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ScienceDirect

Theriogenology 73 (2010) 651–658

Theriogenology

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Equine chorionic gonadotropin and gonadotropin-releasing hormone enhance fertility in a norgestomet-based, timed artificial insemination protocol in suckled Nelore (*Bos indicus*) cows

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Received 18 March 2009; received in revised form 16 October 2009; accepted 5 November 2009

Abstract

Two experiments were conducted to investigate the effects of equine chorionic gonadotropin (eCG) at progestin removal and gonadotropin-releasing hormone (GnRH) at timed artificial insemination (TAI) on ovarian follicular dynamics (Experiment 1) and pregnancy rates (Experiment 2) in suckled Nelore (*Bos indicus*) cows. Both experiments were 2 $\times$ 2 factorials (eCG or No eCG, and GnRH or No GnRH), with identical treatments. In Experiment 1, 50 anestrous cows, 134.5 $\pm$ 2.3 d postpartum, received a 3 mg norgestomet ear implant sc, plus 3 mg norgestomet and 5 mg estradiol valerate im on Day 0. The implant was removed on Day 9, with TAI 54 h later. Cows received 400 IU eCG or no further treatment on Day 9 and GnRH (100 μg gonadorelin) or no further treatment at TAI. Treatment with eCG increased the growth rate of the largest follicle from Days 9 to 11 (means $\pm$ SEM, 1.53 $\pm$ 0.1 vs. 0.48 $\pm$ 0.1 mm/d; $P < 0.0001$), its diameter on Day 11 (11.4 $\pm$ 0.6 vs. 9.3 $\pm$ 0.7 mm; $P = 0.03$), as well as ovulation rate (80.8% vs. 50.0%, $P = 0.02$), whereas GnRH improved the synchrony of ovulation (72.0 $\pm$ 1.1 vs. 71.1 $\pm$ 2.0 h). In Experiment 2 (n = 599 cows, 40 to 120 d postpartum), pregnancy rates differed ($P = 0.004$) among groups (27.6%, 40.1%, 47.7%, and 55.7% for Control, GnRH, eCG, and eCG + GnRH groups). Both eCG and GnRH improved pregnancy rates (51.7% vs. 33.8%, $P = 0.002$; and 48.0% vs 37.6%, $P = 0.02$, respectively), although their effects were not additive (no significant interaction). In conclusion, eCG at norgestomet implant removal increased the growth rate of the largest follicle (LF) from implant removal to TAI, the diameter of the LF at TAI, and rates of ovulation and pregnancy rates. Furthermore, GnRH at TAI improved the synchrony of ovulations and pregnancy rates in postpartum Nelore cows treated with a norgestomet-based TAI protocol.

Keywords: Anestrous; artificial insemination; bovine; eCG; GnRH

1. Introduction

The majority of cattle are located in tropical areas, where *Bos indicus* breeds are predominant, due to their greater tolerance to high temperatures and humidity, as well as their resistance to parasitic infections. In Brazil,

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0093-691X/$ – see front matter
doi:10.1016/j.theriogenology.2009.11.004

the majority of Zebu (*Bos indicus*) beef cattle are of the Nelore breed. Despite the adaptability of this subspecies to heat and nutritional stress, they have a shorter duration of estrus than *Bos taurus* cattle, a propensity to show estrus during the night, and a high incidence of postpartum anestrous that substantially impairs the efficiency of protocols that require detection of estrus to determine the appropriate time for artificial insemination (AI) [1,2]. Thus, hormonal treatments have been designed to control both luteal and follicular functions, providing exciting possibilities for synchronizing estrus and ovulation and, more importantly, to eliminate estrus detection to determine timing of AI [3].

