10 min and centrifuged (6500 µg for 10 min) for moving lipid droplets to one side of the oocytes. Then they were enucleolated by micromanipulation and cultured for 30 h in TCM199 containing 10% fetal calf serum and 0.1 mg/ml sodium pyruvate at 38.5 °C and 5% CO<sub>2</sub> in air. Enucleolated and sham-operated oocytes were fixed at 0, 6, 12, 18, 24, and 30 h after culture, and the nucleus status was examined after staining with 1% aceto-orcein. The data were analyzed by Chi-square test. All of the oocytes were at the GV stage before culture. After 12 h, 46% of enucleolated oocytes underwent germinal vesicle breakdown, and matured up to metaphase II at 30 h in the similar time course as sham-operated oocytes (Table 1). Although 36% of enucleolated oocytes reached to metaphase II, 32% were still at metaphase I. In Experiment 2, denuded GV oocytes were prepared as in Experiment 1. The oocytes were enucleolated or enucleated by micromanipulation, and cultured for up to 30 h. Some enucleolated and sham-operated oocytes which had the first polar body, and enucleated oocytes which showed normal morphology were selected after culture for 30 h, then activated by single electric pulse (100 µs, 1500 V/cm) in medium containing 0.3 M mannitol, 0.05 mM CaCl<sub>2</sub>, 0.1 mM MgSO<sub>4</sub> and 0.01% PVA, and further cultured for 8 h. Oocyte samples were collected at various time intervals during maturation and after electro-activation, and stored at -80 °C before kinase assay. Activities of Cdc2 and MAP kinases of oocytes were measured by phosphorylation of histone H1 and myelin basic protein as in vitro substrates using [ $\gamma$ -<sup>32</sup>P] ATP. Activities of Cdc2 and MAP kinases increased after 12 h of maturation in all enucleolated, enucleated and sham-operated oocytes, and kept high until 30 h. After electro-activation, Cdc2 and MAP kinases in all three groups of oocytes were inactivated. These results indicate that the porcine GV-oocytes without nucleolus are able to mature up to metaphase-II stage with a similar activation pattern of Cdc2 and MAP kinases when compared to sham-operated or enucleated oocytes after electro-stimulation.

Table 1  
The effect of enucleolation upon maturation of porcine oocytes

Oocyte type	Culture time (h)	Total no. of oocytes (n)	MI <sup>1</sup> (%)	MII <sup>1</sup> (%)	2SP <sup>1</sup> (%)	Degeneration (%)
Sham operated	12	30	20 (64) <sup>a</sup>	0 (0)	0 (0)	2 (7) <sup>a</sup>
Enucleolated	12	22	10 (46) <sup>ac</sup>	0 (0)	0 (0)	9 (39) <sup>b</sup>
Sham operated	30	31	1 (3) <sup>b</sup>	15 (48)	7 (23)	2 (6) <sup>a</sup>
Enucleolated	30	28	9 (32) <sup>c</sup>	10 (36)	2 (7)	7 (25) <sup>ab</sup>

Data are pooled from three replicates. Different superscripts within columns denote significant differences ( $P < 0.05$ ).

<sup>1</sup> MI: metaphase I, MII: metaphase II, and 2SP: 2 spindles in an oocyte.

CORTICAL GRANULE TYPES AND NUCLEAR STAGE OF BOVINE OOCYTES  
AFTER EXPOSURE TO ELEVATED TEMPERATURE DURING MATURATION

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Reduced developmental competence of heat-shocked oocytes after the resumption of meiosis [Edwards JL and Hansen PJ, *Biol Reprod* 1996;55:340–346] may be due to alterations in nuclear or cytoplasmic maturation. Objective of this study was to examine cortical granule types and nuclear stage of bovine oocytes matured at an elevated temperature. COC were matured at 38.5 °C for 24 h (Control), 41 °C for first or last 6 h of maturation (HS0-6 and HS18-24, respectively), 41 °C for first or last 12 h of maturation (HS0-12 and HS12-24, respectively), or 41 °C for 24 h (HS0-24). After 24 h of maturation, COC were denuded; number recovered, number lysed, and number having a discernible polar body were recorded. Zona pellucidae were removed (0.5% pronase) and oocytes fixed (3% paraformaldehyde) before staining with 10 µg/ml lens culinaris agglutinin-FITC and 0.5 µg/ml Hoechst 33342. Proportion of oocytes with typical versus atypical nuclear morphologies and cortical granule types I (aggregates), II (aggregates with some dispersion), and III (all granules dispersed) were recorded. Data were collected from a randomized block design and analyzed using mixed models of SAS (2001) after testing for normality. Culture of maturing oocytes at 41 °C for up to 24 h did not alter the proportion recovered, lysed, or having an intact membrane. However, exposure of oocytes to 41 °C for 24 h reduced ( $P < 0.04$ ) proportion having a discernible polar body but did not alter proportion progressing to metaphase II (MII; Table). In fact, greater than 70% of oocytes not having a discernible polar body were arrested at MII (70.7 versus 74.8, 75.8, 81.6, 82.2, and 80.8, respectively for control, HS0-6, HS0-12, HS12-24, HS18-24, and HS0-24, respectively; S.E.M. = 6.4). Moreover, culture of oocytes at 41 °C altered distribution of cortical granules (Table). In conclusion, reduced developmental competence of heat-stressed oocytes may be due to specific alterations occurring in the cytoplasm during maturation.

Temperature (°C)	Duration of heat shock (h)	No. of oocytes	Polar body	MII (%)	CG <sup>a</sup> type I (%)	CG type II (%)	CG type III (%)
38.5	0	208	64.6	82.5	1.9	44.0	54.1
41.0	0–6	221	58.4	83.9	0.9	30.7	68.4
41.0	0–12	205	54.2	86.9	0.5	17.5	81.9 <sup>c</sup>
41.0	12–24	207	59.1	89.5	1.0	19.3	79.6 <sup>c</sup>
41.0	18–24	210	66.0	88.0	0.5	33.3	66.2
41.0	0–24	239	38.3 <sup>c</sup>	83.9	ND <sup>b</sup>	7.9 <sup>c</sup>	92.1 <sup>c</sup>
SEM			6.2	3.2	0.8	7.9	7.7
<i>P</i> -value			0.03	0.60	0.66	0.01	0.02

<sup>a</sup> CG: cortical granule.

<sup>b</sup> ND: none detected.

<sup>c</sup> Differs from other groups.

ROLE OF ESSENTIAL AND NON-ESSENTIAL AMINO ACIDS CONTAINED  
IN MATURATION MEDIUM ON BOVINE OOCYTE MATURATION AND  
SUBSEQUENT FERTILIZATION AND EARLY EMBRYONIC  
DEVELOPMENT IN VITRO

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In vitro milieu during oocyte maturation may affect subsequent embryonic development. However, it is unclear whether the presence of amino acids in maturation medium could influence oocyte nuclear and cytoplasmic maturation, subsequent fertilization and early embryonic development. The objective of this study was to investigate the effect of amino acids on bovine oocyte maturation, subsequent fertilization and embryonic development in vitro. The basic medium for oocyte maturation is a chemically defined protein-free medium supplemented with 0.3% polyvinylpyrrolidone (PVP), 75 mIU/ml FSH and LH. Bovine ovaries were collected from a local abattoir and brought into laboratory. Cumulus–oocyte complexes (COC;  $n = 1198$ ) were aspirated from follicles (2–8 mm in diameter) and randomly assigned to four groups for maturation in culture: (1) Basic medium alone (Control); (2) Basic medium supplemented with 2% MEM essential amino acids solution (GIBCO; 50X); (3) Basic medium supplemented with 1% MEM non-essential amino acids solution (GIBCO; 100X); (4) Basic medium supplemented with 2% MEM essential amino acids solution + 1% MEM non-essential amino acids solution. COC were incubated in 1 ml maturation medium in an Organ culture dish (Falcon; 60 mm × 15 mm) at 38.5 °C under an atmosphere of 5%CO<sub>2</sub> and 95% air with high humidity. After 24 h of culture, some oocytes were fixed to determine maturation rate, and the remaining oocytes were used for in vitro fertilization (IVF). Frozen semen pooled from five bulls was used for IVF. A final sperm concentration was  $1 \times 10^6$  sperm/ml and five oocytes were placed in each 50 µl drop. Following 18 h of insemination, some oocytes were fixed and examined for fertilization and the remaining oocytes were further cultured as described previously [Chian et al., *Reprod Bio Med*, 2000;4:129–134]. The statistical significance of the differences between the means of the groups was determined and compared by analysis of variance with the use of the Newman–Keuls test. There were no differences in maturation rates ( $80.5\% \pm 7.7$ ,  $81.1\% \pm 7.2$ ,  $82.3\% \pm 9.1$ , and  $90.2\% \pm 7.2$ ) and penetration ( $68.8\% \pm 15.9$ ,  $76.5\% \pm 11.4$ ,  $73.1\% \pm 11.7$ , and  $77.4\% \pm 11.1$ ) among the four groups. Although oocyte cleavage rates were not different in the four groups ( $79.4\% \pm 7.3$ ,  $84.6\% \pm 6.8$ ,  $77.2\% \pm 6.5$  and  $82.7\% \pm 5.1$ ), embryo development to the 8-cell stage ( $35.8\% \pm 12.6$ ,  $47.9 \pm 15.1$ ,  $29.2\% \pm 10.1$ ,  $61.2\% \pm 9.0$ ) and blastocyst ( $2.2\% \pm 4.4$ ,  $21.5\% \pm 11.0$ ,  $8.4\% \pm 9.8$ , and  $23.6\% \pm 14.0$ ) were significantly higher ( $P < 0.05$ ) in Group (2) and (4) than in the Control and Group (3). These results indicate that the presences of amino acids, especially essential amino acids in the maturation medium, is beneficial to oocyte cytoplasmic maturation and subsequent early embryonic development in vitro (This research was supported by NSERC of Canada).

EFFECTS OF INVASIVE ADENYLAT CYCLASE, 3-ISOBUTYL  
1-METHYLXANTHINE AND DIBUTYRYL CYCLIC AMP ON IVM,  
IVF AND IVC OF PORCINE OOCYTES

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Invasive adenylate cyclase (iAC), 3-isobutyl 1-methylxanthine (IBMX) and dibutyryl cyclic AMP (db-cAMP) during collection and initiation of maturation of mammalian oocytes maintain intracellular cAMP high and cause arresting of nuclear maturation. Effects of iAC, IBMX and db-cAMP on meiotic maturation, fertilization and subsequent embryonic development were investigated. Oocyte collection was carried out in NCSU-37 solution with 1 mg/ml glucose, 12 mM sorbitol, 50  $\mu$ M  $\beta$ -mercaptoethanol, 25 mM HEPES, 1 mg/ml PVA and supplemented with or without 0.1  $\mu$ g/ml iAC and 0.5 mM IBMX. IVM of cumulus–oocyte complexes (COCs) was performed in NCSU-37 containing 10% (v/v) pig follicular fluid, 0.6 mM cysteine, 10 IU/ml PMSG, 10 IU/ml hCG supplemented with or without 1 mM db-cAMP for 22 h. COCs were subsequently cultured in the medium without hormones and db-cAMP for an additional 24 h. After culture for 46 h, COCs were coincubated with  $1 \times 10^5$ /ml frozen–thawed epididymal spermatozoa for 3 h. In vitro fertilized oocytes were cultured in vitro (IVC) for 6 days by the method described previously [Kikuchi et al., Biol Reprod, 2002;66:1033–1041]. The time-course of meiotic maturation, fertilization status and developmental competence of the zygotes were evaluated after fixation in the whole mounted preparation and staining. Analysis of variance revealed that there was no difference in nuclear status between oocytes collected and/or matured in the presence or absence of cAMP after culture for 12 h. After culture for 22 h, 44% of oocytes underwent germinal vesicle breakdown (GVBD), while a higher rate (95%) of the oocytes cultured with db-cAMP remained in germinal vesicle stage ( $P < 0.01$ ; ANOVA). After culture for 36 h, no difference was observed in the rate of oocytes that underwent GVBD between the control group and the db-cAMP group (93 and 94%, respectively). After culture for 46 h, the rate of oocytes matured to metaphase-II (MII) was higher ( $P < 0.01$ ; ANOVA) when cultured for the first 22 h with db-cAMP (81%) than without db-cAMP (57%). The proportion of oocytes arrested at metaphase-I stage was lower ( $P < 0.01$ ; ANOVA) in case of synchronization (15%) than that without synchronization (31%). Most of the nuclear changes from metaphase-I to MII were observed from 36 to 46 h of culture for the oocytes treated with db-cAMP, whereas it was observed from 22 to 46 h for the oocytes without db-cAMP treatment, showing a synchronization of nuclear maturation with db-cAMP. No significant difference in the proportion of degenerated oocytes was detected between the db-cAMP treated and the control groups (3 and 11%, respectively). The rate of monospermic fertilization after IVF was higher ( $P < 0.05$ ; Chi-square test) when cultured with db-cAMP (21%) than without db-cAMP (9%) and there was no difference in total penetration rates between them (58 and 52%, respectively). The rate to the blastocyst stage on the 6th day was higher ( $P < 0.05$ ;  $\chi^2$  test) when cultured with db-cAMP (32%) than without db-cAMP (19%). Supplementation of collection medium with iAC and IBMX had no effect on these parameters, suggesting that a change of intracellular level of cAMP during collection does not affect further developmental competence of porcine oocytes. These results suggest that synchronization of meiotic maturation using db-cAMP enhances meiotic potential of oocytes by promoting MI–MII transition and results in increased rate of monospermic fertilization and enhanced developmental competence after IVF.

EFFECT OF DONOR AGE AND HORMONAL STIMULATION ON DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES RETRIEVED BY ULTRASOUND-GUIDED TRANSVAGINAL FOLLICULAR ASPIRATION

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Aim of the present study was to investigate the effect of age and exogenous hormonal stimulation on bovine (Murray Grey × Brahman) oocyte developmental competence. Heifers ( $n = 12$ ), young ( $n = 25$ ;  $\leq 3$  calvings; 7–8 years of age) and older ( $n = 22$ ;  $\geq 4$  calvings;  $\leq 14$  years of age) cows were used. Cumulus–oocyte–complexes (COCs) were recovered by ultrasound-guided transvaginal follicular aspiration (TVFA), followed by in vitro maturation (TCM199 + 0.01 mg/ml bLH + 50 ng/ml EGF), fertilization (Tyrode + 2 mg/ml Heparin + PHE) and embryo development (glucose-free HECM-6 + 11 aminoacids for 72 h, then TCM 199 + 10% FCS for 96 h). Three experiments were carried out to test the following: (1) effect of age on oocyte developmental competence. TVFA performed on 12 heifers (104 oocytes and 68 COCs per cow), 15 young (86 oocytes and 51 COCs per cow), and 10 older cows (144 oocytes and 43 COCs per cow); first TVFA performed on day of estrus in heifers and young cows and at random in the cycle in older cows. (2) Effect of FSH on oocyte developmental competence in young cows. Ten young cows (60 oocytes and 37 COCs per cow) served as control first and then treated with two different dosages of FSH (52 oocytes and 33 COCs per cow, 37 oocytes and 25 COCs per cow, respectively). (3) Effect of FSH on oocyte developmental competence in older cows. Six serving as control (173 oocytes and 49 COCs per cow), and 6 being stimulated with FSH (94 oocytes and 38 COCs per cow). Blood samples for estradiol and progesterone values were taken daily starting one month prior to TVFA until the end of the trials. Data were analyzed by ANOVA Paired *t*-test. The results of first experiment show that cleavage and blastocyst rates (CR-BR) were higher in heifers compared to young and older cows (79.4–45.8% versus 62.4–30.9% and 56.0–13.9%, respectively;  $P < 0.05$ ); mean  $E_2$  in heifers was higher compared to young and older cows before ( $1.72 \pm 0.27$  pg/ml versus  $0.78 \pm 0.24$  pg/ml and  $0.26 \pm 0.04$  pg/ml) and during ( $1.05 \pm 0.07$  pg/ml versus  $0.47 \pm 0.05$  pg/ml and  $0.17 \pm 0.02$  pg/ml) TVFA sessions ( $P < 0.05$ ), whereas mean  $P_4$  was lower (before TVFA:  $0.79 \pm 0.12$  ng/ml versus  $2.83 \pm 0.22$  ng/ml and  $3.31 \pm 0.20$  pg/ml; during TVFA:  $0.83 \pm 0.16$  ng/ml versus  $3.01 \pm 0.11$  ng/ml and  $3.44 \pm 0.13$  ng/ml) ( $P < 0.05$ ). In the second experiment young cows receiving the highest dosage of FSH resulted in higher CR and BR compared to a lower FSH dosage and control animals ( $P < 0.05$ ). Mean 2 was not different between control ( $0.52 \pm 0.003$  pg/ml) and treatment groups (lower FSH:  $0.58 \pm 0.02$  pg/ml; higher FSH:  $0.60 \pm 0.03$  pg/ml), but mean  $P_4$  in animals receiving the higher FSH dosage was significantly higher ( $4.35 \pm 0.26$  ng/ml versus  $2.79 \pm 0.22$  ng/ml and  $2.30 \pm 0.34$  ng/ml,  $P < 0.05$ ). In the third experiment older cows receiving FSH resulted in higher CR and BR compared to their control peers (50–33.8% versus 35.4–14.6%, respectively;  $P < 0.05$ ); mean  $E_2$  was elevated during hormone stimulation compared to control animals ( $0.83 \pm 0.09$  pg/ml versus  $0.41 \pm 0.03$  pg/ml,  $P < 0.05$ ), whereas mean  $P_4$  values were found elevated during aspiration sessions compared to the period of hormonal stimulation and to control animals ( $3.96 \pm 0.32$  ng/ml versus  $2.58 \pm 0.31$  ng/ml and  $2.60 \pm 0.13$  ng/ml,  $P < 0.01$ ). In conclusion, age and exogenous hormonal stimulation are important factors regulating the developmental potential of oocytes recovered by TVFA through possibly the effect of blood hormone concentration on follicle development. Higher cleavage and blastocyst rates were obtained from heifers and young cows and from stimulated animals.

THE METABOLIC PROFILES OF BOVINE CUMULUS–OOCYTE  
COMPLEXES: EFFECTS OF OOCYTE-SECRETED FACTORS AND  
STIMULATION OF CUMULUS EXPANSION

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Bi-directional communication and other interactions exist between the mammalian oocyte and surrounding cumulus vestment, with oocyte-secreted factors having profound effects on cell growth and differentiation. The aim of this study was to determine the influence of the oocyte on bovine cumulus–oocyte complex (COCs) metabolism during *in vitro* maturation. COCs were aspirated from antral follicles within abattoir-derived ovaries. Complexes were randomized into three groups: oocyctomized (OOX, aspiration of the ooplasm, leaving the cumulus, oolemma and zona pellucida), OOX plus denuded oocytes (OOX + DO) and intact COC. Complexes were cultured individually in 10 µl drops of IVM medium (TCM199 + pyruvate + BSA + hCG + FSH) at 39 °C in 5% CO<sub>2</sub> in air. Complexes were transferred into fresh medium and oxygen consumption measurements were taken at 1–4 h, 11–14 h and 21–24 h of culture using a non-invasive microfluorescence method [Houghton FD et al., *Mol Reprod Dev* 1996;44:476–485]. Microfluorometric assays were also utilized to determine glucose and pyruvate consumption and L-lactate production in spent medium [Leese HJ and Barton AM, *J Reprod Fertil* 1984;72:9–13]. A further experiment investigated the effect of cumulus expansion on glucose uptake, in which matured COCs incubated for 24 h in ±FSH (Table 1). The DNA content of individual complexes was quantified using PicoGreen fluorescence dye to enable metabolic measurements to be expressed per ng of DNA. Metabolic parameters across treatments and times were compared using ANOVA. There were no significant differences between COC, OOX and OOX + DO in any of the metabolic measurements, suggesting cumulus cell metabolism is not influenced by oocyte-secreted factors. Regardless of treatment, metabolic profiles changed with time over the 24-h maturation period, with significant increases ( $P < 0.05$ ) in the uptake of oxygen, glucose and pyruvate. Oxygen consumption accounted for <2% of glucose uptake. In contrast, glycolytic activity resulting in L-lactate production, accounted for 70 to 100% of the glucose consumed. FSH-stimulated cumulus expansion increased glucose uptake by 150% from unstimulated complexes ( $5.8 \pm 2.2$  versus  $14.5 \pm 3.1$  pmol/ng DNA/h,  $P < 0.01$ ), suggesting that the glycolytic pathway has a significant role in the production of matrix.