Commercial hormonal treatments for synchronization of ovulation include estradiol and natural or synthetic progestins (to synchronize follicular wave emergence), prostaglandin $F_{2\alpha}$ and analogues (PGF; to ensure luteolysis) when a progestin-releasing device is removed, and subsequent administration of a low dose of estradiol concurrent with device removal or 24 h later (to induce synchronized ovulation) [3]. Alternatively, ovulation can be induced with GnRH or luteinizing hormone (LH) given 12 h before [4,5] or concurrently with timed AI (TAI) [5–7]. In addition to facilitating TAI, these progestin-based protocols induced postpartum ovarian activity [2,8,9].

Postpartum anestrous cows have insufficient pulsatile release of LH to support the final stages of ovarian follicular development and ovulation [10], limiting the effectiveness of TAI protocols [11]. Exogenous progestins increased LH pulse frequency during and after treatment and induced ovulation [8]. However, despite progestin-induced postpartum cyclicity, the efficiency of such treatment could be compromised in herds with a high proportion of anestrous cows, those with low body condition scores, or both [2,12]. Thus, gonadotropins could be included in synchronization protocols to improve LH support. For example, equine chorionic gonadotrophin (eCG) has both follicle-stimulating hormone (FSH)- and LH-like activity and stimulates ovarian follicular growth and ovulation in cattle [13]. The high amount of sialic acid present in its molecular composition, especially in the β-subunit, confers a long half-life [14]. Therefore, administration of eCG at progestin removal has also been suggested as an alternative to increase pregnancy rates in TAI programs for suckled cows with a high prevalence of anestrus [2,9,15,16].

Two experiments were conducted to evaluate the effect of eCG at norgestomet implant removal and GnRH at TAI on ovarian follicular dynamics (Experiment 1) and pregnancy rates (Experiment 2) in suckled Nelore (*Bos indicus*) cows. Our hypotheses were, in suckled Nelore cows submitted to a norgestomet-based, TAI protocol, (1) eCG increases rates of ovulation and pregnancy, and (2) GnRH improves the synchrony of ovulations and increases pregnancy rates.

2. Materials and methods

2.1. Experiment 1

2.1.1. Animal management

Experiment 1 was conducted at the São Paulo University Campus (Pirassununga, SP, Brazil) during the summer (March 2004). Fifty (13 multiparous and 37 primiparous) suckled Nelore (*Bos indicus*) beef cows, 134.5 ± 2.3 d postpartum, were used (12 or 13 cows per group). All cows were anestrous [no corpus luteum (CL) detected in three ultrasonographic examinations conducted 7 d apart]. On the first day of the synchronization protocol, the body condition score (BCS) was 2.43 ± 0.04, based on a 1- to 5-point scale (1 = emaciated, 5 = obese) [17]. These cows were maintained on a *Brachiaria brizantha* pasture with free access to water and mineralized salt.

2.1.2. Experimental design

Anestrous cows (no CL detected in three ultrasonographic examinations conducted 7 d apart) were assigned by parity, BCS, and postpartum interval, in a 2 × 2 factorial arrangement, with eCG treatment (eCG vs. No eCG) and GnRH treatment (GnRH vs. No GnRH) as main effects (Fig. 1). Therefore, the groups were No eCG/No GnRH, eCG/No GnRH, No eCG/GnRH, and eCG/GnRH. At the start of the study (designated Day 0), all cows were given 5 mg estradiol valerate and 3 mg norgestomet im (Intervet, Boxmeer, The Netherlands), and concurrently, an implant containing 3 mg norgestomet (Crestar; Intervet) was inserted sc on an ear. On Day 9, the implant was removed, and cows

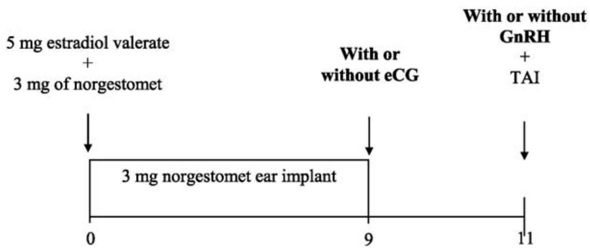

Fig. 1. Schematic diagram of treatments to synchronize ovulation in suckled Nelore cows. GnRH = 100 μg gonadorelin; eCG = 400 IU equine chorionic gonadotropin; TAI = timed artificial insemination, performed 54 h after norgestomet implant removal.