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Table 1  
Cumulus–oocyte complex metabolic profiles over a 24 h IVM period

	Culture time		
	4 h	14 h	24 h
Oxygen (pmol/ng DNA/h)	$50.2 \pm 5.64^a$	$70.1 \pm 1.4^{ab}$	$94.0 \pm 14.9^b$
Glucose (pmol/ng DNA/h)	$23.5 \pm 3.60^a$	$32.0 \pm 5.09^{ab}$	$42.4 \pm 6.38^b$
Pyruvate (pmol/ng DNA/h)	$2.15 \pm 0.22^a$	$2.62 \pm 0.518$	$4.69 \pm 0.85^b$
L-Lactate (pmol/ng DNA/h)	$53.4 \pm 13.6$	$63.6 \pm 17.8$	$63.0 \pm 9.6$

Data are displayed as means ± S.E.M. and means within same row with different superscripts are significantly different ( $P < 0.05$ ).

BOVINE OOCYTE-CUMULUS CELL GAP JUNCTIONAL COMMUNICATION  
DURING IN VITRO MATURATION IN RESPONSE TO  
CELL-SPECIFIC PHOSPHODIESTERASE INHIBITORS

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In the growing follicle, communication between the oocyte and its surrounding follicular cells is essential for normal oocyte and follicular development. Maturation of the fully-grown oocyte in vivo is associated with the loss of oocyte-cumulus cell gap junctional communication (GJC), preventing entry of meiotic-modulating factors such as cAMP into the oocyte. Prolonging communication during in vitro maturation (IVM) may improve post-maturation outcomes, such as embryo development, through improved cytoplasmic maturation. We have previously shown that oocyte and cumulus cell cAMP levels can be independently regulated using inhibitors of cell-specific phosphodiesterase (PDE) isoenzymes [Thomas R et al., *Dev Biol* 2002;244:215–225]. The objectives of this study were to examine the effects of cell type-specific PDE inhibitors on the maintenance of oocyte-cumulus cell gap junction communication and oocyte meiotic progression. Cumulus-oocyte complexes (COCs) were aspirated from 2–8 mm antral follicles of abattoir-derived ovaries. Oocyte-cumulus cell gap junctional communication during oocyte maturation was measured using the fluorescent dye, calcein-AM (Molecular Probes, Eugene, OR). COCs (10–15 per treatment group at each time point; 3–4 replicate experiments) were cultured in the presence of specific PDE inhibitors; milrinone (MR, oocyte PDE3 inhibitor) or rolipram (RP, cumulus cell PDE4 inhibitor), and were pulsed with calcein-AM for 15 min, followed by a further 25 min culture in calcein-AM-free medium to allow dye transfer between the two cell types. Following cumulus cell removal, fluorescence in denuded oocytes was measured by microphotometry and meiotic progression assessed. In control COCs, dye transfer from cumulus cells to the oocyte fell progressively from 0 to 8 h, after which oocyte-cumulus cell GJC was completely lost. Loss of gap junctional communication was significantly ( $P < 0.05$ ) attenuated at 3 and 4 h of culture by increasing oocyte cAMP with MR, while increasing cumulus cell cAMP with RP ( $P < 0.05$ ) attenuated the loss measured at 2 h of culture only. Forskolin (FK)—a stimulator of adenylate cyclase that increases cAMP—maintained gap junctional communication at the initial 0 h level until 3–4 h of culture, while treatment with MR and forskolin together actually increased the level of dye transfer above that of control and forskolin alone. Importantly, all treatments that prolonged GJC also delayed meiotic resumption, with meiosis generally resuming when fluorescence had fallen to ~35% of initial levels. Treatment of COCs with the gap junction inhibitor carbenoxolone reduced GJC by ~80% compared to control levels and completely abolished the attenuation of gap junction loss by treatment with MR, RP and MR + FK, but did not affect GJC levels induced by RP + FK. These results demonstrate that meiotic resumption can be delayed by manipulating oocyte cAMP levels and oocyte-cumulus cell GJC, and that maintaining/elevating cumulus cell and/or oocyte cAMP levels is associated with the maintenance of communication between the two cell types. This could potentially alter the capacity of an oocyte to undergo cytoplasmic maturation and to acquire developmental competence. Supported by NH&MRC.

A NOVEL MAPK FAMILY MEMBER IS INVOLVED IN MEIOTIC MATURATION OF PORCINE OOCYTES: P38 MAPK

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Activation of the extracellular signal-regulated kinase (ERK) during meiotic maturation of oocytes in many animals has been studied and in general its importance is quite accepted for events during the resumption of meiosis. Here we investigated the possible existence of one of the members of the mitogen-activated protein kinase (MAPK) family, p38 MAPK different to RK during meiotic maturation of porcine oocytes. In the first experiment, cumulus–oocytes complexes (COCs) from follicles (4–6 mm) were cultured in modified TCM-199 (TCM199, 10% fetal calf serum, 100 mg/ml sodium pyruvate and 100 ng/ml FSH) for differing periods of time. After removal of cumulus cells some oocytes were used for Western blotting analysis to detect p38 MAPK and its phosphorylated form using anti-p38 (sc-7149, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho p38 (#9211, Cell Signalling Technology, Beverly, MA) antibodies, respectively. Other oocytes were used in kinase assay to evaluate the kinetic activity of p38 MAPK using its substrate, activated transcription factor. In the second experiment, denuded oocytes were cultured in modified TCM-199 (without FSH stimulation) supplemented with different concentrations (0, 5, 10 and 20  $\mu$ M) of SB203580, a specific inhibitor of p38 MAPK, for 44 h. After culture, oocytes were fixed, stained with 1% aceto-orcein, and examined to determine their meiotic stage. Each experiment was repeated a minimum of three times. In immunoblots p38 MAPK was not detected in the oocytes at germinal vesicle stage, but started to accumulate around germinal vesicle breakdown, and was phosphorylated by metaphase I and metaphase II. The kinase assay confirmed that p38 MAPK became activated around the germinal vesicle breakdown and its activity was maintained until metaphase II. The inhibition of p38 MAPK during culture period did not affect the resumption of meiosis, but oocytes stopped their maturation at metaphase I in a dose-dependent manner. These results demonstrate that another member of the MAPK family, p38 MAPK, is present in porcine oocytes after their resumption of meiosis, and suggest that p38 MAPK has a role in the transition from metaphase I to metaphase II.

SB203580 $\mu$ M	No. of oocytes examined	GV number (%)	MI number (%)	A I-T I MII number (%)	II number (%)	Degen number (%)
0	28	5 (17.9)	2 (7.1) <sup>a</sup>	0 <sup>a</sup>	17 (60.7) <sup>a</sup>	4 (14.3)
5	33	3 (9.1)	7 (21.2) <sup>b</sup>	2 (6.0) <sup>b</sup>	18 (54.5) <sup>a</sup>	3 (9.1)
10	33	5 (15.2)	15 (45.5) <sup>c</sup>	1 (3.0) <sup>a,b</sup>	10 (30.3) <sup>b</sup>	2 (6.1)
20	35	5 (14.3)	19 (54.3) <sup>c</sup>	1 (2.9) <sup>a,b</sup>	7 (20.0) <sup>b</sup>	3 (8.6)

GV: germinal vesicle; MI: metaphase I; A I: anaphase I; T I: telophase I; M II: metaphase II; Degen: degeneration. (a,b,c) Values within the same column with different superscripts differ ( $P < 0.05$ ; chi-square test).

BOVINE OOCYTE MATURATION IN CO-CULTURE WITH ESTRADIOL-17 $\beta$   
PRODUCING GRANULOSA CELLS IN CHEMICALLY DEFINED  
CULTURE MEDIUM

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A number of in vitro culture systems have shown that the presence of granulosa cells (GC) in the maturation medium results in better rate of oocyte maturation, fertilization and embryo development. However, most of these systems use luteinized cells maintained in non-defined culture media, preventing the analysis of cells interactions in vitro. Since the oocyte is successfully matured in vivo in a dominant pre-ovulatory follicle that produces 17 $\beta$  Estradiol (E<sub>2</sub>) and low Pro-gesterone (P<sub>4</sub>), we proposed to use E<sub>2</sub>/P<sub>4</sub> producing GC in a defined culture system with polyvinyl alcohol (PVA) as donor of macromolecules to mimic a predominant growing follicle, avoiding contaminants of serum, and to study oocyte nuclear maturation under that condition. Previous results our laboratory pointed out an increase in estradiol production by these cells from 48 h (8.36  $\pm$  1.07 pg/ml) to 144 h of culture (38.27  $\pm$  5.26 pg/ml). Granulosa cells isolated from bovine antral follicles (3–5 mm) were cultured for 96 h in  $\alpha$ MEM medium + PVA with insuline-like growth factor-1 (IGF-1) and insulin [Montrezor LH et al., Biol Reprod 2002;66 (suppl 1):211] at 38.5 °C in a humidified atmosphere of 5%CO<sub>2</sub> in air. Bovine immature cumulus–oocyte complexes (COCs; *n* = 1.289) were aspirated from 2–8 mmfollicles of ovaries obtained from a slaughterhouse and cultured in the presence or absence of GC. COCs were randomly allocated to four IVM treatments:T1,  $\alpha$ MEM + PVA + GC; T2,  $\alpha$ MEM + PVA (negative control); T3, TCM-199 + GC ( $\alpha$ MEM medium replaced by TCM-199 for GC culture after 96 h previous culture period);T4, TCM-199 (positive control sup-plemented with 10% fetal calf serum + FSH + LH). The oocytes were cultured and a study of the time course (8, 16 and 24 h) of nuclear maturation was performed. The chromosome configurations were classified as one of the following stages: germinal vesicle (GV), condensing chromosomes I e II (CCI; CCII), diakinesis or metaphase I (D/MI), anaphase I (AI), telophase I (TI) and metaphase II (MII). At 8 h after the onset of incubation (IVM), in  $\alpha$ MEM + PVA (T2), oocytes showed higher percentage (*P* < 0.05) of CCI configuration (20.3%) than T1 (11.5%), T3 (7.7%) and T4 (3.7%). After 16 h IVM, oocytes showed a similar (*P* > 0.05) MII rate among the groups. At 24 h, the presence of GC in the maturation medium partially and temporarily retarded the resumption of meiosis of COCs (*P* < 0.01), since that were observed high CCI configuration rates:T1 (5.3%), T2 (0.7%), T3 (4%) and T4 (0%). In T1, T2 and T4 no differences (*P*>0.05) in MII rates were noted (65.3%, 67.6% and 73.6%, respectively) after 24 h IVM. However, lower levels (*P* < 0.05) of MII in T3 were observed (54.5%). The in vitro maturation medium in T1 achieved the same nuclear maturation potential as that obtained in classical positive control (T4). The high percentage of CCI configuration observed in the oocytes co-cultured with GC in the culture system standardized suggests an inhibitory effect of GC upon meiosis resumption during IVM. This co-culture model of bovine CG-oocyte since performed under defined media conditions is an alternative to study oocyte competence and help to develop concepts on oocyte maturation.

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## EFFECTS OF REVERSIBLE INHIBITION OF MEIOSIS ON MESSENGER RNA EXPRESSION PATTERNS IN BOVINE OOCYTES

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Ovarian oocytes are arrested at the diplotene stage of the first meiotic prophase, the so-called germinal vesicle (GV) stage. Resumption of meiosis begins immediately upon removal from the follicle, but full maturation only occurs if the oocyte has completed the complex process of capacitation. When submitted to *in vitro* maturation, fertilization and culture, the majority of GV stage oocytes fail to reach the blastocyst stage, despite their inherent meiotic competency. Both nuclear and cytoplasmic maturation are required to ensure regular fertilization and subsequent embryonic development. The hypothesis is that if oocytes are cultured *in vitro* prior to maturation under conditions that maintain the arrest at the GV stage, they may have greater chances to complete capacitation and thus acquire a greater developmental competence [Ponderato et al., 2001;MRD, 60:579–585]. The objectives of the present study were to investigate the effects of various inhibitors of meiotic resumption on the expression pattern of selected gene transcripts in bovine oocytes. The four analyzed gene transcripts are thought to play important roles in RNA transcription and further processing [RNA polymerase I (RNA pol I), upstream binding factor (UBF), poly(A) polymerase (PolyA)] as well as in adaptation to (oxidative) stress [heat-shock protein (Hsp)]. Cumulus–oocyte complexes (COCs) were collected from slaughterhouse ovaries (immature control group) and matured *in vitro* in medium M199 supplemented with or without EGF for 24 h [mature control group; Holmet al., Theriogenology 1999;52:683–700] or cultured in inhibition medium containing (1) 2  $\mu$ M Flavopirridol, (2) 100  $\mu$ M Butyrolactone, (3) 100  $\mu$ M Roscovitine or (4) 6.25  $\mu$ M Butyrolactone and 12.5  $\mu$ M Roscovitine, which are known to be potent inhibitors of cell cycle kinases, for 21–22 h. After inhibition, the COC were placed in the regular maturation medium. Subsequently, the COC were mechanically denuded by pipetting. After washing three times in PBS supplemented with 0.1% polyvinyl alcohol (PVA), single oocytes were frozen at  $-80^{\circ}\text{C}$  until assayed. A highly sensitive semi-quantitative RT-PCR assay [Wrenzycki et al., Mol Reprod Dev 1999;53:8–18] was used to determine the relative abundances (RA) of the gene transcripts in single oocytes. Assays were repeated at least ten times. Data were analyzed using the generalized linear models (GLM) procedure with EGF supplementation, status (immature, inhibited, matured, inhibited and matured) and inhibitor as main effects. Differences of  $P \leq 0.05$  were considered to be significant. EGF supplementation significantly decreased the RA of PolyA and RNA pol I transcripts indicating a possible effect on transcription, while the RA of Hsp and UBF transcripts were not affected. The RA of PolyA, RNA pol I and Hsp were also decreased after maturation, possibly due to the general reduction of transcription. The mRNA expression of Hsp and RNA pol I were increased or decreased depending on the inhibitor. Only when the combination of Butyrolactone and Roscovitine was used, a less pronounced effect was seen. These data suggest that the combination of two inhibitors seems to be the most suitable approach for a reversible inhibition of meiosis in bovine oocytes. Supported by the European Union (QLK3-CT1999-00104 # Ex Ovo Omnia).

COMPARISON OF THE DEVELOPMENTAL COMPETENC OF IN VITRO FERTILIZED BOVINE OOCYTES AFTER MATURATION IN M199 MEDIUM SUPPLEMENTED WITH GROWTH FACTOR AND GONADOTROPINS

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The objective of the current study was to compare the developmental competence of in vitro produced bovine embryos after oocytes maturation in M199 supplemented with gonadotropins or/and epidermal growth factor (EGF) in our production of high-quantity blastocysts suitable for cryo-preservation and embryo transfer. Cumulus–oocytes complexes (COCs) were aspirated from slaughterhouse ovaries, and those with at least 4 layers of cumulus cells were selected for culture for 22–24 h at 39 °C, 5%CO<sub>2</sub> in humidified air in M199 medium supplemented with 7.5% FBS, 0.25 mM Sodium pyruvate, 5.0 µg/ml luteinizing hormone (LH), 2 µg/ml estradiol and different combinations of follicle- stimulating hormone (FSH, 1:1 and 1:10) and EGF. The treatments are as follows: 1) 20 ng/ml EGF (EGF); 2) FSH 0.5 µg/ml (FSH 1:1); 3) FSH 0.05 µg/ml (FSH 1:10); and 4) FSH 0.5 µg/ml and 20 ng/ml EGF (EGF + FSH). After maturation, the COCs were subjected to in vitro fertilization (IVF) using standard BO fertilization procedure. At 20–22 h post maturation each 25 COCs were fertilized in a 100 µl droplet for 6 h. Cumulus cells were then stripped off from oocytes and presumptive zygotes were cultured in standard CR1aa plus 6 mg/ml BSA for 48 h at 39 °C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. Cleaved embryos were subsequently co-cultured in 10% FBS CR1aa medium with cumulus-cell monolayer for 5 days. On day 6.5, development was recorded and those expanded (Grade I) blastocysts were selected. The experiment was replicated 3 times and data were analyzed with General Linear Model (GLM), SPSS 9.0 (SPSS, Inc., 1999, Chicago, IL, 60606). The results (Table 1) showed that there were no significant differences among treatments with respect to the rates of cleavage and eight-cell embryos. However, the FSH 1:1 treatment resulted in highest blastocyst rates on day 6.5 (42% versus 19–32%, *P* < 0.05). A significantly higher rate of Grade I blastocysts was also achieved in FSH 1:1 group compared to all other treatments (30% versus 14–23%, *P* < 0.05). A ten-fold dilution of FSH concentration in maturation medium negatively affected the competence of development in IVF embryos. Interestingly, although EGF alone treatment showed an intermediate rate of development similar to that of FSH 1:10 treatment, the combined treatment (FSH + EGF) group showed lowest blastocyst rates in contrast to groups of FSH 1:1, and EGF (*P* < 0.05). It is suggested that EGF has stimulatory effect on embryonic development; however, current combination of FSH and EGF in the EGF + FSH treatment group in fact suppresses embryo development up to day 6.5 blastocyst stage. In conclusion, our experiment demonstrated that the FSH 1:1 maturation protocol best supports embryo development in the CR1aa culture system. The fact of inferior development due to low concentration FSH treatment suggests that sufficient FSH hormone is imperative during maturation for promoting future development of IVF derived embryos. Future research will focus on the optimization of EGF and FSH combination during in vitro maturation and to study the roles of EGF and FSH in the bovine oocyte maturation process.

Table 1  
Development competence of in vitro produced embryos after maturation with gonadotropins and EGF

% Embryos developed to					
Treatment	Number	Cleaved (%, n)	8-cell (% , n)	Day 6.5 BL (%, n)	Day 6.5 Grade I BL (%, n)
EGF	1110	80 (944)	57 (642)	32 (363) <sup>a</sup>	23 (259) <sup>a</sup>
FSH, 1:1	901	76 (732)	66 (591)	42 (352) <sup>b</sup>	30 (262) <sup>b</sup>
FSH, 1:10	867	75 (642)	62 (524)	26 (226) <sup>ac</sup>	20 (165) <sup>ac</sup>
FSH + EGF	665	90 (587)	55 (392)	19 (124) <sup>c</sup>	14 (91) <sup>c</sup>

<sup>a,b,c</sup>Values in the same column with different superscripts are significantly different (*P* < 0.05).