received either no further treatment (Control, n = 12 cows) or treatment with eCG (400 IU, Folligon; Intervet). In all cows, TAI was done on Day 9 (54 h after implant on removal), and cows concurrently received either GnRH im (100 μg gonadorelin; Fertagyl; Intervet) or no treatment. Frozen-thawed semen from the same ejaculate of a single bull was used for all TAI.

2.2. Experiment 2

2.2.1. Animal management

Experiment 2 was conducted on two commercial farms (Farm 1 = 309 and Farm 2 = 290 cows) located in Mato Grosso do Sul and São Paulo states, Brazil, respectively, during the spring–summer breeding season (December 2003 to January 2004). These were suckled Nelore (*Bos indicus*) cows, 40 to 120 d postpartum, maintained on a *Brachiaria brizantha* pasture with free access to water and mineralized salt supplementation.

2.2.2. Experimental design

At unknown stages of the estrous cycle, cows were assigned to the same treatments described in Experiment 1. All cows were TAI at 54 h after implant removal. On the first day of the synchronization protocol, BCS was determined as described for Experiment 1. To determine the effect of BCS on pregnancy rates, cows were classified as low versus moderate to high (<3.0 vs. ≥3.0).

2.3. Ultrasonographic examinations

In Experiment 1, transrectal ultrasonography was done with a 6.0/8.0 MHz linear-array transducer (Falco Vet; Pie Medical, Maastricht, The Netherlands). Both ovaries were examined on Days –14, –7, and 0, to confirm the absence of a CL. Ovarian ultrasonographic examinations were performed once daily, from Days 0 to 9, and then twice daily from Days 10 to 13, to evaluate ovarian follicular dynamics and interval from implant removal to ovulation, respectively. Once-daily ultrasonographic examinations were performed every morning. Both ovaries were visualized to identify and measure the two largest follicles present on each ovary. The diameter of the largest follicle (LF) at implant removal (Day 9) and the diameter of the LF at TAI (Day 11) were recorded. Final follicular growth rate (mm/day) was defined as the difference between the LF recorded on Day 11 and on Day 9, divided by two. Ovulation was considered to have occurred when a large follicle, previously observed, was no longer present.

In both experiments, pregnancy diagnosis was performed by ultrasonography 30 d after TAI. Pregnancy rate was defined as the number of pregnant cows divided by the total number of cows submitted to TAI in each treatment (Control, eCG, GnRH, or eCG + GnRH).

2.4. Statistical analysis

Statistical analysis was performed using the SAS System for Windows (SAS, 2003; SAS Institute, Cary, NC, USA). Parametric data (i.e., diameter of the LF at implant removal on Day 9; diameter of the LF at Day 11; growth rate of LF from Days 9 to 11; and the interval from implant removal to ovulation) were tested for normality of their residuals, using Guided Data analysis. Data transformation ($Log_{10}X$; SQRT X) was employed whenever necessary. Growth rate of the LF follicle from Day 9 to 11 and the interval from implant removal to ovulation were analyzed by ANOVA (PROC GLM). The effects of eCG, GnRH, and the interaction between eCG and GnRH were included in the statistical model. Profiles of the diameter of the LF on Day 9 and on Day 11 were analyzed using PROC MIXED to detect the effects of day, eCG, GnRH, and the interaction between day and treatments. For the interval from implant removal to ovulation, the equality of variances among treatment groups was analyzed by Levene's test and the distribution curves compared using standardized kurtosis ($Z_{Kurtosis}$ = Kurtosis – 0/Standing error$_{Kurtosis}$). The kurtosis value provides information regarding the distribution curve shape of the response variable. The normal distribution presents kurtosis values between −1 and 1. However, positive numbers greater than 1 represent a heavy or long-tailed shape that is often called leptokurtic distribution [18].

A binomial distribution was assumed for the categorical response variables, ovulation (Experiment 1) and pregnancy rates (Experiments 1 and 2). Pregnancy rate was analyzed using the procedure GLIMMIX of SAS, with the effect of cows treated as a random effect. In Experiment 2, the variables initially included in the models were the effects of eCG, GnRH, farm, BCS (categorized as <3 or ≥3), and interactions. For the final logistic regression model, variables were removed by backward elimination, based on the Wald statistics criterion when $P > 0.20$. Variables that were included in the final model for analysis of pregnancy rate were eCG, GnRH, and BCS category. However, in Experiment 1, only the effects of eCG and GnRH were included in the statistical model. All results are presented as means ± SEM.

Table 1
Effects of eCG at norgestomet ear implant removal and of GnRH at TAI on ovarian follicular dynamics and pregnancy outcomes in suckled anestrous *Bos indicus* (Nelore) cows (Experiment 1).

	Treatments[1]				P values[2]		
	No eCG		eCG				
	No GnRH	GnRH	No GnRH	GnRH	eCG	GnRH	eCG*GnRH
Number of cows	12	12	13	13	—	—	—
Diameter of the largest follicle at Day 9 (implant removal), mm	8.3 ± 1.0	8.5 ± 0.9	8.2 ± 0.9	8.5 ± 0.8	0.94	0.78	0.97
Diameter of the largest follicle at Day 11 (TAI), mm	9.4 ± 1.1	9.3 ± 0.9	11.2 ± 0.9	11.6 ± 0.7	0.03	0.87	0.78
Growth rate of the largest follicle from Days 9 to 11, mm/d	0.56 ± 0.2	0.40 ± 0.2	1.50 ± 0.1	1.56 ± 0.2	<0.0001	0.89	0.85
Interval from implant removal to ovulation, h	72.0 ± 3.1	69.6 ± 2.4	70.5 ± 2.7	73.1 ± 1.1	0.63	0.70	0.29
Ovulation rate, % (n)	50.0 (6/12)	50.0 (6/12)	76.9 (10/13)	84.6 (11/13)	0.03	0.75	0.70
Pregnancy rate, % (n)	25.0 (3/12)	16.7 (2/12)	46.2 (6/13)	46.2 (6/13)	0.07	0.76	0.76

[1]Cows received a 3 mg norgestomet ear implant and 5 mg estradiol valerate plus 3 mg norgestomet at implant insertion on the first day of the protocol for synchronization of ovulation. The implant was removed 9 d later with or without eCG (equine chorionic gonadotropin = 400 IU) and with or without GnRH (gonadorelin = 100 μg) at fixed time AI.
[2]eCG = effect of eCG treatment (eCG vs. No eCG); GnRH = effect of GnRH (GnRH vs. No GnRH); eCG*GnRH = interaction between eCG and GnRH.

3. Results

3.1. Experiment 1

There were no significant interactions between eCG and GnRH for any response variable (Table 1). There was an effect of eCG for diameter of the LF on Day 11 ($P = 0.03$), growth rate of the LF from Days 9 to 11 ($P < 0.0001$), and ovulation rate ($P = 0.03$), and a tendency ($P = 0.07$) for a higher pregnancy rate (Table 1). Furthermore, there was a day by treatment interaction for diameter of the LF on Day 11 ($P = 0.002$; Fig. 2). For the interval from implant removal to ovulation, the kurtosis value for the eCG and No eCG groups were 2.71 ± 1.01 and 1.86 ± 1.28 and $Z_{Kurtosis}$ values (kurtosis standardized) were 2.68 and 1.45, respectively. Finally, GnRH increased the synchrony of the interval from implant removal to ovulation (GnRH, 72.0 ± 1.1 h; No GnRH, 71.1 ± 2.0 h; Levene's tests, $P = 0.07$). For the interval from implant removal to ovulation, the kurtosis value for the GnRH and No GnRH groups were 7.50 ± 1.09 and 0.30 ± 1.15, and $Z_{Kurtosis}$ values were 6.87 and 0.26.