## Sexing

### EFFECT OF CONCENTRATION OF SEXED BOVINE SPERM SORTED AT 40 AND 50 PSI ON DEVELOPMENTAL CAPACITY OF IN VITRO PRODUCED EMBRYOS

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Frozen sex sorted sperm undergo a series of procedures [Schenk JL et al., Theriogenology 1999;52:1393–1405] that compromise fertility with IVF. The objective of this experiment was to determine whether there was a difference in fertilization and blastocyst rates with sperm sorted at either 40 or 50 psi. To test this at critical sperm numbers, a dose response of sperm concentration in the fertilization medium was done with X- and Y-sorted sperm in each subclass. Thus, an unreplicated factorial experiment was designed with two sorting pressures, three sperm concentrations (1, 0.33 and  $0.11 \times 10^6$  sperm/ml), six bulls and two sexes. About 2000 oocytes were aspirated from 2 to 8 mm follicles from slaughterhouse ovaries. Chemically defined media (CDM) were used throughout [Olson SE and Seidel GE, J Anim Sci 2000;78:152–157]. Maturation took place in M-CDM supplemented with 0.5% fatty acid free (FAF)-BSA, 15 ng/ml NIDDK-oFSH-20, 1  $\mu\text{g/ml}$  USDA-LH-B-5, 0.1  $\mu\text{g/ml}$  E<sub>2</sub>, 50 ng/ $\mu\text{l}$  EGF and 0.1 mM cysteamine for 23 h at 38.8 °C and 5% CO<sub>2</sub> in air. Sorted sperm frozen with  $2 \times 10^6$  cells per straw were thawed and centrifuged at  $400 \times g$  through 2 ml 45% and 2 ml 90% Percoll gradient for 20 min. Then, the supernatant was discarded and 2 ml of FCDM supplemented with 0.5% FAF-BSA, 2 mM caffeine and 0.02% heparin was added to the sperm pellet and centrifuged at  $400 \times g$  for 5 min. The supernatant was discarded leaving approximately 50  $\mu\text{l}$  of sperm suspension. Matured oocytes were washed once in FCDM and transferred in groups of 15 in 5  $\mu\text{l}$  into 25  $\mu\text{l}$  drops of FCDM under mineral oil. Fertilization took place by adding 10  $\mu\text{l}$  of sperm suspension per drop for 18 h at 38.8 °C, 5% CO<sub>2</sub> in air. Presumptive zygotes were cultured in CDM1 for 2 days and CDM2 for 4.5 days at 38.5 °C, 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>. On Day 7.5, blastocyst development was evaluated: Quality 1–4 (1 = excellent and 4 = poor) and stage of development, 6–8 (6 full, 6.5 expanding, 7 expanded, 7.5 hatching and 8 hatched blastocysts). Data (Table 1) were analyzed by ANOVA and Tukey's HSD after arc sin transformation.

Cleavage (53.6 and 43.6%) and blastocyst (18.2 and 14.7%) rates were higher for 40 than 50 psi ( $P < 0.01$ ). There was no interaction between dose and pressure; therefore, there was a similar advantage to lower pressure at each sperm concentration. A clear dose response of sperm concentration for cleavage and blastocyst production was found. Also, there were large differences among bulls ( $P < 0.01$ ) for both responses (Table 1), and there was a bull  $\times$  dose interaction ( $P < 0.01$ ) for percentage cleaved. The data indicate that the sperm dose should be  $>1.0 \times 10^6/\text{ml}$  for some bulls. Embryo quality was higher ( $P < 0.01$ ) for Y-sperm than X-sperm (1.12 versus 1.57). Others have noted this for IVF embryos when embryos were sexed, and this effect now is confirmed with sexed sperm.

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Table 1  
Cleavage (% C) and blastocysts (% B) per oocyte data presented by bull

Bull	H023	H024	H025	H026	H027	H028	Average	
	C/B	C/B	C/B	C/B	C/B	C/B	C	B
0.11	18/1	6/1	4/11	36/15	20/7	54/15	30 <sup>a</sup>	8 <sup>a</sup>
0.33	44/4	7/2	72/31	62/21	29/11	68/18	47 <sup>b</sup>	14 <sup>b</sup>
1.0	56/18	35/14	85/43	83/34	72/27	85/29	69 <sup>c</sup>	28 <sup>c</sup>

Values without common superscripts (a, b, c) within groups differ,  $P < 0.01$ .

## USE OF HETEROSPERMIC INSEMINATION AND GENOTYPING EMBRYOS TO COMPARE FERTILITY OF FLOW-SORTED SPERM FROM INDIVIDUAL BULLS

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Current *in vitro* tests of sperm function are not highly correlated with male fertility, and homo-spermic inseminations require hundreds of inseminations per treatment to obtain accurate fertility data. Heterospermic insemination, mixing the sperm of two or more males, provides an accurate estimation of relative fertility in most species examined. In this study, a heterospermic method was developed to test *in vivo* fertility of bulls rapidly. Frozen, flow-sorted sperm from four groups of four bulls were thawed. Sperm from three bulls within each group were combined in all possible combinations (ABC, ABD, ACD, BCD) and inseminated into heifers 12 or 24 h following onset of estrus. Equal numbers of progressively motile sperm were inseminated from each bull, totaling 600,000 motile sperm post-thaw. Half of each inseminate was deposited into each uterine horn. Embryos were collected non-surgically 14.5–20 days following estrus. Polymorphic DNA markers were used to genotype embryos to determine the sire of each embryo biopsy. Heterospermic indices for ranking each bull within groups were calculated using the maximum likelihood analysis theorem (Table 1) so that average fertility gave an index of 1.0. The heterospermic index is based on the probability that sperm from a particular bull will fertilize the embryo in competition with sperm from other bulls in the group. Collections yielded 165 elongating embryos from 332 Heifers (48%) with no difference between AI 12 or 24 h post-estrus. After genotyping, 118 of the 165 embryos could be assigned a specific sire. In group 1, the fertility of the poorest bull was significantly lower ( $P < 0.05$ ) than two other bulls. In group 2, the dominant bull had the highest index ( $P < 0.05$ ), and therefore, the highest fertility in the group. Similar distinctions could be made in groups 3 and 4. However, in three of the groups the fertility of some bulls was not clearly high or low ( $P > 0.05$ ).

With these procedures, an average of 30 genotyped embryos was needed to compare fertility among four bulls. This *in vivo* test requiring the insemination of approximately 60 females rapidly provides information concerning which bulls have relatively high or low fertility. In addition, sperm treatments could be evaluated with this technique.

This research was supported by XY, Inc., Fort Collins, CO, USA.

Table 1  
Heterospermic indices  $\pm$  S.E. for individual bulls within groups

Group 1	Group 2	Group 3	Group 4
1.47 $\pm$ 0.41 <sup>a</sup>	2.43 $\pm$ 0.43 <sup>a</sup>	1.68 $\pm$ 0.44 <sup>a</sup>	0.92 $\pm$ 0.36 <sup>a,b</sup>
0.44 $\pm$ 0.27 <sup>a,b</sup>	0.22 $\pm$ 0.15 <sup>b</sup>	1.09 $\pm$ 0.39 <sup>a,b</sup>	0.46 $\pm$ 0.20 <sup>a</sup>
1.84 $\pm$ 0.46 <sup>a</sup>	0.90 $\pm$ 0.35 <sup>b</sup>	0.83 $\pm$ 0.31 <sup>a,b</sup>	2.02 $\pm$ 0.40 <sup>b</sup>
0.25 $\pm$ 0.17 <sup>b</sup>	0.45 $\pm$ 0.23 <sup>b</sup>	0.40 $\pm$ 0.22 <sup>b</sup>	0.59 $\pm$ 0.24 <sup>a</sup>

Indices without common superscripts (a, b) differ,  $P < 0.05$ .

## RAPID SEXING OF PRE-IMPLANTATION BOVINE EMBRYOS USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

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Loop-mediated isothermal amplification “LAMP” is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency and rapidity under isothermal condition [Notomi et al., *Nucleic Acids Res* 2000;28:e63]. This study evaluated the usefulness of the LAMP for sexing of bovine embryos. The LAMP reaction was performed in the reaction mixture containing DNA sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature of 63.5 or 65 °C for 30–40 min. DNA fragments synthesized were detected by yielding white precipitate of magnesium pyrophosphate in the reaction mixture. The absorbance of reaction mixture at 650 nm was measured sequentially or at the end point. The primers to amplify the tandem repeat sequence on Y chromosome were used for sexing of bovine embryos, and the control reaction was performed using the male-female common primers to amplify 1.715 satellite DNA sequence. Sex of the embryo was judged as male, when both reactions were positive, while sex of the embryo was judged as female, when control reaction alone was positive. In Experiment 1, the efficiencies of three DNA extraction methods (Tris-HCl, NaOH and Proteinase K-Tween 20) were compared using single blastomeres dissociated from in vivo derived male morulae of which sex had been determined by PCR. There was no significant difference in the number of samples judged as male by the LAMP among three extraction methods (Tris: 16/18, 89%; NaOH: 18/20, 90%; Proteinase K-Tween 20: 17/18, 94%). In Experiment 2, the sensitivity and accuracy of LAMP-based embryo sexing method was determined using 1–5 embryonic cells. Embryonic cells were biopsied ( $n = 15–28$  for each cell number) with a microblade attached to a micromanipulator. The number of cells in biopsied samples was counted by Hoechst 33342 staining before sexing, and then the samples were treated with Tris-HCl. The sex of in vivo and in vitro derived embryos used for the experiment was determined by PCR. In 1–5 cells, the determination efficiencies were 80, 93, 81, 100 and 100%, and the accuracies were 75, 88, 100, 100 and 100%, respectively. In Experiment 3, embryo sexing by the LAMP and embryo transfer were performed to verify the applicability of this assay. Of 113 in vivo derived embryos biopsied by the microblade for sexing, the sex of all embryos was determined. Following the transfer of 61 fresh embryos sexed (male; 23, female; 38) into recipient cows, 35 (57%) pregnancies were established at Day 39–60. They are going to deliver by December 2002, and all of the sex of 15 calves (male; 9, female; 6) delivered by July 2002 were consistent with the sex determined by LAMP. These results show that sexing of bovine embryos by LAMP is an efficient and accurate method of sex diagnosis of pre-implantation bovine embryos. We gratefully acknowledge the skilled assistance of M. Ashino, N. Kitano and C. Horikawa.

## HYPERGLYCEMIA-INDUCED APOPTOSIS AT PRE-IMPLANTATION STAGES AFFECTS SEX RATIO OF MICE FOETUSES

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We have reported that exposure to high concentrations of glucose (20 mM) before implantation decreases expression of glucose transporters. This leads to a drop in intra-embryonic free glucose levels in blastocysts that show a decrease of cell number and an increase in the frequency of apoptotic cells. In the present study, we have evaluated the effect of high concentrations of glucose (20 mM) in the *in vitro* culture medium on the sex ratio of bovine blastocysts derived from *in vitro*-matured and *in vitro*-fertilized oocytes and on sex ratio of mice foetuses. We have also determined whether blastocyst production of the X-linked inhibitor of apoptosis protein (XIAP) differs between sexes. Bovine oocytes were matured, inseminated, and cultured *in vitro* in mSOF medium with 10% FCS with or without glucose supplementation. Two-cell mice embryos were obtained from B6CBAF1 superovulated donors and were cultured in KSOM medium with or without glucose. At the blastocyst stage embryos were transferred into pseudo-pregnant recipients. A significantly higher proportion of females was found among those embryos that developed under hyperglycemic conditions *in vitro* both in bovine embryos and mice foetuses (Day 14). Total mRNA was isolated from two pools (15/pool) of male and female blastocysts sexed by PCR and a quantitative reverse transcription was performed using specific reverse primers to XIAP plus  $\beta$ -actin. The expression of  $\beta$ -actin was used as a reference value to quantify XIAP in the real time-quantitative PCR (Corbett Research) using SYBR green as a double-stranded DNA-specific fluorescent dye, and using the relative standard curve method for quantification of XIAP expression mRNA. Our results show that female blastocysts produce significantly higher amounts of XIAP mRNA than males and this could be crucial to explaining the higher proportion of female bovine blastocysts and mice foetuses observed following *in vitro* culture under hyperglycemic-induced apoptosis. This mechanism provides an explanation to the significant reduction of male children born to diabetic mothers.

EFFECTS OF CAFFEINE STIMULATION ON STALLION SPERM MOTION CHARACTERISTICS FOLLOWING 18-H STORAGE, FLOW-SORTING, AND CRYOPRESERVATION

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Foals of pre-determined sex have been accurately and reliably produced in a research setting [Lindsey et al., Equine Vet J 2002;34:128–132]. Sex-sorted sperm would be more efficiently utilized by the industry, however, if frozen/thawed sex-sorted sperm were available. The objective of this study was to compare the motion characteristics of sperm that had been stored for 18 h at 15 °C, flow-sorted, and then frozen, to sperm that had been cryopreserved immediately following shipment for 18 h at 15 °C. Two ejaculates were used from each of five stallions. Following collection, sperm for both treatments were extended to  $25 \times 10^6$ /ml in a Kenney + modified Tyrodes (KMT) medium and stored in a water bath at 15 °C for 18 h. After storage, sperm were allowed to reach ambient temperature (~22 °C) prior to centrifugation at  $600 \times g$  for 10 min. Seminal plasma was removed and the sperm pellet resuspended to  $\sim 500 \times 10^6$ /ml in KMT. An aliquot of sperm was removed (control) from this sample, extended to  $87 \times 10^6$ /ml in a skim-milk, egg yolk freezing extender (4% glycerol; FR5), and allowed to slow cool to 5 °C for 90 min before freezing in liquid nitrogen vapor. A second aliquot (flow-sorted) of sperm was extended to  $100 \times 10^6$ /ml in KMT, stained with Hoechst 33342 (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA), incubated for 30 min, and subsequently sorted by flow-cytometry. Sorted sperm was centrifuged at  $850 \times g$  for 20 min, resuspended to  $87 \times 10^6$ /ml in FR5, and allowed to cool slowly to 5 °C for 90 min prior to cryopreservation. Sperm for both treatments was packaged in 0.25-ml straws, and each straw contained 20 million sperm. Sperm were evaluated (blindly) for visual progressive motility (two observers) at 30 and 90 min post-thaw. An aliquot of sperm from each straw was diluted in both KMT and in KMT containing 2 mM caffeine. Samples were allowed to equilibrate for 5–10 min at 37 °C prior to evaluation. A second straw of each treatment was evaluated (with and without caffeine) using the Hamilton–Thorne Motility Analyzer (CASA). Results are in Table 1. Differences in motion parameters were determined by ANOVA. According to most measured responses, flow-sorting was detrimental to sperm motility. Additionally, 2 mM caffeine improved many sperm responses. There was an interaction whereby caffeine improved some responses more for sorted sperm than for control sperm. Therefore, the damage caused by sorting can be partially compensated for by caffeine. It is possible that similar compensation may occur in the mare reproductive tract. Studies are currently in progress to compare the fertility of stored, cryopreserved stallion sperm to that of sperm that has been stored and sorted prior to cryopreservation.

Table 1  
Motion characteristics of stallion sperm following storage, sorting, and cryopreservation

Treatment	Vis 30	Vis 90	CASA Tot	CASA Prog	VAP	VSL	VCL	ALH	BCF	STR	LIN
C-control	50 <sup>a</sup>	47 <sup>a</sup>	64 <sup>a</sup>	60 <sup>a</sup>	94 <sup>a</sup>	80 <sup>a</sup>	164 <sup>a</sup>	6.19 <sup>a</sup>	33 <sup>a</sup>	83 <sup>a</sup>	50 <sup>a</sup>
Control	45 <sup>a</sup>	40 <sup>b</sup>	50 <sup>b</sup>	44 <sup>b</sup>	82 <sup>a</sup>	69 <sup>a</sup>	144 <sup>a</sup>	5.73 <sup>a</sup>	33 <sup>a</sup>	82 <sup>a</sup>	50 <sup>a</sup>
C-sorted	32 <sup>b</sup>	31 <sup>c</sup>	32 <sup>c</sup>	21 <sup>c</sup>	48 <sup>b</sup>	38 <sup>b</sup>	98 <sup>b</sup>	4.75 <sup>b</sup>	41 <sup>b</sup>	73 <sup>b</sup>	39 <sup>b</sup>
Sorted	18 <sup>c</sup>	16 <sup>d</sup>	24 <sup>d</sup>	12 <sup>d</sup>	39 <sup>b</sup>	30 <sup>b</sup>	80 <sup>b</sup>	4.51 <sup>b</sup>	37 <sup>c</sup>	69 <sup>b</sup>	38 <sup>b</sup>

Values in the same column without common superscripts (a, b, c, d) differ ( $P < 0.05$ ). C-treatments stimulated with 2 mM caffeine.

## EFFECT OF DOSE OF SPERM PROCESSED FOR SEX-SORTING AND CRYOPRESERVATION ON FERTILITY IN EWES

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Lambs have been produced after artificial insemination (AI) with low numbers ( $2\text{--}4 \times 10^6$ ) of cryo-preserved sex-sorted sperm [Hollinshead FK et al., *Reprod Fertil Dev* 2002, in press]. Fewer ewes were pregnant after AI with X- or Y-sorted frozen-thawed (25 and 15%, respectively) than with a commercial dose of unsorted frozen-thawed sperm (54%). The object of the present study was to determine the minimum numbers of sorted frozen-thawed sperm required to obtain pregnancy rates similar to those obtained with unsorted sperm.

A sample of sperm from single ejaculates of two rams was stained, incubated, analyzed and sorted using a modified high-speed cell sorter (MoFlo<sup>®</sup>, Cytomation, Fort Collins, CO, USA) as previously described [Hollinshead FK et al., *Reprod Fertil Dev* 2002, in press]. Sperm were processed at 15,000–18,000/s without sex-sorting into 10-ml centrifuge tubes pre-soaked with 1% BSA in sheath fluid containing 0.2 ml Tris-buffered medium and 20% egg yolk (v/v). For every sample,  $1.3 \times 10^6$  sperm were sex-sorted and analyzed to determine purity [Johnson LA and Welch GR, *Theriogenology* 1999;52:1323–1341]. Sorted and unsorted (control) samples were extended with a zwitterion-buffered diluent containing 13.5% egg yolk and 6% glycerol [Molinia FC et al., *Reprod Nut Dev* 1996;36:21–29] and frozen as 250  $\mu$ l pellets containing  $5 \times 10^6$  sperm. The time of oestrus was controlled in 144 Merino ewes by progestagen sponges (FGA, Vetrepharm A/Asia, Sydney) inserted intra-vaginally for 12 days and an injection of 400 IU of PMSG (Pregnecol, Vetrepharm A/Asia) at sponge removal (SR). Thirty-six hours after SR 134 ewes were injected with 40 mg GnRH (Fertagyl<sup>®</sup>, Intervet) to control the time of ovulation. One hundred and eleven ewes were inseminated in the uterus by laparoscopy 57–60 h after SR with 5, 10, 20 or  $40 \times 10^6$  sorted or unsorted frozen-thawed sperm. Thirteen ewes not given GnRH were inseminated with  $50 \times 10^6$  unsorted frozen-thawed sperm 57–58 h after SR as a control treatment typical of commercial practice. Pregnancy was diagnosed by ultrasound on Day 53. The data were analyzed by  $\chi^2$ .

Sperm motility after thawing was  $37.8 \pm 1.78\%$  (sorted) and  $42.9 \pm 0.93\%$  (unsorted). Seven of thirteen (53.8%) ewes not given GnRH were pregnant. Of the GnRH-treated ewes the proportion pregnant was affected by the number of sperm inseminated ( $P < 0.05$ ) but not by ram or type of sperm ( $P > 0.05$ ). For ewes inseminated with sorted or unsorted (control) frozen-thawed sperm, pregnancy rate was higher for inseminates of 10 and  $40 \times 10^6$  than for 5 and  $20 \times 10^6$  sperm (Table 1). The results are inconclusive as to the required dose for commercially acceptable AI with sex-sorted semen, but suggest that a minimum dose of between 10 and  $40 \times 10^6$  sorted frozen-thawed sperm inseminated close to the time of ovulation would be appropriate.