3.2. Experiment 2

The average BCS for cows from Farms 1 and 2 were 3.14 ± 0.28 and 3.16 ± 0.28, respectively. On Farm 1, 10.0% and 90.0% of the cows had BCS <3.0 and ≥3.0, respectively, whereas on Farm 2, these proportions were 19.7% and 80.3%. There was no effect of farm ($P = 0.77$) nor interactions between farms and BCS ($P = 0.80$) or between farms and treatments ($P = 0.25$) on pregnancy rates. Although BCS on Day 0 affected pregnancy rates (32.5% vs. 44.4% for BCS <3 vs. ≥ 3;

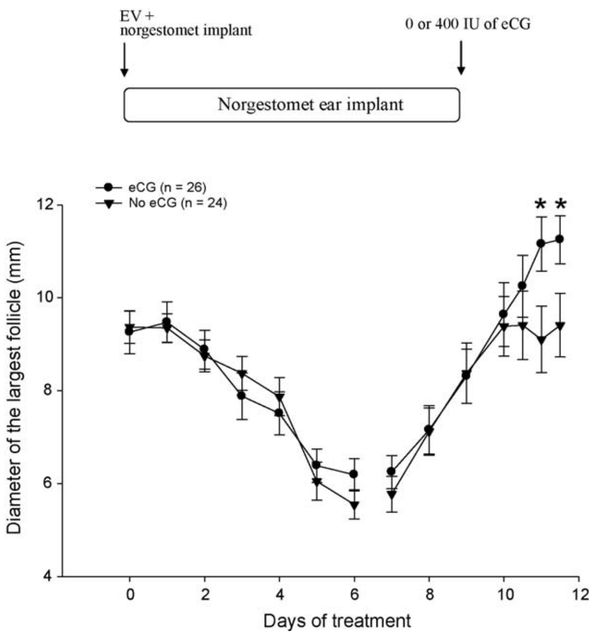

Fig. 2. Effects of eCG (400 IU) on ovarian follicular dynamics of suckled anestrous Nelore (*Bos indicus*) cows given a 3 mg norgestomet ear implant and 5 mg estradiol valerate plus 3 mg norgestomet. *Effect of eCG treatment on the diameter of the largest follicle at Day 11 ($P < 0.05$).

Table 2
Effects of eCG at the time of norgestomet ear implant removal and of GnRH at TAI on pregnancy outcomes in suckled anestrous *Bos indicus* (Nelore) cows (Experiment 2).

	Treatments[1]				P values[2]		
	No eCG		eCG				
	No GnRH	GnRH	No GnRH	GnRH	eCG	GnRH	eCG*GnRH
Number of cows	152	147	151	149	—	—	—
Pregnancy rate, %	27.6	40.1	47.7	55.7	0.002	0.02	0.75

[1]Cows received a 3 mg norgestomet ear implant and 5 mg estradiol valerate plus 3 mg norgestomet at implant insertion on the first day of the protocol for synchronization of ovulation. The implant was removed 9 d later with or without eCG (equine chorionic gonadotropin = 400 IU) and with or without GnRH (gonadorelin = 100 μg) at fixed time AI.
[2]eCG = effect of eCG treatment (eCG vs. No eCG); GnRH = effect of GnRH (GnRH vs. No GnRH); eCG*GnRH = interaction between eCG and GnRH.

P = 0.04), there was no interaction between BCS and treatments (P = 0.83). In the absence of a significant interaction between eCG and GnRH treatments (P = 0.75, Table 2), the main effects of these treatments were analyzed. Treatment with eCG or GnRH improved pregnancy rates (51.7% vs. 33.8%, P = 0.002; and 48.0% vs. 37.6% P = 0.02, respectively).