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Table 1  
Pregnancy after intra-uterine insemination of ewes with frozen-thawed control and sorted ram sperm

Dose ( $\times 10^6$ sperm)	Number of ewes inseminated	Number of ewes pregnant	Percentage of ewes pregnant
5	30	10	33.3 <sup>a</sup>
10	28	16	57.1 <sup>b</sup>
20	29	10	34.5 <sup>a</sup>
40	23	16	69.6 <sup>b</sup>

Within columns different superscripts (a, b) differ ( $P < 0.05$ ).

THE LONGEVITY AND ACROSOME STATUS OF STALLION FLOW  
SORTED SPERMATOZOA

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The recent development of flow cytometric separation of stallion spermatozoa has resulted in the production of normal foals of pre-selected sex (Lindsey C, Morris LH, Allen WR, Schenk JL, Squires EL, Bruemmer JE *Equine Vet J* 2002;34:128–132). For this technology to be accessible, semen will be transported from the flow cytometer to the mare. This study examined the longevity and acrosome status of fresh stallion spermatozoa after sex pre-selection. Three ejaculates from each of seven stallions were collected by artificial vagina and shipped to the laboratory at 20 °C for 2–6 h in a skim milk-glucose extender (1:1 v/v). The semen was centrifuged at  $400 \times g$  for 10 min and the seminal plasma removed. The sperm pellet was resuspended to  $100 \times 10^6/\text{ml}$  in Kenneys modified Tyrodes medium (KMT), stained with Hoechst 33342 (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA), incubated for 30 min and subjected to flow cytometry. The sorted spermatozoa were centrifuged and resuspended to  $40 \times 10^6/\text{ml}$  in KMT in 250- $\mu\text{l}$  aliquots for 48 h storage at either 4 or 20 °C. The total progressive motility (TPM) and the acrosome status of the spermatozoa were evaluated prior to sorting and at 0, 2, 12, 24, 36 and 48 h after sorting. The TPM was evaluated microscopically and acrosomes stained with FITC-PNA (Sigma-Aldrich) and classified as intact, patchy or lost. The effect of stallions, time and storage temperatures were analyzed using the Proc GLM procedure and least means comparisons made (SAS Institute). There was an effect of stallion ( $P = 0.03$ ) on sperm motility and on the proportion of intact acrosomes over time. Staining and incubating the spermatozoa with Hoechst 33342 resulted in a decrease in the proportion of intact acrosomes (Table 1). However, the proportion of intact acrosomes observed after sorting was higher than in the sperm population prior to sorting. The proportion of intact acrosomes declined ( $P < 0.0001$ ) as the lost acrosomes increased ( $P < 0.0001$ ) during 48 h after sorting, but there was no effect of time on the proportion of patchy acrosomes. There was a significant effect of sperm storage temperature after sorting such that storage for 12 h at 20 °C resulted in higher motility than storage at 4 °C. Sex-sorting spermatozoa by flow cytometry results in the selection of a population of spermatozoa which can maintain acrosome integrity for 24 h, equivalent to fresh spermatozoa. The maintenance of sperm longevity for 12 h after sorting should enable sex-sorted spermatozoa to be shipped to mares located some distance from the site of the flow cytometer.

Table 1  
The motility and acrosome status of flow sorted spermatozoa over time

Time	Temperature (°C)	TPM	Acrosome intact (%)
Pre-stain	20	50.7 ± 10.2	57.1 ± 28.2
Post-stain	20	42.0 ± 17.1	42.3 ± 26.7
Post-incubation	20	48.0 ± 15.7	34.9 ± 27.1 <sup>a</sup>
Post-sort 0 h	20	49.9 ± 18.3	60.2 ± 22.3 <sup>b</sup>
2 h	4	34.3 ± 19.3	47.1 ± 28.3
2 h	20	40.8 ± 21.8	53.9 ± 24.5
12 h	4	8.5 ± 12.8 <sup>a</sup>	59.8 ± 19.7
12 h	20	27.0 ± 21.07 <sup>b</sup>	66.6 ± 12.0
24 h	4	4.8 ± 12.9	56.2 ± 16.8
24 h	20	17.2 ± 17.9	64.3 ± 16.4
48 h	4	0.0 ± 0.0	32.7 ± 25.0
48 h	20	5.1 ± 8.6	41.6 ± 24.0

Values within a column with different superscripts (a, b, c) are significantly different ( $P < 0.05$ ).

## EMBRYO PRODUCTION USING SEXED SEMEN IN SUPEROVULATED COWS AND HEIFERS

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Obtaining pre-selected sex embryos for use in an embryo transfer program can improve the potential use of resources in dairy and beef production systems. The most reliable technique to achieve this goal has been to apply the PCR technology to the biopsy of an embryo. However frozen embryos after biopsy have low pregnancy rates (37%) (Shea, Theriogenology 1999;51:841–854), almost half of the embryos have to be discarded if you are selecting only one sex, it is a time consuming test, and cost can be another disadvantage. The sex of embryos collected can now be efficiently pre-selected (90–95% accuracy) using frozen sperm sorted by flow cytometry to inseminate the donors in a embryo transfer program. The aim of this study was to evaluate the embryo production in cattle using sexed semen in different categories (cows and heifers) and breeds (beef and dairy) in MOET programs. Fifty-two animals were superovulated using a 7-day protocol. At Day 0 they were injected with estradiol benzoate 2.5 mg IM; progesterone 100 mg IM, and a low-releasing progesterone intra-vaginal device was introduced (CIDR<sup>®</sup>). On Day 4, the superovulation began in an eighth decreasing dosis schedule of FSHp (Folltropin<sup>®</sup>, Vetrepharm) during 4 days, (360 mg in cows and 290 mg in heifers). On the third day of FSHp injection, the donors were injected with prostaglandin (clorprostenol) two dosis (one AM and the other PM). Then the heat was checked and the AI performed. Forty animals (twenty-six cows and fourteen heifers) were inseminated with frozen sexed semen at 0, 12 years 24 h after heat detection, with one dose of 10 million spermatozoas each time. Sixteen animals were inseminated with conventional semen (control group). Thawing was performed at 35 °C during 30 s for sexed semen and 40 s for conventional. The semen was deposited in the uterine body. The embryo collection was performed 7 days after insemination. The flushing media was ICP<sup>®</sup> (New Zealand), using 1 l to flush each uterine horn. The eggs obtained were classified into three categories: unfertilized, degenerated and viable embryos. Results are shown in the table below.

There were no statistical differences between non-sexed and sexed semen groups for embryo production (Table). These results imply that sexed semen can be used successfully in an embryo transfer program to produce pre-selected sexed embryos. There were no statistical differences between using sexed semen in different categories (cow versus heifer), while embryo production was higher in beef cattle.

	N	UFO	DEG	EMB	TE
Non-sexed semen	16	1.6 ± 3.0 <sup>a</sup>	1.7 ± 2.3 <sup>a</sup>	5.4 ± 4.4 <sup>a</sup>	8.8 ± 7.3 <sup>a</sup>
Sexed semen	40	4.3 ± 4.0 <sup>b</sup>	1.7 ± 2.5 <sup>a</sup>	4.7 ± 3.6 <sup>a</sup>	10.7 ± 6.2 <sup>a</sup>
Cows	26	4.3 ± 3.9 <sup>a</sup>	0.7 ± 0.9 <sup>a</sup>	4.2 ± 3.0 <sup>a</sup>	9.2 ± 5.5 <sup>a</sup>
Heifers	14	4.3 ± 4.2 <sup>a</sup>	3.6 ± 3.3 <sup>b</sup>	5.5 ± 4.6 <sup>a</sup>	13.4 ± 6.6 <sup>b</sup>
Dry dairy cows and heifers	21	4.8 ± 4.2 <sup>a</sup>	0.6 ± 0.9 <sup>a</sup>	3.5 ± 2.5 <sup>a</sup>	8.9 ± 5.8 <sup>a</sup>
Dry beef cows and heifers	19	3.8 ± 3.7 <sup>a</sup>	2.9 ± 3.0 <sup>b</sup>	5.9 ± 4.3 <sup>b</sup>	12.6 ± 6.1 <sup>b</sup>

Values with different superscripts (a, b) differ ( $P < 0.05$ , *t*-test).

References: UFO: unfertilized oocyte, DEG: degenerated non-viable embryos, EMB: viable embryos, TE:

## INSEMINATION OF COW ELK WITH SEXED FROZEN SEMEN

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Cow elk 3–6 years of age in Colorado and Minnesota were synchronized for estrus in September by insertion of a protesterone CIDR into the vagina for 12–14 days. Upon removal of the CIDR, 200 IU of eCG were administered IM and elk were time-inseminated 60 h later. Fresh semen was collected via electro-ejaculation from a 5-year-old bull elk and slowly cooled over 4 h to 20 °C for transportation as a neat ejaculate to the sperm-sorting laboratory. The ejaculate was concentrated to  $1 \times 10^9$  sperm/ml for staining by centrifuging 1.5 ml aliquots for 10 s at  $15,000 \times g$ . Semen was incubated in 112  $\mu$ M Hoechst 33342 at  $200 \times 10^6$  sperm/ml in a TALP medium for 45 min at 34 °C, and then diluted to  $100 \times 10^6$  ml<sup>-1</sup> for sorting. Sperm were sorted on the basis of differing DNA content of X- and Y-chromosome-bearing sperm. X-chromosome-bearing elk sperm contained 3.8% more DNA than Y-chromosome-bearing sperm. Sperm were flow-sorted over a 4-h period using a MoFlo<sup>®</sup>SX operating at 50 psi with a TRIS-based sheath fluid. The 351 and 364 bands of an argon laser, emitting 150 mW, excited Hoechst 33342 dye bound to DNA. Both X- and Y-chromosome-bearing sperm were collected (~92% purity as verified by reanalyzing sonicated sperm aliquots for DNA) at 4700 sperm/s into tubes containing 2 ml of 20% egg yolk–TRIS extender. Sorted volumes of 15 ml were sequentially collected. Approximately  $110 \times 10^6$  sperm of each sex were sorted and cooled to 5 °C over 90 min. An equal volume of glycerol (12%) containing extender was added to the sorted volume at 5 °C. Sorted sperm aliquots containing 30 ml were concentrated by centrifugation at 4 °C for 20 min at  $850 \times g$ . Sperm pellets were pooled, adjusted to  $21.7 \times 10^6$  sperm/ml and loaded into 0.25-ml straws. Each straw, containing  $5 \times 10^6$  total sperm, were frozen in liquid nitrogen vapor. As a control,  $5 \times 10^6$  total sperm from the same ejaculate were frozen in 0.25-ml straws at the same time as the sexed sperm. After thawing for 30 s at 37 °C, 65 and 60% of sperm (control and sexed, respectively) were progressively motile as determined by visual estimates. Cows at three different locations and management schemes were inseminated using routine transcervical semen deposition into the uterine body. Pregnancy was determined 40 days postinsemination by assaying blood for Pregnancy-Specific Protein B (Bio Tracking, Moscow, Idaho). Ten cows at one location were in poor condition at the time of insemination and no pregnancies were achieved with sexed or control sperm. The pregnancy rate at the other locations with sexed sperm (61%; 11/18) was similar to that for control inseminates (50%; 3/6). These pregnancy rates (sexed and controls) resulted from fewer sperm than are used in normal elk artificial insemination. Nine of eleven (82%) sexed calves were of the predicted sex.

## USE OF HETEROSPERMIC INSEMINATION WITH FETAL SEX AS THE GENETIC MARKER TO STUDY FERTILITY OF SEXED SPERM

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Testing hypotheses involving binomial responses such as pregnant/not pregnant require huge numbers of animals per treatment to obtain statistical significance unless treatment differences are fairly large. One approach to amplifying treatment differences in fertility is competitive, or heterospermic, fertilization, mixing sperm of different treatments or males before insemination, and determining the proportion of embryos, fetuses or offspring derived from each male or treatment. Here we compare fertility after sexing sperm by flow cytometry/cell sorting for DNA content at two sorter pressures using a heterospermic approach with sex as the genetic marker.

Sperm from each of two bulls was sorted into X- and Y-chromosome populations at 95% accuracy with the pressure of the sorter at either 30 or 50 psi. After concentrating sperm postsorting by centrifugation,  $10^6$  X-sperm sorted at 30 psi were placed in 0.25-ml straws with  $10^6$  Y-sperm sorted at 50 psi within each bull, as well as the converse in other straws:  $10^6$  Y-sperm at 30 psi plus  $10^6$  X-sperm at 50 psi. These sperm, along with unsorted controls, were then frozen, thawed some months later, and inseminated into the body of the uterus of 85 Holstein heifers either 12 or 24 h after observed estrus with subgroups balanced across two inseminators. Two months postinsemination, 81% of the 43 heifers becoming pregnant had fetuses of the sex (determined by ultrasound) corresponding to the sex of sperm processed at 30 psi. This differed from the 50:50 sex ratio expected ( $P < 0.01$ ) if there was no difference in fertility of sperm sorted at the two pressures. A disadvantage of the lower pressure is that rates of sorting sperm are slightly lower. The pregnancy rate with sexed sperm at  $2 \times 10^6$  sperm per dose was 51% (43/85); this was similar to the controls of  $20 \times 10^6$  unsexed sperm per dose from the same ejaculates, 39% (9/23). This procedure using sex as the genetic marker also can be used to rank fertility of males and to rank fertility of sperm treatments not involving sperm-sexing if the males or treatments do not interact with sperm-sexing procedures. One disadvantage of this approach to testing fertility is that no estimates of the magnitude of the treatment or male to male differences are produced. Another disadvantage is 5% errors in assigning parentage via fetal sex when sperm are sorted at 95% accuracy. Nevertheless, this heterospermic approach was rapid, sensitive, and noninvasive, and required relatively few animals to detect a significant treatment difference.

PRESSURE DURING FLOW SORTING OF BULL SPERM AFFECTS POST-THAW  
MOTILITY CHARACTERISTICS

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For rapid flow cytometric sorting of sperm to produce sexed inseminates at commercially viable rates, sperm are propelled through the system at accelerating speeds reaching nearly 90 km/h when they exit the nozzle at the routine sorting pressure of 50 psi. This high pressure and sequelae may compromise the viability and motility of sperm. Here we compared post-thaw sperm quality after sexing by flow cytometer at three sorter pressures. Sperm from each of six bulls was stained with 125  $\mu\text{m}$  Hoechst 33342 for 45 min at 34 °C, bulk-sorted or sorted into X- and Y-chromosome populations at 95% accuracy with the pressure of sorters at 30, 40 or 50 psi, cooled to 5 °C and concentrated by centrifugation, loaded into 0.25-ml straws with  $2 \times 10^6$  total sperm per 100  $\mu\text{l}$  column, and frozen using a vapor freezing method along with unsorted controls. The internal diameter of nozzle used for sorting was 70  $\mu\text{m}$ . Sperm were evaluated subjectively (blindly) by two observers at 30 and 120 min post-thaw for progressive motility, as well as by flow cytometry 10<sup>5</sup> min post-thaw for percent live by PI stain, and by CASA analysis 120 min post-thaw using the Hamilton Thorne system. The entire experiment was replicated twice. Factorial ANOVA indicated that both bull and pressure effects were significant ( $P < 0.05$ ) for all responses in the table.

There were the usual major differences among bulls in all responses; bull by treatment interactions were small with one exception, meaning findings apply similarly to most bulls in the population. There was no difference between bulk sorting and actual sorting. There was a large improvement going from 50 to 40 psi, and then a much smaller improvement dropping to 30 psi, indicating that effects were not linear. At 30 psi, sperm quality was similar to the nonsorted controls. Similar results were observed with stallion sperm in a separate experiment. In conclusion, lowering the system pressure during sperm sorting under conditions tested greatly improved quality of sperm post-thaw. Lowering the pressure reduced sorting rate by 2–3%.

Response	Pressure (psi)			Unsorted control
	50	40	30	
30 min motility (%)	44.7 <sup>a</sup>	48.6 <sup>b</sup>	49.6 <sup>bc</sup>	52.1 <sup>c</sup>
120 min motility (%)	34.5 <sup>a</sup>	40.8 <sup>b</sup>	42.7 <sup>b</sup>	40.8 <sup>b</sup>
Live sperm (%)	51.7 <sup>a</sup>	55.7 <sup>b</sup>	57.8 <sup>b</sup>	58.5 <sup>b</sup>
CASA total motility (%)	25.1 <sup>a</sup>	37.2 <sup>b</sup>	40.9 <sup>b</sup>	34.8 <sup>b</sup>
CASA ALH <sup>1</sup>	6.0 <sup>a</sup>	7.6 <sup>b</sup>	7.8 <sup>bc</sup>	8.8 <sup>c</sup>

Means within rows without common superscripts (a, b, and c) differ ( $P < 0.05$ ).

<sup>1</sup> Amplitude of lateral head displacement. Higher numbers mean less stiff (more normal) motility.

## NORMALITY OF CALVES RESULTING FROM SEXED SPERM

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Flow cytometric sorting of sperm has made sex preselection of offspring possible in many domestic species. The objective of this study was to determine normality of calves from AI with sexed versus control sperm in nine recent breeding trials. Sperm from 22 bulls were sexed on the basis of DNA content by flow cytometry/cell sorting after staining with H33342, and then cryopreserved [Theriogenology 52:1375]. Estrus was synchronized in heifers and cows of various beef and dairy breeds, either by feeding 0.5 mg melengestrol acetate (MGA) daily for 14 days followed by 25 mg prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) i.m. 17–19 days later or injection of 25 mg PGF<sub>2α</sub> i.m. at 12-day intervals. Insemination with either sexed or control frozen–thawed sperm from the same ejaculate took place 12 or 24 h after initial observation of estrus. For each trial, about two-third of the inseminations were with sexed sperm, and one-third with control sperm. Pregnancy and fetal sex were diagnosed by ultrasound 2 months later. Cattle were managed at 13 farms through calving and weaning under differing levels of management ( $N = 49$ – $228$  per farm). Data collected included gestation length, birth weight, calving ease (1 = normal to 4 = Caesarian), weaning weight, neonatal deaths, and deaths from birth to weaning. Not all farms recorded birth and weaning weights. Data were subjected to factorial ANOVA with factors: management groups, sorted versus control sperm, and sex of calves. The arc sin transformation was used for percentage data. Least-square means are in Table 1.

There were no differences ( $P > 0.1$ ) between calves from sexed versus control groups for any response studied, nor were there significant interactions. There were significant effects of management groups for all responses studied ( $P < 0.001$  for all except percent alive at weaning,  $P < 0.02$ ). Also, there were significant differences (all  $P < 0.001$ ) between female and male calves for birth weight: 32.2 and 35.5 kg; weaning weight: 232 and 246 kg; calving difficulty: 1.20 and 1.42; and gestation length: 278 and 280 days. The sex ratio of the control calves was 51.0% males ( $N = 382$ ; three unrecorded sexes at birth). X sort sperm resulted in 87.7% females, while the Y sort sperm produced 93.6% males ( $N = 94$ ). A few calves that were dead at birth did not have sex recorded and are not included (three control, eight sexed).

We conclude that the population of calves from sexed sperm is virtually identical to the control population from unsexed sperm. Furthermore, this sperm sexing procedure resulted in approximately 90% of calves of the planned sex.