4. Discussion

In the current study, treatment with eCG increased rates of ovarian follicular growth and ovulation after implant removal, treatment with GnRH improved the synchrony of ovulation, and treatment with either hormone significantly improved pregnancy rates in suckled Nelore cows submitted to TAI. Therefore, hypotheses that eCG treatment increases ovarian responses and pregnancy rates and that GnRH improved the synchrony of ovulation and pregnancy rates were supported. However, it was noteworthy that the effects of eCG and GnRH were not additive (no significant interaction).

Growth rate of the LF from Days 9 to 11, diameter of the LF at TAI, and the ovulation rate were all significantly higher for eCG-treated cows. However, these findings were not consistent with those previously reported in suckled beef cows treated with either intravaginal progesterone devices (CIDR) [7] or norgestomet implants [19,20]. Similar studies conducted with dairy cows also found no effect of eCG on follicular diameter or ovulation rate [21,22]. However, the current results corroborated data from lactating crossbred *Bos indicus* beef cows reported by Maraña et al. [23]. In that study, eCG significantly improved growth rate of the ovulatory follicle (1.1 ± 0.1 vs. 0.6 ± 0.1 mm/d). In addition, eCG increased the ovulation rate in *Bos indicus* heifers treated with CIDR devices (16 of 21, 76.2% vs. 10 of 20, 50%) [24] and with norgestomet implants (85 of 90, 94.4% vs. 64 of 87, 73.6%) [25]. These apparently conflicting results may be related to differences in cyclic status, BCS, and breed of the cows, or perhaps the experimental environment. It was noteworthy that only anestrous cows were used in Experiment 1. Perhaps this specific physiologic status resulted in the more pronounced effects of eCG effects on preovulatory follicular development and ovulation rates.

It was noteworthy that neither the mean interval from implant removal to ovulation nor its synchrony were significantly influenced by eCG treatment. This outcome was similar to previous reports by Baruselli et al. [24] in Nelore heifers (eCG = 72.0 ± 2.5 h vs. No eCG = 72.0 ± 3.1 h; $P > 0.05$) and Marques et al. [7] in crossbred *Bos indicus* × *Bos taurus* suckled beef cows (eCG = 74.2 ± 4.0 h vs. No eCG = 78.0 ± 3.1 h; $P > 0.05$). Duffy et al. [19], Bergamaschi [20], and Maraña et al. [23] also reported no significant effect of eCG treatment on the interval from removal of a progestin source to ovulation in *Bos taurus, Bos indicus,* and crossbred cattle, respectively. Conversely, one study with nonlactating *Bos indicus* cows reported an improvement in the synchrony of ovulation in cows treated with eCG at norgestomet implant removal compared with those treated only with norgestomet (eCG = 67.3 h [range, 60.8 to 82.5 h] vs. No eCG = 68.8 h [65.8 to 114.0 h]) [26]. In a recent report, Souza et al. [22] tested the effect of eCG at removal of an intravaginal progesterone-releasing device in high-producing dairy cows and, similar to the current study, found no effect of eCG on the timing of ovulation. Thus, the current findings reaffirmed previous reports that giving eCG at progestin did not alter the interval to ovulation in cows.

The positive effect of eCG treatment on pregnancy rates in the current study supported results previously reported in postpartum beef cows synchronized for TAI

[15,16,23,27,28]. The fertility improvement in eCG-treated cows may be explained by three main effects: (1) eCG increased diameter of the preovulatory follicle at TAI in anestrous cows; (2) eCG improved ovulation rate; and (3) eCG improved plasma progesterone concentrations during the ensuing luteal phase.