Table 1  
Calving results from sexed and control calves

Treatment	<i>N</i>	Gestation length (days)	Neonatal death (%)	Calving ease	Birth weight (kg)	Live at weaning (%)	Weaning weight (kg)
Sexed <sup>a</sup>	574	279	3.9	1.31	34.3	92.0	239
Control	385	279	5.9	1.30	34.1	88.9	239

<sup>a</sup> No significant differences ( $P > 0.1$ ) for any response.

# Sperm Injection

## EFFECT OF ELECTRICAL ACTIVATION ON EMBRYONIC DEVELOPMENT FOLLOWING INTRACYTOPLASMIC SPERM INJECTION (ICSI) IN PIG

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This study was performed to assess pronuclear formation, the chromosomal constitution, and the developmental capacity of porcine zygotes fertilized by intracytoplasmic sperm injection (ICSI). Oocytes collected from slaughterhouse ovaries were matured in BSA-free NCSU 23 medium supplemented with 10% porcine follicular fluid, 0.1 mg/ml cystein, 0.5  $\mu$ /ml LH, 0.5  $\mu$ /ml FSH, 1  $\mu$ /ml E<sub>2</sub> and 10 ng/ml EGF. After 22 h, the oocytes were further cultured for 22 h in the same maturation medium without hormone supplementation. Expanded cumulus cells were removed in NCSU 23 medium containing 0.1% hyaluronidase by voltexing for 3 min. Oocytes with an extruded first polar body and dense cytoplasm were selected and then mature sperm was injected into the ooplasm. The injected oocytes were randomly divided into two groups, activation and nonactivation. Each 15–20 injected oocytes were cultured in a 50# droplet of NCSU 23 medium supplemented with 0.4% BSA for 168 h at 39 in a humidified atmosphere of 5% CO<sub>2</sub> in air. Pronucleus formation of the eggs injected with sperm was compared to that of in vitro fertilization (control). The proportion of two pronuclei formation in the eggs after injection of sperm was significantly higher than that of IVF-derived eggs (96.2 and 93.5% versus 64.5%, respectively,  $P < 0.05$ ). The cleavage rate in an electrical activation group (78.6%) was significantly higher than those of both of control and nonactivated group (51.8 and 46.0%, respectively,  $P < 0.05$ ). The percentage of oocytes developed to blastocysts at 7 days was significantly higher in electrical activation group than those in both of control group and nonactivation group (18.9% versus 11.6% and 9.1%, respectively,  $P < 0.05$ ). Chromosome analysis showed that most of blastocysts were diploid regardless of IVF or ICSI. At Day 8, the average cell numbers of blastocyst following IVF and with or without activation after ICSI was  $46.7 \pm 2.9$ ,  $44.7 \pm 4.2$ , and  $41.9 \pm 4.6$ , respectively. After embryo transfer (3166 embryos), 9 of 38 recipients showed pregnancies at Days 25–50 determined by ultrasound, but none results in development to term. These results show that oocyte activation after ICSI is effective for elevating cleavage rate and subsequent blastocyst development. However, experiments are in progress to give birth of piglets.

## INTRACYTOPLASMIC SPERM INJECTION OF PORCINE OOCYTES WITH TWO DIFFERENT IN VITRO CAPACITATION SYSTEMS

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The intracytoplasmic sperm injection (ICSI) in pigs is a relatively new in vitro fertilization approach to get viable embryos, which is being developed in many laboratories with increasing success [Martin, *Biol Reprod* 2000;63:109–112; Lai et al., *Zygote* 2001;9:339–346; Probst et al., *Theriogenology* 2002;57:752]. One of the main problems is the low or even null rate of blastocyst obtained when in vitro matured oocytes are employed [Klocke, 1999; Hannover, Tierärztliche Hochschule, Dissertation]. Moreover, the basic conditions to develop the technique (including culture media, sperm treatments, and oocyte activation) are variable among labs, and one of the first steps to get standardized results should be to know more about the effect of such conditions. The objective of the present experiment was to compare the effect of two different in vitro capacitation systems on the ICSI results. With this purpose, porcine oocytes were matured in NCSU-37 for 44 h and sperm injected on DPBS with 10% FCS. Fresh ejaculated spermatozoa were obtained by the glove hand method from a fertility-tested boar once a week. Then, sperm samples were processed to ICSI as follows: Group 1, rich fraction of sperm was resuspended in PBS-serum solution; Group 2, sperm were layered on a discontinuous (45–90%) Percoll gradient. The pellet was resuspended to give a concentration of  $5 \times 10^5$  cells/ml in both groups. ICSI was conducted on a heated microscope at  $200\times$  magnification using a Nikon Diaphot 300 inverted microscope with attached micromanipulators. One microliter of diluted spermatozoa was added to the  $4 \mu\text{l}$  microdrops of the ICSI dishes. A single sperm was immobilized and injection was performed as described by Martin. Injected oocytes were transferred to TALP medium [Rath et al., *J Anim Sci* 1999;77:3346–3352] until 18 h when they were either fixed and stained in orcein to check fertilization or transferred again to NCSU-23 medium [Machaty et al., *Biol Reprod* 1998;59:451–455] for further embryo culture. Sham-injected oocytes were used as controls. Results (data were analyzed by one-way ANOVA) show that both sperm treatments gave similar rates of oocyte activation (82.7% versus 80%), and putative zygotes formation at 18 h ( $54.17 \pm 7.27\%$  versus  $51.92 \pm 7.00\%$ ). No differences were observed either in the proportion of cleaved embryos at 48 h between the two groups (55.36% versus 63.79%). After 7 days of culture the proportion of blastocysts was 17.74% versus 20.27% with an average cell count of 20.90% versus 29.91%,  $P > 0.05$ . Data show that in vitro matured pig oocytes can be injected with PBS- or Percoll-treated spermatozoa and give rise to blastocyst formation at similar levels as IVF [Coy et al., *Reproduction* 2002;24:279–288] without any exogenous activation. Supported by Ministerio de Ciencia y Tecnología (AGL2000-0485-C02-01).

THE EFFECTS OF CYSTEINE SUPPLEMENT ON THE FORMATION OF  
MALE PRONUCLEUS AFTER INTRACYTOPLASMIC  
SPERM INJECTION (ICSI) IN PORCINE OOCYTES

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Supplement of cysteine (Cys) during IVM improves the formation of male pronucleus (MPN) in porcine oocytes after IVF [Yoshida et al., *Biol Reprod* 1993;49:89–94]. This study was performed to examine whether Cys-supplement in maturation media improves the MPN formation after ICSI in porcine oocytes or not. After ICSI, sperm-injected oocytes were cultured in the medium with or without Cys, the effects of Cys-supplement during in vitro culture (IVC) of presumed zygotes were also examined on the formation of MPN and blastocyst (BLC). Ejaculated boar spermatozoa were cultured for 6 h in BO solution and treated with 1 mg/ml progesterone (P4) for 10 min to induce the acrosome reaction. The oocyte–cumulus–granulosa cell complexes (OCGCs) were collected from porcine follicles by the dissection method. In Experiment 1, to examine the effect of Cys-supplement in maturation media, OCGCs were cultured with follicle shells in TCM-199 including 10% FCS, 0.1 IU/ml hMG and 9.08 mM Na-pyruvate (mTCM-199) with or without 0.57 mM Cys for 42 or 48 h, and oocytes, which had extruded the first polar body, were injected with the P4-treated spermatozoon ([42 h, Cys–], [42 h, Cys+], [48 h, Cys–], [48 h, Cys+]). After 10 h culture in NCSU23, presumed zygotes were fixed and MPN formation was examined. In Experiment 2, to examine the effects of Cys-supplement during IVC of presumed zygotes, oocytes matured in mTCM-199 with 0.57 mM Cys for 42 h were injected with the P4-treated spermatozoon, and presumed zygotes were cultured in NCSU23 with or without 0.57 mM Cys for 10 h ([10 h+], [10 h–]). Some of them were fixed and MPN formation was examined. The others were sequentially cultured in NCSU23 with or without 0.57 mM Cys to 168 h ([10 h–, 158 h–], [10 h+, 158 h–], [10 h+, 158 h+]), in vitro development of presumed zygotes was observed. Data were analyzed by ANOVA and *t*-test. In Experiment 1, there was no difference in the rate of matured oocytes in four groups. The Cys-supplement in maturation media significantly increased the rate of MPN formation in the oocytes matured for 42 and 48 h compared with those matured without Cys (55% [42 h, Cys+] and 57% [48 h, Cys+] versus 13% [42 h, Cys–] and 26% [48 h, Cys–];  $P < 0.05$ ). In Experiment 2, Cys-supplement in NCSU23 for 10 h after ICSI increased the rates of MPN formation, although not significantly (63% [10 h+] versus 52% [10 h–]). However, at 24 h, this culture condition significantly increased the rate of 2-cell embryos (22% [10 h+, 158 h–], 25% [10 h+, 158 h+] versus 13% [10 h–, 158 h–];  $P < 0.05$ ). The Cys-supplement in NCSU23 during 168 h accelerated the formation of morulae and BLC; however, there was no difference in the rate of BLC formation at 168 h (29% [10 h+, 158 h+] versus 19% [10 h–, 158 h–], 22% [10 h+, 158 h–]). The rate of expanded BLC was significantly increased (22% [10 h+, 158 h+] versus 10% [10 h–, 158 h–] and 8% [10 h+, 158 h–];  $P < 0.05$ ) by the Cys-supplement during 168 h. The Cys-supplement in maturation media improves more efficiently the formation of MPN after ICSI than extended culture periods. Although Cys-supplement during IVC does not affect the formation of MPN and BLC, it accelerates the cleavage of embryos and induces the expansion of BLC.

Condition of IVC after ICSI	No. of oocytes with MPN (%)	No. of embryos (%)		
		2-cell stage at 24 h	BLC stage at 168 h	
			Total	Expanded
10 h–	16/31 (52)	–	–	–
10 h+	20/32 (63)	–	–	–
10 h–, 158 h–	–	9/68 (13) <sup>a</sup>	13/68 (19)	7/68 (10) <sup>a</sup>
10 h+, 158 h–	–	11/51 (22) <sup>b</sup>	11/51 (22)	4/51 (8) <sup>a</sup>
10 h+, 158 h+	–	16/63 (25) <sup>b</sup>	18/63 (29)	14/63 (22) <sup>b</sup>

Values with different superscripts (a and b) are significantly different ( $P < 0.05$ ).

## MICROINSEMINATION WITH FIRST-WAVE SPERMATOGENIC CELLS FROM IMMATURE MALE MICE

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In mice, primary spermatocytes and round spermatids can be used to produce normal offspring using microinsemination techniques. In this study, we examined whether these spermatogenic cells retrieved from immature testes, which are undergoing the first wave of spermatogenesis before forming the normal seminiferous epithelium, have already acquired fertilizing ability comparable to cells from mature testes. Experiment 1 (Round spermatid injection): Metaphase II (MII)-stage oocytes were collected from the oviducts of B6D2F1 superovulated females 15 h after hCG injection. At 40–50 min after activation with 3 mM SrCl<sub>2</sub>, round spermatids from ICR or JF1 males were injected into Telophase II oocytes. Experiment 2 (Primary spermatocyte injection): GV stage oocytes were collected from the follicles of B6D2F1 females 44 h after eCG injection. Primary spermatocytes from JF1 males were injected into proMI to MI oocytes after 3–4 h in culture with alpha-MEM. Injected oocytes were matured in vitro for about 17 h in the same medium. The chromosomes of the oocytes that reached MII were transferred into the ooplasm of enucleated fresh MII oocytes by electrofusion. Oocytes thus reconstructed were activated with 3 mM SrCl<sub>2</sub>. In both experiments, microinjection was performed using a piezo-driven micromanipulator (Prime Tech. Co., Ibaraki, Japan). Embryos were cultured in KSOM until the four to eight cell stage (48 h) or the morula/blastocyst stage (72 h) at 37 °C under 5% CO<sub>2</sub> in air, and transferred into the oviducts (0.5 day post-coitum, dpc) and uteri (2.5 dpc), respectively, of ICR pseudopregnant females. At 19.5 dpc of pregnancy, recipient females were examined for fetuses and implantation sites by Caesarian section. Live fetuses were raised by lactating ICR foster mothers. Experiment 1: More than 80% of oocytes survived injection with round spermatids. After 72 h in culture, 36.8–83.5% of oocytes developed into the morula/blastocyst stage, irrespective of the day of the experiment and the age of the males used. In all, 9.2% (21/229) of transferred embryos developed to term when round spermatids from 20-, 21-, and 22-day-old males were used. However, only one (0.5%, 1/214) live fetus was obtained from round spermatids at 17 days, the stage at which the first-round spermatids are expected to appear. Experiment 2: When primary spermatocytes from 20-day-old males were injected, 52.9% (27/51) developed to the four to eight cell stage and 7.4% (2/27) developed to normal fetuses. Similarly, embryos from primary spermatocytes at 15 days 63.6% (35/55) developed to the four to eight cell stage, but no fetuses (0/35) reached term after embryo transfer. These results suggest that at least some first-wave spermatogenic cells have the ability to support full-term embryo development, like spermatogenic cells from mature males. However, the efficiency, as measured by embryos developed to term, was low when the youngest spermatogenic cells were used. It is important to discover whether this has a biological cause, such as incomplete paternal genomic imprinting, or is for some technical reason. The use of male germ cells from immature animals may rescue male-factor sterility of early onset and save time in the production of congenic strains.

## FERTILIZATION AND DEVELOPMENT TO PIGLETS BY INTRACYTOPLASMIC SPERM HEAD INJECTION INTO PORCINE OOCYTES MATURED IN VITRO

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Intracytoplasmic sperm injection (ICSI) of a nonmotile cell into the ooplasm for assisted fertilization is a highly specialized procedure for producing the next generation. The production of piglets by ICSI has been succeeded when *in vivo* matured oocytes have been used as recipients; however, any viable piglet generated from ICSI using *in vitro* matured oocytes (IVM) has not been reported yet. The objective of the present study was to generate viable piglets by using porcine oocytes matured *in vitro* and fertilized by ICSI, after evaluating the efficacy of using donor spermatozoa in which the acrosome had been artificially removed by treatment with calcium ionophore A23187 (Ca-I). The status of acrosome was evaluated by a triple-stain technique. The rate of acrosomal loss in spermatozoa was increased significantly as the duration of treatment with 10  $\mu$ M Ca-I was prolonged for 30–120 min (Ca-I-treated; 55.6–78.6%), whereas the rate was not different as the duration of incubation without Ca-I was prolonged for 30–120 min (control; 45.3–58.4%). Porcine cumulus–oocyte complexes were matured *in vitro* for 46 h under 5% O<sub>2</sub> at 39 °C [Kikuchi et al., *Biol Reprod.*, 2002;66:1033–1041]. Oocytes with the first polar body were collected as matured oocytes after treatment with 150 IU/ml hyaluronidase and gentle pipetting. The Ca-I-treated and control spermatozoa that were incubated for 120 min were injected into the matured oocyte with the aid of a piezo-driven micromanipulator. The oocytes injected with the sperm heads were subsequently stimulated with an electrical pulse (1.5 kV/cm DC, 20  $\mu$ s) at 6 h post-ICSI, cultured for 6 days at 38.5 °C under 5% O<sub>2</sub> [Kikuchi et al.], fixed and stained for the evaluation of embryonic development. The rates of blastocyst formation were not significantly different between the two groups: the rates for oocytes injected with Ca-I-treated sperm heads and for those injected with control sperm heads were 8.6 and 4.0%, respectively. The mean cell numbers of the blastocysts were not significantly different between the two groups (25.6 and 22.7, respectively). Within 2 h after the stimulation, the injected oocytes were transferred into the oviducts of recipient gilts, of which estrous had been synchronized. The three recipients that received oocytes injected with Ca-I-treated sperm heads (77–150 oocytes per recipient) were not pregnant, whereas two of the four recipients given oocytes injected with control sperm heads (55–100 oocytes per recipient) were pregnant. On Day 117 post-transfer, three (a male and two female) healthy piglets farrowed by Caesarian-section. The results demonstrate clearly that *in vitro* matured oocytes injected with sperm heads are developmentally competent and can produce viable piglets. They also suggest that removal of the acrosome from the spermatozoan before injection does not affect the development of the blastocyst *in vitro*. This might not improve the production of piglets *in vivo*. Using IVM oocytes that can be prepared less costly and time-consuming than *in vivo* matured oocytes, IVM-ICSI is now expected to apply for the generation not only in scarce animals but also in transgenic animals.

## EVALUATION OF ACTIVATION PROTOCOLS FOR BOVINE INTRACYTOPLASMIC SPERM INJECTION

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For bovine intracytoplasmic sperm injection (ICSI) embryos, additional stimuli are generally required to induce oocyte activation, male pronuclear formation and embryo development [Keefer et al., 1990]. Activation protocols for ICSI differ from those effective for nuclear transfer embryos, in that second polar body (SPB) extrusion is a requisite for normal development following ICSI. Bovine oocytes can be activated by chemical or physical methods, which may be combined with treatments including cycloheximide (CHX) and 6-dimethylaminopurine (6-DMAP). We compared several activation regimes for bovine oocytes in order to determine: (a) their ability to induce haploid development of parthenotes and (b) developmental potential of haploid parthenotes in vitro. Bovine oocytes matured in vitro for 22–24 h, using methods described previously [Daniels et al., 2000], were denuded with 0.1% hyaluronidase and subjected to one of the following treatments: (1) activated with 5  $\mu$ M calcium ionophore A23187 for 5 min (Ca-I); (2) activated with Ca-I and immediately incubated for 4 h in presence of 2 mM 6-DMAP (DMAP-I); (3) a 4-h incubation with 2 mM 6-DMAP commenced 3.5 h after Ca-I activation (DMAP-II); (4) activated with Ca-I followed by a 5-h incubation in the presence of 10  $\mu$ g/ml cycloheximide (CHX); (5) injected with approximately 10  $\mu$ l of a 7 mg/ml solution of sperm factor (SF, per kind favour of Dr. Nancy Ruddock); (6) sham injected with medium alone (SHI). Following activation oocytes were examined for extrusion of the second polar body (SPB), cultured in SOF medium supplemented with 5% FCS for 7 days and development was recorded on Days 2, 4, 6 and 7. Results are presented in Table 1. All treatments tested, except DMAP-I, were able to induce SPB extrusion. SPB extrusion was reduced in DMAP-II group compared Ca-I and CHX groups ( $P < 0.1$ ). The rate of SPB expulsion was lower in SHI group than all other groups ( $P < 0.01$ ). The low cleavage was observed in SHI and Ca-I alone groups and none of the activated oocytes developed beyond the 8-cell stage. Injection of SF resulted in rates of cleavage similar to those seen in DMAP-II and CHX groups. Also, the rates of oocytes capable of developing to the 32-cell stage were similar in all three groups. Only oocytes from DMAP-I, DMAP-II and CHX groups were able to develop to the compact morula stage. The rate of compact morulae was higher in DMAP-I group ( $P < 0.01$ ) than in DMAP-II and CHX groups, which were not significantly different from each other. Only DMAP-I and CHX group oocytes developed to blastocysts although in the CHX group the rate of blastocyst development was extremely low (1.8%) compared with DMAP-II group ( $P < 0.01$ ). In conclusion the data suggest, that of the activation regimes tested the CHX protocol is most suitable for bovine ICSI. Also a combination of SF injection and CHX activation might facilitate the outcome of ICSI in cattle.

Table 1

In vitro development of bovine oocytes following various parthenogenetic activation protocols

Group	Treatment	<i>n</i>	Extrusion of SPB (%)	Cleaved (<8; %)	Cleaved (8–32; %)	Compact morula (%)	Blastocyst (%)
1	Ca-I	75	69 (92) <sup>a</sup>	1 (1.3) <sup>a</sup>	0		
2	DMAP-I	71	0	38 (53.5) <sup>b</sup>	23 (32.4) <sup>a</sup>	21 (29.6) <sup>a</sup>	15 (21.1) <sup>a</sup>
3	DMAP-II	56	49 (88) <sup>ab</sup>	8 (14.3) <sup>ab</sup>	3 (5.4) <sup>b</sup>	3 (5.4) <sup>b</sup>	0
4	CHX	56	55 (98.2) <sup>a</sup>	11 (19.6) <sup>ab</sup>	5 (8.9) <sup>b</sup>	4 (7.1) <sup>b</sup>	1 (1.8) <sup>b</sup>
5	SF	49	18 (36.7) <sup>b</sup>	7 (14.3) <sup>ab</sup>	2 (4.1) <sup>b</sup>	0	0
6	SHI	70	9 (12.9) <sup>c</sup>	1 (1.4) <sup>a</sup>	0		

Values in columns with different superscripts (a, b, and c) differ  $P < 0.01$ .

# Superovulation

## DOSE-RESPONSE TRIAL IN *BOS TAURUS* VS. *BOS INDICUS* COWS SUPERSTIMULATED WITH FSH, ASSOCIATED WITH CONTROLLED LH SURGE AND FIXED-TIME ARTIFICIAL INSEMINATION

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The aim of this study was to compare the optimal dose of FSH necessary to superstimulate *Bos taurus* versus *Bos indicus* cows, using a superstimulation protocol with controlled LH surge and fixed-time AI [Nogueira et al., Theriogenology 2002;57:1625–1634]. In Experiment 1, Simmental (*Bos taurus*,  $n = 6$ ) and Nelore (*Bos indicus*,  $n = 6$ ) received different doses of FSH (100, 200 or 300 mg) in a complete block design. Cows in the three groups were treated with a progesterone intravaginal device (CIDR-B<sup>®</sup>, 1.9 g progesterone, InterAG, Hamilton, New Zealand) plus estradiol benzoate (EB, Estrogin<sup>®</sup> 3 mg, i.m., Farmavet, Sao Paulo, Brazil) at unknown stages of the estrous cycle. Five days later (D0) the animals were superstimulated with pFSH (Folltropin<sup>®</sup>-V 100, 200 or 300 mg, Vetrepharm, Ont., Canada) administered twice daily in decreasing doses (40, 30, 20 and 10% of the total) from Days 0 to 3. All cows were treated with a luteolytic dose of prostaglandin F<sub>2α</sub> on Day 2 (a.m.) and the CIDR-B devices were removed 36 h later (D3, p.m.), just after the last injection of FSH. Ovulations were induced by pLH (Lutropin<sup>®</sup> 25 mg, Vetrepharm, Ont., Canada) on D4 (a.m., 48 h after PGF<sub>2α</sub>), and cows received fixed-time AI 12 and 24 h after the LH injection. This protocol was called “P36” because progesterone source (CIDR-B) was removed 36 h after PGF<sub>2α</sub> administration. The embryos/ova were recovered on Day 11 and graded according [Lindner and Wright, Theriogenology 1983;20:407–416]. Results are shown in Table 1. In Experiment 2, the dose of FSH that produced the higher superovulatory response in Nelore cows (200 mg) was tested in a commercial embryo transfer program, in which the semen quality varied among the sires used. Nelore cows were superstimulated (total dose is 200 mg) using the same protocol (P36) described in Experiment 1, except that the number of semen straws varied according to the quality of the semen (ranging from one to two straws each AI session) and the AI schedule was adjusted to the semen quality (double or triple AI sessions; at 12 and 24 h or 12, 24 and 36 h post-LH administration, respectively). Thus, the cows inseminated with poor-quality semen received a total of five straws in three AI sessions, while animals inseminated with good-quality semen received a total of two straws in two AI sessions. The number of total structures and transferable embryos recovered from 27 cows treated with 200 mg of FSH (protocol P36) was  $13.0 \pm 1.47$  and  $10.1 \pm 1.33$ , respectively. In conclusion, in spite of the reduced number of animals used in Experiment 1, the results indicate that the optimal superstimulatory dose of FSH may be lower for Nelore (*Bos indicus*, 200 mg) when compared to Simmental cows (*Bos taurus*, 300 mg). Additionally, the exogenous LH administration in P36 protocol allowed fixed-time AI, and yield embryo rates comparable to conventional embryo transfer programs with estrus detection [Nogueira et al.]. In Experiment 2, the effectiveness of FSH dose (200 mg) to superstimulate. Nelore cows was confirmed in a commercial ET program.

Table 1

Embryo production in Simmental (S;  $n = 6$ ) and Nelore (N;  $n = 6$ ) cows superstimulated with 100, 200 or 300 mg of FSH (Folltropin<sup>®</sup>-V; mean  $\pm$  S.E.M.)

	100 mg		200 mg		300 mg	
	S	N	S	N	S	N
Total structures (embryos/ova)	$3.2 \pm 0.95^a$	$3.0 \pm 0.86^*$	$13.5 \pm 3.45^{a,b}$	$12.2 \pm 5.05^*$	$15.32 \pm 0.14^b$	$9.0 \pm 2.13^*$
Transferable embryos	$1.5 \pm 0.56^a$	$2.5 \pm 0.81^*$	$6.3 \pm 1.91^a$	$7.8 \pm 2.86^*$	$10.0 \pm 3.02^b$	$4.5 \pm 1.29^*$

Values with different superscripts (a and b), within rows (in the same breed) differ ( $P \leq 0.05$ ; Kruskal–Wallis test).

\*  $P = 0.068$ .

## SUCCESSFUL CLINICAL MANAGEMENT OF REPEAT SUPERSTIMULATED BRAHMAN DONORS EXHIBITING CHRONIC CYSTIC OVARIAN DEGENERATION

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Cystic Ovarian Degeneration (COD) is a major cause of wastage in valuable Brahman donors in Central Queensland, Australia. Cows have shown COD after two to seven repeat superovulation programs resulting in their being dropped from commercial programs and, in many cases, eventual culling for slaughter. A treatment protocol needs to be user friendly, require no specialist skills or equipment, use readily available drugs, and not need to differentiate between follicular and luteal cysts or their hormone production states. The chronic COD Brahman donor cows, 4–8 years of age, ( $n = 4$ ) had all exhibited anestrus or nymphomania with diagnosed COD for a least the previous season with recurrence in spite of GnRH and LH treatments. These COD donors were synchronized with Crestar<sup>®</sup> (Intervet (Australia) Pty Ltd., Castle Hill, NSW 2154, Australia) using two ear implants each of 3 mg norgestomet and one intramuscular injection of 3 mg norgestomet and 5 mg oestradiol valerate. At this time the ovaries were examined ultrasonographically using a 6.0 MHz rectal probe (100 Falco Vet, Pie Medical, The Netherlands) to locate and measure luteal and follicular cysts. As many as possible anechoic ovarian structures greater than 10 mm diameter were drained using an ovum pickup needle operated blindly (not ultrasound guided) or manually ruptured if large with a fragile wall. Superstimulation commenced 7 days later using FSH (Folltropin<sup>®</sup>-V, Vetrepharm Canada Inc., Ont., Canada) injected i.m. twice daily during 4 days in decreasing amounts (50/50, 40/40, 30/30, 20 mg). The donors ears were checked at the time of the first injection to confirm that both implants were still present. If one was missing it was replaced immediately and if both were missing that donor was rejected from the program. On the first or second day of FSH treatment all anechoic structures greater than 10 mm diameter were drained blindly. Dinoprost (Lutalyse, Pharmacia (Australia) Pty Ltd., Rydalmere, NSW 2116, Australia) was injected (25 mg, i.m.) at the time of the fifth FSH treatment. The two ear implants were removed at the time of the sixth FSH treatment. Donors were in standing estrus on the beginning of the fifth day and inseminated at both 12 and 24 h later. Gonadorelin (Fertagyl, Intervet) was injected (0.5 mg, i.m.) at the time of the first insemination. The donors were flushed 7 days after estrus and transferable embryos were transferred non-surgically to synchronized recipients. A total of 24 embryos (7, 5, 5, 7) were collected of which 12 (4, 4, 2, 2) embryos were of transferrable quality. Excess transferrable embryos (2) were frozen and 10 embryos (4, 2, 2, 2) transferred, resulting in three pregnancies (1, 1, 1, 0). This compares with 15 pregnant from 35 transfers over seven flushes in the control group. All anechoic cystic structures were eliminated. Blood progesterone at the time of first FSH injection varied between 0.39 and 5.05 ng/ml in similar studies. The donor was under the influence of progesterone (or an analog) for at least 9 days. It is important that all anechoic structures greater than 10 mm diameter are eliminated by the second day of FSH treatment. Some of the larger follicular cysts refilled with fluid after the first needle drainage. This protocol is now used as a commercial procedure and has gained an extra 2 years production from these chronic COD donors.

CHANGE IN CONCENTRATION OF PORCINE FSH IN BOVINE PERIPHERAL  
BLOOD FOLLOWING A SINGLE INTRAMUSCULAR INJECTION  
WITH ALUMINUM HYDROXIDE GEL SUSPENSION

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In cattle, conventional superovulation is induced by serial injections of FSH for 2–3 days, which is time- and labor-consuming, and gives strong stress to donor cows. Aluminum hydroxide is extensively used as adjuvant and excipient as it has an ability to adsorb large molecules. The objective of this study was to examine the ability of aluminum hydroxide gel for a sustained release of FSH to simplify the induction method of superovulation in cattle. Aluminum hydroxide gel was suspended in 10 mM PBS (pH 7.2) at 1.6–1.8 mg/ml aluminum. In Experiment 1, 30 Armour unit (AU) of lyophilized porcine FSH (Antrin; Denka Pharmaceutical Co.) was dissolved in 1 ml of physiological saline. The FSH solution was gently mixed with 4 ml of aluminum hydroxide gel suspension in a 5-ml glass tube. The mixture was centrifuged at  $1500 \times g$  for 15 min and 4.5 ml of supernatant was extracted from the tube. The precipitate was re-suspended with 4.5 ml of 1% BSA PBS. Then, 4.5 ml of the supernatant was collected after centrifugation. Porcine FSH in the supernatant was measured by radioimmunoassay. Anti-porcine FSH rabbit serum and purified porcine FSH for iodination and reference standard were purchased from UCB Bioproducts (Belgium). In Experiment 2, 20 or 30 AU of FSH was dissolved in 1 ml of physiological saline. The FSH solution was gently mixed with 4 ml of aluminum hydroxide gel suspension in a 5-ml disposable syringe. The mixture was administered to Japanese Black cows into gluteal muscle by a single injection. Blood was collected from jugular vein at 0 (before injection), 5, 10, 15, 20, 30 and 40 min, 1, 1.5, 2, 3, 4, 6, 8 and 12 h, 1, 2, 3, 4, 5, 6 and 7 days after injection. Harvested plasma was stored at  $-35^{\circ}\text{C}$  until assayed. Porcine FSH concentrations were measured by radioimmunoassay with 100  $\mu\text{l}$  of the plasma. In Experiment 1, 0.090 and 74.2% of total FSH were included in the first and second supernatant, respectively. These results mean that 99.9% of FSH were adsorbed to the gel under protein free condition and a greater dose of BSA replaced and released FSH from the gel. In Experiment 2, as shown in the figure, porcine FSH was not detected in bovine circulation during first 2 h after injection. The subsequent concentration gradually increased to a peak at 12 h after injection, then gradually decreased. It was still detected at 2 days after injection but not detected at the third day. The mean level of porcine FSH at 12 h was 1.5 times higher in cows injected 30 AU FSH than in cows with 20 AU injection. In conclusion, the results of the present study suggest that aluminum hydroxide gel has an ability to adsorb FSH and it is useful for injectable sustained-release preparation of FSH for ovarian superstimulation.

## AMINO ACID CONCENTRATIONS IN OVIDUCT FLUID OF SUPEROVULATED EWES DURING THE PERI-OVULAR PERIOD

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This study examined the concentrations of amino acids in oviduct fluid during the peri-ovular period as a reference for the establishment of optimal *in vitro* culture conditions for sheep embryos. Measurements were made in superovulated ewes because of the ability to control the time of ovulation (using GnRH) compared with spontaneously cycling ewes [Walker et al., *J Reprod Fertil* 1986;77:135–142]. Six mature ewes (4–5 years, 58–67 kg) of comparable body condition were fed a standard diet for 2 weeks before the start of collection. Ewes were superovulated using conventional treatment involving a progestagen, FSH and GnRH treatment. Oviducts were catheterized 18 days before collection and fluid was collected every 24 h, commencing 1 day (Day-1) before the time of ovulation (Day 0) and continuing until 5 days after ovulation (Day 5). The mean daily collection volume was  $1.37 \pm 0.13$  ml. Amino acid analyses were performed by high-performance liquid chromatography methods (HPLC; Waters Alliance 2690XE Separation module) involving automated precolumn derivatization using an AccQfluor reagent (Waters AccQTag kit) and fluorescent detection (474 Scanning Fluorescence detector and 150 mm AccQTag Column). The data were analyzed by ANOVA. The number of corpora lutea per ewe (range: 3–19) did not significantly influence the concentrations of the 17 amino acids measured. From the repeated measures ANOVA it can be concluded that the concentrations of arginine, aspartic acid, cystine, methionine, serine, proline, threonine and tyrosine did not change significantly over time. However, for glycine, histidine, isoleucine, leucine, phenylalanine and valine, the effect of day was significant ( $P < 0.05$ ). Glycine, alanine, lysine, leucine, histidine, valine and glutamic acid were present in high concentrations (Table 1) compared with published plasma levels [Nancarrow et al., *Proc Aust Soc Reprod Biol* 1992;71 (abstract)]. These concentrations were significantly ( $P < 0.05$ ) decreased between Day-1 and Day 1 (range: of decrease 13–53%) and significantly ( $P < 0.05$ ) increased between Days 1 and 3 inclusively (range of increase 32–159%), the latter period corresponding with the time embryos normally reside in the oviducts. The significance of these high and changing concentrations of amino acids is not known but further investigations are warranted to better characterize the oviductal environment of the superovulated ewe. It is noteworthy that the concentrations reported in this study are substantially higher than those reported in spontaneously cycling ewes [Nancarrow et al.] thus raising questions about the significance of amino acid concentrations and the developmental ability of embryos in superovulated ewes.

Table 1

Amino acids concentrations ( $\mu\text{M}$ ) in ovine oviductal fluid from Day-1 to Day 5 after the expected time of ovulation (Day 0)

	Glycine	Histidine	Isoleucine	Leucine	Valine	Phenylalanine
Day 1	3261 $\pm$ 512	170.0 $\pm$ 7.1	78.3 $\pm$ 9.5	200.8 $\pm$ 19.5	185.7 $\pm$ 15.4	52.4 $\pm$ 6.6
Day 0	2752 $\pm$ 454	147.7 $\pm$ 11.4	66.7 $\pm$ 9.8	174.4 $\pm$ 23.4	154.7 $\pm$ 12.1	49.2 $\pm$ 12.1
Day 1	1528 $\pm$ 280	118.3 $\pm$ 11.4	54.3 $\pm$ 6.2	157.4 $\pm$ 13.8	125.7 $\pm$ 14.3	45.6 $\pm$ 12.8
Day 2	2021 $\pm$ 327	136.1 $\pm$ 18.6	69.6 $\pm$ 9.6	188.6 $\pm$ 24.2	180.3 $\pm$ 24.2	54.0 $\pm$ 18.1
Day 3	3960 $\pm$ 860	197.0 $\pm$ 18.3	106.0 $\pm$ 14.7	263.0 $\pm$ 33.1	245.0 $\pm$ 24.1	60.3 $\pm$ 13.1
Day 4	5769 $\pm$ 1738	224.3 $\pm$ 33.5	107.5 $\pm$ 11.4	267.5 $\pm$ 25.3	257.2 $\pm$ 118.3	62.2 $\pm$ 10.1
Day 5	6868 $\pm$ 1611	261.4 $\pm$ 44.1	107.1 $\pm$ 14.7	272.0 $\pm$ 31.9	240.9 $\pm$ 27.7	116.1 $\pm$ 19.9.

Values are mean  $\pm$  S.E.M.

OVARIAN SUPERSTIMULATION BY A SINGLE ADMINISTRATION OF  
PORCINE FSH AND ALUMINUM HYDROXIDE GEL SUSPENSION IN RATS:  
COMPARISON WITH CONVENTIONAL SERIAL INJECTION AND  
POLYVINYLPIRROLIDONE EMULSION METHODS

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To develop a simple induction method of superovulation, aluminum hydroxide gel suspension was employed for sustained release preparation of FSH. Aluminum hydroxide is extensively used as adjuvant and excipient as it has an ability to adsorb large molecules. The objective of this study was to compare the efficacy of ovarian superstimulation by aluminum hydroxide gel suspension of FSH with conventional serial injection and polyvinylpyrrolidone emulsion methods. Each method was evaluated by *in vivo* induction of ovarian weight gain in female Wistar–Imamichi rats at 3 weeks of age [Steelman and Pohley, *Endocrinology* 1953;69:604–616]. Antrin R10 and Gestron 1, 500 (Denka Pharmaceutical Co.) were utilized as porcine FSH and hCG, respectively. Aluminum hydroxide gel suspension was made by reaction of  $AlCl_3$  and NaOH. In Groups A (negative control) and B (positive control), animals were subcutaneously injected 0.5 ml of physiological saline twice daily in the morning and the evening for 3 days from Day 0. In Group B, 0.1 (B1) or 0.2 (B2) Armour unit (AU) of FSH and 40 IU hCG were administered with the saline in total of six injection. In Groups C (FSH or hCG with aluminum hydroxide gel), D (aluminum hydroxide gel adsorption), (PVP emulsion) and F (vehicle free control). Animals were administered 1 ml of the following drugs by single subcutaneous injection in the morning on Day 0; 0.3 AU FSH (C1) or 40 IU hCG (C2) with aluminum hydroxide gel containing 1.28 mg/ml aluminum in Group C, the mixture (six combinations) of 0.2 or 0.3 AU FSH, 40 or 60 IU hCG and aluminum hydroxide gel containing 0.56, 0.72, 1.28 or 1.44 mg/ml aluminum in Group D, 0.3 AU FSH and 60 IU hCG suspended in 24% (w/v) PVP K30 (Group E) or dissolved in physiological saline (Group F). Experiments in Groups A, B and were repeated twice. Ovarian weights were measured in the afternoon on Day 3 and statistically analyzed using Student's *t*-test. The weights (mean  $\pm$  S.D.,  $N = 5$ ) were  $24.9 \pm 5.5$  and  $22.6 \pm 4.1$  mg in Group A, whereas those were  $110.3 \pm 20.2$  and  $106.2 \pm 10.3$  mg in Group B1, and  $147.5 \pm 17.2$  and  $132.6 \pm 22.2$  mg in Group B2. Ovaries were  $36.8 \pm 11.3$ ,  $52.5 \pm 4.6$  and  $50.8 \pm 7.9$  mg in Groups C1, C2 and F, respectively. In Group E, the weights were  $108.8 \pm 10.4$  and  $122.9 \pm 25.1$  mg, which were similar to the weights in Group B1 and significantly heavier than the weights in Groups A, C and F. In Group D, although 0.3 AU FSH and 60 IU hCG were administered, weights of ovaries were  $59.6 \pm 11.3$  and  $58.0 \pm 4.5$  mg in rats injected 0.56 and 0.72 mg/ml aluminum, respectively. They were similar to Groups C and F but significantly lighter than Group B. The weights in 1.28 and 1.44 mg/ml aluminum groups were from  $88.5 \pm 19.8$  to  $109.8 \pm 10.8$  mg. They were significantly heavier than the weights in Groups A, C and F, and similar to the weights in Group B1 as well as in Group E. These results demonstrate that a single injection of FSH with aluminum hydroxide gel suspension at 1.28–1.44 mg/ml aluminum as well as 24% PVP emulsion had an effect on rat ovary similar to the conventional serial injection method. In conclusion, a single injection of aluminum hydroxide gel suspension of FSH has an ability to induce follicular growth similar to conventional and PVP emulsion methods in rats.