Consistent with these ideas, reducing the preovulatory follicle diameter, by manipulating the interval from follicle deviation to the induction of ovulation, reduced pregnancy rates [29]. Thus, in the current study, the increased pregnancy rate obtained in eCG-treated anestrous Nelore cows may be related to the increased follicular diameter at TAI. Also, in previous studies, increased duration of exposure to estradiol during follicular development immediately preceding ovulation was critical to an enhanced luteal life span [30]. In support of this idea, when the duration of exposure to estradiol during proestrous was increased with exogenous estradiol cypionate, conception rates in *Bos indicus* × *Bos taurus* crossbred embryo recipient heifers were enhanced [31]. In addition, Mann and Lamming [32] detected a positive effect of estradiol production during proestrous on progesterone production in the subsequent luteal phase and on embryonic survival. Furthermore, recent studies speculated that the beneficial effects of estradiol supplementation on conception rate were likely caused by postfertilization processes [33,34]. In the current study, anestrous suckled Nelore cows that ovulated larger follicles probably had higher concentrations of estradiol, which could have enhanced the subsequent luteal phase and pregnancy rates.

The effects of eCG on pregnancy rate after TAI in cattle have been inconsistent. The positive effect of eCG was more evident in cows that were anestrus or under nutritional stress [2,16,22,28,35,36]. In a meta-analysis (n = 1984 TAI), Baruselli et al. [37] evaluated the interaction of BCS and eCG on pregnancy rate. In that analysis, pregnancy rate was only improved by eCG in cows with lower BCS ($\leq$2.5; No eCG = 40.7% vs. eCG = 55.4%; $P < 0.05$) when compared with cows with higher BCS ($\geq$2.5; No eCG = 54.3% vs. eCG = 56.6%; $P > 0.05$). Similarly, Souza et al. [22] only detected improved fertility in high-producing dairy cows when eCG was given to those with low BCS (<2.75). Additionally, other experiments have reported an effect of eCG on pregnancy outcomes only in noncyclic cows (i.e., absence of a CL at the beginning of the protocol for synchronization of ovulation) [15,16,28,35] or in primiparous cows [36]. Therefore, eCG-induced increases in fertility were mainly due to increased pregnancy rates in noncyclic postpartum cows or cows under nutritional stress (i.e., low BCS cows). Under these circumstances, treatment with eCG at progestin removal can provide additional gonadotropic support, increasing growth of the preovulatory follicle. Thus, use of eCG in TAI protocols would be most effective in herds with a high proportion of anestrous cows, low BCS, or both.

Treatment with GnRH increased both the synchrony of ovulation and pregnancy rates to TAI in Experiment 2. This result agrees with those reported by Vasconcelos et al. [38] when GnRH treatment was administered 30 h after removal of a norgestomet implant. Improved synchronization of ovulation by GnRH treatment was probably due to two major factors: (1) the induction of a more uniform preovulatory LH surge, and (2) hastening ovulation in cows in which it would have otherwise been delayed. Troxel et al. [39] reported that GnRH administration 30 h after norgestomet implant removal increased pregnancy rates to TAI in *Bos taurus* cows (46% vs. 18% in GnRH-treated vs. control cows, respectively). Finally, in a meta-analyses study (40 trials involving 19,000 inseminations), Morgan and Lean [40] reported that GnRH at AI increased the risk of pregnancy by 8% in normal cows and by 22.5% in cows classified as repeat breeders.

In conclusion, giving eCG concurrent with removal of the norgestomet ear implant increased growth rate of the LF from implant removal (Day 9) to TAI (Day 11), diameter of the LF at TAI, and rates of ovulation and the pregnancy in postpartum Nelore *Bos indicus* cows. In addition, GnRH treatment at TAI resulted in more synchronous ovulation and better pregnancy rates in postpartum *Bos indicus* cows synchronized for TAI. Therefore, giving eCG at implant removal, or GnRH at TAI, should be considered to enhance reproductive efficiency in norgestomet-based TAI programs in suckled *Bos indicus* cows.

Acknowledgments

The authors are grateful to the owners and employees of Agropecuária Hora, Brasilândia/MS, and Agropecuária NSA for infrastructure and animal care and to Intervet (Brazil) for supporting this research.

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