## SUPEROVULATION IN CATTLE USING SUBCUTANEOUS OSMOTIC PUMPS

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Continuous gonadotropins infusion via subcutaneous osmotic pumps has been considered a method of superovulation in cows and it requires less than twice daily injections [Wubishet et al., *Theriogenology* 1986;25–26:809–812; *Theriogenology* 1991;35–32:451–457]. Twice daily injection gave higher results than continuous infusion; however, different doses of gonadotropins were used. To our knowledge, no investigation has been done to prove which of the two methods is more efficient using equal doses of gonadotropins. The aim of this study was to compare the two different superovulation treatments administering equal doses of gonadotropins over the same period. Holstein–Friesian cows with functional CL received an i.m. injection of 0.15 mg PGF<sub>2α</sub> [Veteglan, Laboratorios Calier, Barcelona, Spain]. Total 72 h after PGF<sub>2α</sub>, cows were checked for estrus (Day 0) and 10 were assigned to one of the two treatments: T1 (five cows) declining doses of pFSH-LH (Pluset, Laboratorios Calier, Barcelona, Spain) by i.m. injection twice daily for 5 days (750 IU); T2 (five cows) continuous infusion of gonadotropins (Pluset) 150 IU per day using a subcutaneous osmotic pump (Alzet Model 2 ml, Alza Corp., CA); pumps were removed after 5 days of treatment. To reduce the variability among animals, all follicles ≥5 mm in diameter were removed by ultrasound-guided transvaginal aspiration 24 h before the beginning of superovulation (Day 9). On Day 14 (8 a.m. and 8 p.m.) all cows received 0.15 mg PGF<sub>2α</sub> (Veteglan) by i.m. injection and were inseminated twice, 48 and 60 h after the first PGF<sub>2α</sub> injection, with frozen–thawed semen from the same bull. Embryo recovery was performed 7 days after AI (Day 23). Data from T1 and T2 were compared by chi-square test; the number of viable embryos per cow of T1 and T2 were compared using a non-parametric test (Wald Wolfowitz Runs Test). Descriptive analysis (mean and standard deviation) and tests were elaborated applying a software package (Statistica for Windows, Stat. Soft Inc., Tusla, USA). Results are shown in Table 1. There were no significant differences ( $P > 0.05$ ) between the two treatments in the number of unfertilized oocytes, viable embryos and in the number of viable embryos per cow. The number of degenerate embryos between treatments was at the limit of significance ( $P = 0.05$ ). Significance has been assessed for  $P < 0.05$ . These results indicate that, even if subcutaneous osmotic pumps did not improve the total number of embryos as well as the variability of the superovulatory response, they can be an effective alternative to i.m. treatment to induce superovulation in cows and could be considered the optimal method in animals difficult to handle.

Table 1  
Superovulatory results from cows treated with two different methods

S.O. treatment	Unfertilized oocytes (%)	Degenerate embryos (%)	Viable embryos	Mean ± S.D. viable embryos per cow
T1	48 (54.5)	7 (8.0) <sup>a</sup>	33 (37.5)	6.6 ± 7.7
T2	26 (55.3)	9 (19.2) <sup>b</sup>	12 (25.5)	2.4 ± 2.2

a vs. b:  $P = 0.05$  T1 regular 10× administration of pFSH-LH; T2 continuous administration of pFSH-LH; 750 IU total dose for T1 and T2.

SUPEROVULATORY RESPONSE DURING THE FIRST FOLLICULAR WAVE  
IN NELORE (*BOS INDICUS*) DONORS

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Superstimulatory (SPO) treatments initiated during the first follicular wave in *Bos taurus* cattle, have been shown to induce a response similar to those initiated during mid-cycle. It has also been demonstrated that optimal SPO responses will be obtained when treatments were initiated on the day of ovulation [Nasser et al., Theriogenology 1993;40:713–724]. The purpose of this study was to evaluate the responsiveness of Zebu (*Bos indicus*) donors to SPO treatments administered during the first follicular wave. Eighteen Nelore donor cows were randomly allocated to one of three treatment groups. Cows in Groups 1 and 2 were SPO during the first follicular wave and cows in Group 3 were superstimulated after synchronized follicular wave emergence. All cows received a CIDR-B device (1.9 g of P4, Pharmacia, Brazil) and an i.m. injection of 50 mg of progesterone (P4, Index Farmaceutica, Brazil) and 2.5 mg of estradiol benzoate (EB; Estradiol 10, Lab. Río de Janeiro, Argentina) on Day 0 (randomstages of the estrous cycle). Cows in Groups 1 and 2 were also treated with 125 µg D(+) cloprostenol (PGF, Preloban, Hoechst, Brazil) on Day 4 and 12.5 mg Armour of pLH (Lutropin-V, Vetrepharm Canada Inc.) 24 h after CIDR-B removal (Day 10), to synchronize ovulation [Martinez et al., Theriogenology 2002;57:1049–1059]. Superstimulatory treatments were initiated on Day 11 (expected time of ovulation); donor cows in Group 2 also received a new CIDR-B device at the time of the first FSH injection. Donor cows in Group 3 (controls) were SPO starting on Day 4 (expected time of emergence following treatment with EB and P4). Donors in the three groups were SPO with a total dose of 133 mg NIH-FSH-P1 of Folltropin<sup>®</sup>-V (FSH, Vetrepharm Canada Inc.) divided into twice daily injections of the same dosage (13.3 mg diluted in 1 ml) over 5 days. On the last day of FSH treatment, all animals received PGF after each FSH injection and cows in Groups 2 and 3 had their CIDR-B removed at the time of the last FSH injection. All cows received 25 mg of pLH 24 h after the last FSH treatment and were artificially inseminated 12 and 24 h later. Ova/embryo collection and evaluation were done 7 days after LH by the same veterinarian. Results, summarized in the table, indicate that there was no difference in the superovulatory response in CIDR-B-treated donor cows when SPO treatments were initiated at the time of emergence of either the first follicular wave (Group 2) or following synchronization of follicular wave emergence with EB + P4 (Group 3), but both were greater than when SPO treatments were initiated at the time of emergence of the first follicular wave without the use of a CIDR-B device ( $P < 0.05$ ). Results suggest that the addition of progesterone improves embryo quality in Nelore donor cows superstimulated during the first follicular wave.

Groups	N	Total	Fertilized	Transferable
1	6	8.3 ± 7.0	1.3 ± 3.3 <sup>a</sup>	0.2 ± 0.4 <sup>a</sup>
2	6	11.7 ± 7.1	9.2 ± 5.0 <sup>b</sup>	8.0 ± 4.5 <sup>b</sup>
3	6	9.2 ± 5.6	6.7 ± 4.8 <sup>ab</sup>	6.7 ± 4.8 <sup>b</sup>

Means within columns with different superscripts (a and b) differ ( $P < 0.05$ ).

EFFECT OF THE INTERVAL BETWEEN CIDR AND ESTRADIOL BENZOATE ADMINISTRATION AND INITIATION OF FSH INJECTION ON THE SUPEROVULATORY RESPONSE IN JAPANESE BLACK CATTLE

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Conventional superovulation programs begin with the injection of FSH at 8–12 days after estrus. Recently, intentional superovulation became possible by using CIDR and estradiol benzoate (EB), without the necessity of estrus detection. The objective of this study was to investigate the effect of the interval between CIDR and EB administration and the initiation of FSH injection on the superovulatory response in Japanese Black cattle. Japanese Black cows ( $n = 27$ ) were randomly allocated to one of three treatment groups. Cows received an intravaginal progesterone device (CIDR-B, InterAg, New Zealand) combined with 2 mg EB at a random stage of the estrous cycle (Day 0). Superstimulatory treatments were initiated on Days 5 (Group A), 6 (Group B), or 7 (Group C) with a total dose of 18 or 24 mg FSH via twice daily i.m. injections for 3 days in decreasing doses. PGF<sub>2α</sub> (PG) was administered in the morning (25 mg) and afternoon (15 mg) of the last day of FSH injection. The CIDR-B was removed at the time of the second PG injection. Two days after the PG injection, cows were injected i.m. with 100 μg GnRH, and were artificially inseminated (AI) the next morning. Ova/embryos were collected non-surgically 7 days after AI. The follicular dynamics of the ovaries were observed during FSH injection and at the time of AI by means of ultrasonography. Follicles were classified according to diameter into small ( $\geq 3$  mm), medium (4–7 mm), and large ( $\leq 8$  mm) categories. Data were analyzed using ANOVA. The results are presented in Table 1. There were no differences in the mean number of follicles. However, the number of small and middle follicles at the time of FSH injection in Groups B and C was fewer than those of Group A ( $P < 0.05$ ), and the number of large follicles at the time of AI in Groups B and C showed a tendency to be more numerous than those of Group A. There were no differences among groups regarding the mean number of CL. Total ova/embryos and the number of viable embryos the Group B showed a tendency of being more numerous than those of Groups A and C. These results suggest that superovulatory response in cows may be superior when the interval between FSH and CIDR-B/EB is 6 days.

Table 1

Effect of interval between CIDR-B and EB administration and initiation of FSH injection on superovulatory response in Japanese Black cattle

Group	No. of donors	No. of corpora lutea	Total ova/embryos	No. of viable embryos	No. of unfertilized ova	No. of degenerated embryos
A	9	7.9 ± 2.1	5.9 ± 2.6 <sup>b</sup>	3.2 ± 1.2 <sup>b</sup>	0.9 ± 0.5	2.0 ± 1.5
B	9	11.9 ± 1.5	10.8 ± 1.6 <sup>a</sup>	5.6 ± 0.9 <sup>a</sup>	2.8 ± 1.8	2.7 ± 0.9
C	9	11.1 ± 1.9	6.6 ± 1.4 <sup>b</sup>	2.6 ± 1.2 <sup>c</sup>	0.9 ± 0.5	3.1 ± 1.1

Values were means ± S.D. Values with different superscripts (a, b and c) tended to differ ( $P < 0.1$ ).

EFFECT OF ESTRADIOL BENZOATE AND GnRH ON SUPEROVULATORY RESPONSE AND SUBSEQUENT REPRODUCTIVE PERFORMANCE IN POSTPARTUM SUCKLING JAPANESE BLACK CATTLE

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To improve the reproductive efficiency of Japanese Black cattle, superovulation using CIDR has been attempted in postpartum suckled cows shortly after calving. The objective of this study was to investigate the effect of estradiol benzoate (EB) and GnRH on superovulatory response and subsequent reproductive performance in postpartum suckled Japanese Black cows. Nineteen suckling postpartum Japanese Black cows aged between 2.8 and 10.9 years old that were 40 or 41 days after calving were divided into three groups: seven cows in EB and GnRH-treatment group (EB/GnRH), six cows in GnRH-treatment group (GnRH) and six cows in the control group. In all groups, CIDR (Easy-Breed; InterAg, Hamilton, New Zealand) were inserted with 5 mg EB (Denka Pharmaceutical, Kawasaki, Japan) injection at 40 or 41 days after calving. FSH (Antrin 40; Denka Pharmaceutical) was injected in gradually decreasing doses in total 20 IU from Day 5 (Day 0 = the day of CIDR insertion) to Day 7. CIDR removal and 0.75 mg cloprostenol (PG; Estrumate; Takeda SPAH, Osaka, Japan) injections were carried out on Day 7. In EB/GnRH group, 5 mg EB was injected on Day 8. After PG injection, all animals were observed several times daily to detect estrus, and artificial insemination (AI) was carried out twice on the basis of onset of estrus (The first and second AI were carried out 8–16 and 24 h, respectively, after onset of estrus.) In EB/GnRH and GnRH groups 0.1 mg GnRH (Conceral; Takada SPAH) was injected directly after the first AI. Embryos were non-surgically collected on Day 7 or 8 after estrus. PG injection (0.75 mg) and 2% povidone-iodine (Meiji Seika, Tokyo, Japan) intra-uterine injections were carried out after flushing. The collected embryos were examined for quality under an inverted microscope. The ovaries were examined by ultrasonography and the number of CL and the remaining follicles (RF) were counted at the time of flushing. Cows that showed estrus after flushing were artificially inseminated. Cows that showed recurrence of estrus were artificially inseminated once again. Pregnancy diagnosis was performed 30–40 days after AI by ultrasonography. Data were analyzed by one-way ANOVA and Tukey–Kramer's HSD. There were no significant differences in the number of CL (EB/GnRH:  $13.0 \pm 1.2$ ; GnRH:  $18.5 \pm 10.3$ ; Control:  $16.8 \pm 7.1$ ), RF (EB/GnRH:  $2.6 \pm 2.4$ ; GnRH:  $5.5 \pm 2.2$ ; Control:  $5.2 \pm 4.3$ ), the recovered ova or embryos (EB/GnRH:  $8.4 \pm 3.6$ ; GnRH:  $15.5 \pm 9.5$ ; Control:  $14.5 \pm 6.7$ ) and transferable embryos (EB/GnRH:  $5.7 \pm 3.0$ ; GnRH:  $7.7 \pm 2.9$ ; Control:  $3.2 \pm 4.2$ ). However, in the number of freezable embryos (EB/GnRH:  $3.7 \pm 2.8$ ; GnRH:  $6.8 \pm 2.7$ ; Control:  $1.7 \pm 3.1$ ), GnRH was significant higher ( $P < 0.05$ ) than control and EB/GnRH higher than control although there was no significant difference. The days of the first estrus detection after flushing were  $12.6 \pm 9.6$ ,  $7.5 \pm 1.8$  and  $7.2 \pm 1.5$  in EB/GnRH, GnRH and control, respectively, with no significant difference. The times of AI that were needed to impregnate after flushing was higher ( $P < 0.05$ ) in EB/GnRH ( $1.7 \pm 0.5$ ) than those of others (GnRH:  $1.0 \pm 0$ ; Control:  $1.2 \pm 0.4$ ). The days that were needed to impregnate after flushing were  $97.4 \pm 37.3$ ,  $68.3 \pm 8.3$  and  $79.3 \pm 37.1$  in EB/GnRH, GnRH and control, respectively, with no significant difference. The results suggest that EB and/or GnRH treatment on superovulation may be effective for the recovery of freezable embryos in postpartum suckling Japanese Black cows shortly after calving, and the superovulation treatment and flushing gave no detrimental effects on subsequent fertility.

FERTILIZATION RATE AND EMBRYO QUALITY IN SUPEROVULATED  
HOLSTEIN HEIFERS AND LACTATING COWS ARTIFICIALLY INSEMINATED  
IN THE BODY OF THE UTERUS OR IN THE TIP OF THE UTERINE HORNS

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Two studies compared deep uterine horn AI versus uterine body AI in superovulated Holstein heifers and lactating cows. The heifer study also evaluated the superovulatory response to two different doses of FSH. Fourteen lactating cows and 20 heifers (12–16 months) underwent two superovulatory treatments. Cows and heifers received decreasing doses of FSH for 5 days. All cows received a total equivalent of 300 mg NIH-FSH-P1. Heifers received either 150 or 300 mg of FSH in a cross-over design. Ovulation was induced with GnRH 12 h after the last FSH injection and AI was performed with frozen-thawed commercial semen 12 h after GnRH. Embryos/ova were recovered 7 days after GnRH, counted, and graded. Number of ovulations was estimated by ultrasound. Treatment effects were compared using *t*-test or chi-square analyses. In cows, percentage of fertilized ova ( $60.3 \pm 10.0\%$  versus  $39.6 \pm 9.4\%$ ), and number of transferable embryos ( $3.7 \pm 1.0$  versus  $1.6 \pm 0.6$ ) recovered per flush were greater ( $P < 0.07$ ) for uterine body AI. However, in heifers, percentage of fertilized ova ( $53.2 \pm 12.3\%$  versus  $68.1 \pm 7.9\%$ ), and number of transferable embryos ( $1.2 \pm 0.4$  versus  $2.4 \pm 0.8$ ) recovered per flush for uterine body AI and deep horn AI, respectively, did not differ ( $P > 0.1$ ). The higher dose of FSH produced more ovulations per heifer ( $14.1 \pm 2.1$  versus  $7.5 \pm 0.9$ ;  $P < 0.05$ ); however, fewer embryos/ova per CL per flush were recovered from heifers receiving the higher FSH dose ( $30.2 \pm 5.8\%$  versus  $44.2 \pm 5.9\%$ ;  $P < 0.05$ ). Number or percentage of fertilized ova, and number or percentage of transferable embryos recovered per heifer did not differ ( $P > 0.10$ ) between heifers receiving different doses of FSH. In conclusion, deep horn AI unexpectedly produced a lower fertilization rate in superovulated lactating cows. In addition, decreasing the dose of FSH to 37.5% of the recommended dose reduced the number of ovulations but did not reduce the number or percentage of transferable embryos recovered per heifer.

## A SIMPLE ULTRASOUND-TEST TO PREDICT SUPERSTIMULATORY RESPONSE IN CATTLE

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We tested the hypotheses that (1) the superstimulatory response is related to the intrinsic number of follicles recruited into a follicular wave, and (2) the number of follicles recruited into a wave is correlated to the number of follicles recruited into the successive wave. A positive correlation will form the basis of a simple ultrasound-based, non-invasive test for predicting the superstimulatory response of individual cows.

A group of 141 postpartum beef cows were treated with 2.5 mg estradiol-17 $\beta$  and 50 mg progesterone in 2 ml canola oil i.m. to induce a new follicular wave (first synchronization). On the day after anticipated wave emergence (i.e. 5 days after hormone treatment), the number of follicles  $\geq 2$  mm was recorded during transrectal ultrasonography using a 7.5 MHz ultrasound transducer (Aloka SSD 900). Cows were ranked according to the total number of follicles in the 2–3 and 4–6 mm categories to select the upper and lower 10% of the herd. Ten days after the E/P treatment, the high-end ( $n = 16$ ) and low-end ( $n = 20$ ) groups underwent transvaginal ultrasound-guided ablation of all follicles  $\geq 5$  mm to induce the emergence of a new follicular wave (second synchronization). Beginning 36 h after follicle ablation, cows were treated with FSH twice daily for 3 days (total dose of 200 mg Folltropin<sup>®</sup>-V i.m.; Vetrepharm, Canada) to induce ovarian superstimulation. The ovarian response was assessed by ultrasonography 1 day and 5 days after ablation. The number of follicles in  $\geq 2$ , 2–3, 4–6, 5–7,  $\geq 5$  and  $\geq 8$  mm categories was compared between the high-end and low-end groups by *t*-test. The relationship between the number of follicles at the beginning of the first and second synchronizations, and at the end of superstimulation was examined using Pearson's correlation coefficient (*r*).

At the beginning of both the first and second synchronizations, high-end cows had a greater number of follicles ( $P < 0.001$ ) than low-end cows in the 2–3 mm category ( $21.6 \pm 2.6$  versus  $9.5 \pm 1.4$  and  $32.8 \pm 2.8$  versus  $15.8 \pm 2.1$ ), and the 4–6 mm category ( $23.1 \pm 2.4$  versus  $7.0 \pm 0.9$  and  $17.6 \pm 2.6$  versus  $11.4 \pm 1.7$ ). The total follicle number of follicles  $\geq 2$  mm at wave emergence (data combined for first and second synchronizations) was greater ( $P < 0.0001$ ) in the high-end than in the low-end group ( $48.6 \pm 2.0$  versus  $23.1 \pm 1.6$ ). The number of follicles in the 2–3 and 4–6 mm categories at the time of wave emergence after the first and second synchronizations were positively correlated ( $P < 0.001$ ;  $r = 0.77$  and  $0.71$ , respectively). Superstimulatory treatment resulted in a greater number ( $P < 0.003$ ) of follicles in the 5–7 and  $\geq 8$  mm categories in the high-end group than in the low-end group ( $16.8 \pm 2.2$  versus  $8.1 \pm 0.9$  and  $22.7 \pm 4.1$  versus  $9.7 \pm 1.6$ ). The number of follicles  $\geq 5$  and  $\geq 8$  mm at the end of superstimulation was positively correlated ( $P < 0.001$ ) with the total number of follicles  $\geq 2$  mm at the time of wave emergence after the first ( $r = 0.64$  and  $0.54$ ) and second ( $r = 0.65$  and  $0.5$ ) synchronizations.

Data supported the stated hypotheses. The number of follicles recruited into successive waves was correlated, and the superstimulatory response was predicted by the number of follicles  $> 2$  mm at wave emergence. This simple test will permit the selection of donors expected to provide a greater superstimulatory response.

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# Tissue Culture

## EFFECT OF DONOR CELL AGE ON NUMBER OF POPULATION DOUBLINGS OF FELINE FIBROBLASTS CULTURED IN VITRO

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Somatic donor cells play a key role in successful cloning. Both the age of the cell donor animal and the number of doublings a cell population undergoes in culture are important factors governing developmental rates of reconstructed embryos [Julia et al., *Prostate* 1999;38:110–117]. Previous observations have shown that in several species, the proliferative lifespan of cells in culture reflects the aging changes in the donor from which the cells were originally obtained [Bierman, *In Vitro* 1978;14:951–955 (abstract)]. Although long-term cultured cells can produce offspring in cattle [Kubota et al., *PNAS* 2000;97:990–995], cloning efficiency appears to decrease more quickly for cells from adults than those from fetuses [Poothapillai et al., *Biol Reprod* 2001;64:1487–1493]. The aim of this study was to determine whether animal age could affect the number of population doublings (PD) for fibroblasts derived from skin of fetal or different-aged domestic cats. Fibroblast cell lines were established from the skin of a 5-week feline fetus, as well as a 6-month-old kitten, a 1-year-old and an 8-year-old adult domestic cat. Cells were cultured in DMEM plus 10% FBS and 2 mM L-glutamine. The cell lines were passaged to senescence. For each passage, there were two estimated PDs. Monoclonal antibodies directed against cytoskeletal filaments, vimentin (for fibroblasts) or cytokeratin (for epithelial cells) were used to confirm the phenotype of cultures. Our results showed that cell lines from the 5-week cat fetus, 6-month, 1 year-old and 8-year-old cats showed a gradual decline in proliferative potential. Fibroblasts from fetus had the longest in vitro lifespan with about 36 PDs, whereas cells from older cats had a gradually shortened life span, which were 14 PDs for the 6-month cat, 10 PDs for the 2-year-old cat, 8 PDs for the 8-year-old cat. Cell-specific markers staining showed vimentin was expressed in all the cell lines, but the expression of cytokeratin appeared to be negative, which demonstrated that these cells were fibroblasts. We conclude that the in vitro lifespan of primary fibroblast cell lines is animal age dependent in cats. Due to the long lifespan, feline fetal fibroblasts may be more suitable for genetic manipulations prior to cloning.

# Transgenesis

## USE OF ADENO-ASSOCIATED VIRUS FOR TRANSFECTION OF MALE GERM CELLS FOR TRANSPLANTATION IN PIGS

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To date, transgenic pigs have been produced by pronuclear microinjection and by somatic cell nuclear transfer. However, the efficiency of these techniques is low, and abnormal development in cloned animals often results in fetal loss and health problems in offspring. As an alternate, potentially more efficient approach, transplantation of transfected spermatogonial stem cells could be used to generate transgenic founder males. We developed a technique for germ cell transplantation in pigs and goats, allowing us to now explore manipulation of the male germ line in large animals. Gene transfer into spermatogenic cells has been unrewarding until recently successful retroviral transfection of mouse spermatogonial stem cells was reported. However, efficiency was low as retroviral vectors transfect only replicating cells and, unlike differentiating spermatogonia, stem cells are less actively dividing. Recombinant adeno-associated virus (rAAV) is a replication-defective, non-pathogenic human parvovirus that stably and site-specifically integrates into dividing and non-dividing cells. The objective of this study was to investigate rAAV for transduction of porcine germ cells. Testis cells were isolated by a two-step enzymatic digestion from testes of 1-week or 10-week-old pigs. In Experiment 1, cells were incubated in vitro with an rAAV-2 vector containing the green fluorescent protein (GFP) reporter gene and cytomegalovirus (CMV) promoter at 1, 10, 20, 50, 100, 1000 or 10000 infectious units (iu)/cell for 1 or 12 h before washing ( $n = 3$  replications). Expression of GFP was monitored daily for 1 week and weekly for 4 months by fluorescence microscopy. Germ cells were identified by immunohistochemistry for germ cell nuclear antigen 1 (GCNA-1). We could demonstrate GFP expression in germ cells, and we observed a high degree of stable GFP expression in a mixed population of porcine testis cells starting 2 days post-transfection, increasing for 2 weeks, and remaining high for at least 4 months in culture. There was a positive correlation between the virus titer and the level of GFP expression, with strong expression in more than 90% of testis cells when 50 or more IU/cell were used. In Experiment 2, we exposed porcine testis cells to the viral vector in vitro (50 IU/cell, for 1 h at 37 °C). Cells were washed two to three times and introduced into the seminiferous tubules of prepubertal recipient pigs by ultrasound-guided infusion into the rete testis ( $n = 9$  testes). To control for the presence of residual viral vectors in the medium, supernatant from the last wash was infused into the rete testis of a recipient pig (control). Recipient pigs were castrated 3–6 months after transplantation and the testes examined by whole mount fluorescence microscopy. Colonies of germ cells expressing GFP were observed in the recipient testes that had received transfected germ cells but not in the control testis. Immunocytochemistry for GCNA-1 in cells recovered from the colonized areas confirmed that germ cells expressed the marker gene. These results indicate that rAAV can be used as a vector to transfect porcine germ cells prior to transplantation, resulting in stable gene expression in donor-derived germ cells in the recipient testis. Studies are now directed at demonstrating trans-gene transmission using sperm recovered from recipient animals for fertilization in vitro or in vivo. Support: NIH HD3964, RR17359-01; USDA 99-35205-8620.

## RODENT MODEL OF GENE AND CELL TRANSPLANTATION TO FETAL RAT LIVER BY IN-UTERO MANIPULATION FOR FETAL THERAPY

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Fetal gene and cell therapy should ideally achieve the correction of genetic diseases before the appearance of any clinical manifestations. In this study, we aimed to develop rodent models of gene and cell transplantation for severe hereditary disorders of liver metabolism by in utero manipulation. Inbred LEW rats were used throughout experiments. In utero manipulation was performed on pregnant rats after Day 15 of gestation, when the fetal liver could be visualized through the uterine wall. After ventral laparotomy, fetuses in the uterine horn were manipulated in situ, and then the uterine horn was put back into the abdomen, which could allow natural delivery. Gene transfer to the fetal liver was examined by intrahepatic injection of adenoviral vectors encoding lacZ gene driven by CMV promoter (AdexlacZ,  $10^7$  pfu/10  $\mu$ l). Transplantation of the donor cells ( $1-8 \times 10^6$  cells/10–25  $\mu$ l) infected with AdexlacZ was done by intrahepatic injection to the liver between Days 19 and 21 of gestation and was done via an umbilical vein of neonates obtained by cesarean section. As the donor cells, fetal hepatocytes and amniotic epithelial cells, which were hoped to be a gene carrier to hepatic tissue, were used. The efficacy of gene and cell transplantation was determined by staining for lacZ activity in the liver and other tissues from neonates and thereafter. Intrahepatic injection of AdexlacZ was done on a total of 273 fetuses from Days 15 to 22 of gestation and 78 offspring were delivered. This treatment resulted in an appearance of lacZ-positive hepatocytes at all gestational stages. LacZ-positive hepatocytes increased as the treatment was done on the later gestational stages during Days 18–22. No lacZ-positive cell was observed in other organs including testis and ovary. However, such lacZ-positive hepatocytes gradually diminished after birth and disappeared about 1 month later. Because of no increase of antibody valence against adenoviral antigens and no cellular infiltration, this disappearance seemed not to be the elimination by immune response. On the other hand, cell transplantation resulted in low survival of the donor hepatocytes in the neonatal liver of 22 offspring or no survival of the amniotic epithelial cells in 16 neonatal liver. As the donor cells transplanted to the adult liver showed long-term survival, our results might indicate that they were eliminated in the process of fetal liver development. Cell transplantation via the umbilical vein was examined as an extension of the fetal therapy. Because the umbilical vein forks into a portal vein and an inferior vena cava, the donor cells were expected to reach the hepatic tissue. The results showed that the donor cells were observed in the neonatal liver, or rather distributed in the heart (70 perinatal fetuses were treated in total). However, those cells in the heart disappeared rapidly. In conclusion, the present study demonstrated that the in utero manipulation could be harnessed to the fetal gene and cell therapy. Further investigation such as the use of the retroviral vectors is required to improve the efficacy of these therapeutic methods.

THE EFFECTS OF EXOGENOUS DNA CONCENTRATION AND SPERM TREATMENTS ON THE DNA ASSOCIATION WITH SPERMATOZOA IN GOAT

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Mammalian spermatozoa have the ability to bind and internalize exogenous DNA. It has been demonstrated that using spermatozoa as a DNA carrier is a promising means of transgenesis in various mammalian species. In this study, we examined the factors affecting DNA association with spermatozoa using a fluorescence labeled DNA construct. Semen was collected from a Nigerian Dwarf buck and kept at room temperature in the dark for 1 h. The semen was assigned to two treatments: Percoll wash (PW) or Percoll wash followed by capacitation treatment (PWC). For the PW treatment, motile spermatozoa were separated by centrifugation through a 45%:90% Percoll gradient followed by washing in DM [Brackett et al., BOR 1975;12:260–274] supplemented with 6 mg/ml fatty acid free BSA and 100 µg/ml gentamicin sulphate (mDM). For the PWC treatment, spermatozoa were incubated in mDM containing 0.5 mM 8 Br-cAMP, 10 µg/ml heparin and 200 nM ionomycin for 15 min after PW treatment. The sperm concentrations in both groups were adjusted to  $1 \times 10^6 \text{ ml}^{-1}$ . The DNA construct used was a 5.7 kb Rhodamine labeled circular plasmid (50 µg/50 µl, pGeneGrip, Gene Therapy System, CA). The DNA was diluted to different concentrations with 10 mM Tris–HCl buffer (pH 7.5). Equal volume of DNA solution and sperm suspension were mixed and incubated at room temperature for 30 min. The spermatozoa were then washed twice with mDM. Five microliters of the sperm suspension was used for observation on glass slides under fluorescence microscope. Experiments were designed as a  $2 \times 4$  factorial with sperm treatments of PW versus PWC and with final DNA concentrations of 25, 5, 1 and 0 µg/ml. The trials were repeated four times. The percentage data were transformed by arcsin and analyzed with two-way ANOVA followed by Bonferroni multiple comparisons. The results indicated higher ratios of spermatozoa carrying exogenous DNA in PW treatment compared to PWC treatment (63% in PW, 25 µg/ml versus 16.8% in PWC, 25 µg/ml;  $P < 0.01$ ). Furthermore, more Rhodamine positive spermatozoa were observed in higher DNA concentration groups (63% in PW, 25 µg/ml versus 5% in PW, 1 µg/ml;  $P < 0.01$ ). In conclusion, exogenous DNA concentration and the changes of sperm membrane integrity are crucial factors affecting exogenous DNA binding to sperm heads.

# Ultrasonography

## ULTRASOUND-GUIDED TRANSVAGINAL COLLECTION OF AMNIOTIC AND/OR ALLANTOIC FLUID FROM CATTLE

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Serial transvaginal ultrasound sampling of fetal fluids in cattle would enable regular monitoring of fetal/uterine health following various embryo manipulations. We report here a technique which permits serial sampling of amniotic and/or allantoic fluids between Days 52 and 255 of gestation.

Fluids were sampled using ultrasound-guided transvaginal puncture. Sampling was attempted at approximately 30-day intervals from Day 60 of gestation. Sedative (0.2–0.25 ml) xylazine hydrochloride 20 mg/ml (Rompun<sup>®</sup>, Bayer) or 0.1–0.18 ml detomidine hydrochloride 10 mg/ml (Domosedan, Pfizer Animal Health) i.v. and caudal epidural anaesthesia (3.5–4.0 ml lignocaine hydrochloride 2%, Bomacaine) were administered prior to sampling and during the process cattle stood restrained with front feet on a raised platform. Alubricated, 7.5 MHz transducer in a transvaginal oocyte recovery probe with a needle guide holding a 0.70 mm × 90 mm (3.5 in., 22 gauge) disposable spinal needle was used. A stylet in the needle reduced the risk of material being transferred from the vagina to amniotic/allantoic cavities. Rectal palpation of the uterine horn positioned the targeted cavity within the needle path displayed on the ultrasound screen. The needle was advanced through the vaginal and uterine walls to collect 2–5 ml of sample from the amniotic or allantoic cavity. If the allantoamniotic membrane was identified, the needle was advanced through the membrane and a sample withdrawn from the second cavity.

Fetal fluids were obtained on 92% (81/88) of attempted sampling sessions and more than 100 samples were collected and frozen for electrolyte and steroid analysis. The earliest attempt at collection was Day 52 of gestation ( $n = 4$ ; 50% successful) and collection of samples was more successful after Day 66. Serial sampling of fetal fluids generated 100 samples from 21 animals over gestational age of 52–255 days with the retention of animals for future studies. In contrast, if the same 21 animals had been slaughtered for sample recovery as in other studies, it would have resulted in a total of only 42 samples. The procedure, however, was not without risk to fetal viability. Five animals (5/21; 23.8%) failed to maintain the pregnancy after the first sampling; losses were recorded by 7, 8, 14 and up to 30 ( $n = 2$ ) days after sampling. Of the remaining 16 cows, 1 or 2 failed at each subsequent sampling session; overall pregnancy loss was 52.4%. There was a 0.87 probability of the pregnancy continuing following each sampling session. Nine healthy calves were born after 4 ( $n = 2$ ), 5 ( $n = 1$ ), 6 ( $n = 2$ ) or 7 ( $n = 4$ ) sampling sessions.

The high percentage of loss after the first sampling may have been due to a combination of factors including (i) inexperience during procedure development, (ii) stage of gestation (Days 52–66), (iii) manipulation of the pregnant uterus, (iv) susceptibility of individual cows to the procedure (i.e. individual variation), (v) natural losses, or (vi) bacterial contamination.

The serial sampling technique is currently being used on cattle carrying cloned fetuses to investigate differences between normal (AI generated), clone pregnancies and cows exhibiting hydroallantois. The aim is to identify diagnostic marker(s) for predicting pregnancy failure and abnormal placental development in early gestation.

REPEAT OVUM PICK-UP IN PREPUBERTAL SWAMP BUFFALO  
(*BUBALUS BUBALIS*)

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Transvaginal ultrasound-guided oocyte pick-up (OPU) is a relatively non-invasive procedure that allows repeated collection of oocytes with minimal trauma to ovaries and reproductive tract. For buffalo, OPU has been used in older anestrus and cyclic cows and young postpubertal heifers, but there are no reports of OPU in juvenile buffalo heifers. Our objective was to investigate the effect of repeated oocyte collection by transvaginal ultrasound-guidance (OPU) on ovarian responses and recovery rates in prepubertal swamp buffaloes. A total of nine juvenile swamp buffalo calves (8–12-month-old and 100–150 kg) from a buffalo breeding center received 2 kg concentrate daily and ad libitum of roughage and water. Superstimulation of ovarian follicular growth entailed treatment with 180 mg FSH (Follitropin<sup>®</sup>-V, Vetrepharm Canada Inc., London, Ont., Canada). On Day 0, animals received a norgestomet ear implant and injection of norgestomet/estradiol valerate (Crestar<sup>®</sup>, Intervet, The Netherlands). Stimulation with FSH was from Days 7 to 9 (40 + 40, 30 + 30, 20 + 20 mg, i.m. a.m./p.m.), 100 µg GnRH (Cystorelin<sup>®</sup>, Sanofi, France) was given on Day 10, and using an Aloka SSD-550V ultrasound unit equipped with a 5 MHz real time-B mode transducer, OPU was carried out on Day 11. Animals were prepared for OPU by administering 0.5 ml of Xylazine HCl (Rompun<sup>®</sup>, Bayer Korea Ltd., Seoul, Korea 10 mg/100 kg, i.m.) and, 10 min later, with 1 ml 2% lidocaine hydrochloride epidurally (OLIC, Sweden). For OPU, a convex array transvaginal transducer was fitted with a 34.5 cm, 17-g single lumen needle, that in turn was connected to a vacuum pump. The pressure of aspiration was 80–100 mmHg. Oocytes were aspirated by using a close system of aspiration (Cook, Australia) and recovered in phosphate buffer saline (PBS) supplemented with 2% fetal calf serum. This schedule was repeated five times at intervals of 2 weeks. The number of follicles and oocytes were expressed as means ( $\pm$ S.D.) and were compared by ANOVA. Over five schedules of FSH followed by OPU, 39/42 (92.9%) animals responded and had  $6.6 \pm 3.6$  follicles with a follicular diameter of  $5.0 \pm 2.0$  mm. Oocyte recovery rate was  $5.4 \pm 3.7$  and averaged 83% oocytes except for Schedule 4 when oocyte recovery was around 69% (Table 1). Most oocytes were denuded (39.5%,  $1.95 \pm 2.2$  per animal), while 28.8% had a substantial cumulus mass (cumulus–oocyte complexes,  $0.35 \pm 0.6$  per animal and single layers cumulus oocytes,  $1.2 \pm 1.7$  per animal). There were no differences in ovarian responses and recovery rates among the collections. It was concluded that five repeat cycles of FSH and OPU did not influence the follicular response to superstimulation or number of oocytes recovered from prepubertal buffalo heifers.

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Table 1  
Results of oocyte recovery by OPU method in prepubertal swamp buffalo

Times of OPU	No. of buffaloes	No. of follicles		No. of oocytes		Recovery rate (%)
		<i>n</i>	Mean $\pm$ S.D.	<i>n</i>	Mean $\pm$ S.D.	
First	8	50	6.3 $\pm$ 6.0	43	5.4 $\pm$ 6.0	83.2
Second	8	66	8.3 $\pm$ 2.8	54	6.8 $\pm$ 3.0	82.5
Third	7	52	7.4 $\pm$ 1.9	42	6.0 $\pm$ 2.3	81.3
Fourth	8	36	4.5 $\pm$ 1.2	27	3.4 $\pm$ 2.1	68.8
Fifth	8	52	6.5 $\pm$ 3.5	46	5.8 $\pm$ 3.6	87.9