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Dynamics of follicular growth and progesterone concentrations in cyclic and anestrous suckling Nelore cows (*Bos indicus*) treated with progesterone, equine chorionic gonadotropin, or temporary calf removal

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ABSTRACT

The objective of this study was to investigate the effects of eCG and temporary calf removal (TCR) associated with progesterone (P4) treatment on the dynamics of follicular growth, CL size, and P4 concentrations in cyclic ($n = 36$) and anestrous ($n = 30$) Nelore cows. Cyclic (C) and anestrous (A) cows were divided into three groups. The control group received 2 mg of estradiol benzoate via intramuscular (IM) injection and an intravaginal device containing 1.9 g of P4 on Day 0. On Day 8, the device was removed, and the animals received 12.5 mg of dinoprost tromethamine IM. After 24 hours, the animals received 1 mg of estradiol benzoate IM. In the eCG group, cows received the same treatment described for the control group but also received 400 UI of eCG at the time of device removal. In the TCR group, calves were separated from the cows for 56 hours after device removal. Ultrasound exams were performed every 24 hours after device removal until the time of ovulation and 12 days after ovulation to measure the size of the CL. On the same day as the CL measurement, blood was collected to determine the plasma P4 level. Statistical analyses were performed with a significance level of $P \leq 0.05$. In cyclic cows, the presence of the CL at the beginning of protocol resulted in a smaller follicle diameter at the time of device removal (7.4 ± 0.3 mm in cows with CL vs. 8.9 ± 0.4 mm in cows without CL; $P = 0.03$). All cows ovulated within 72 hours after device removal. Anestrous cows treated with eCG or TCR showed follicle diameter at fixed-timed artificial insemination (A-eCG 10.2 ± 0.3 and A-TCR 10.3 ± 0.5 mm) and follicular growth rate (A-eCG 1.5 ± 0.2 and A-TCR 1.3 ± 0.1 mm/day) similar to cyclic cows (C-eCG 11.0 ± 0.6 and C-TCR 12.0 ± 0.5 mm) and (C-eCG 1.4 ± 0.2 and C-TCR 1.6 ± 0.2 mm/day, respectively; $P \leq 0.05$). Despite the similarities in CL size, the average P4 concentration was higher in the A-TCR (9.6 ± 1.4 ng/mL) than in the A-control (4.0 ± 1.0 ng/mL) and C-TCR (4.4 ± 1.0 ng/mL) groups ($P < 0.05$). From these results, we conclude that eCG treatment and TCR improved the fertility of anestrous cows by providing follicular growth rates and size of dominant follicles similar to cyclic cows. Additionally, TCR increases the plasma concentrations of P4 in anestrous cows.

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1. Introduction

Despite the clear benefits of artificial insemination (AI) for reproductive efficiency, this technique is not widely used in many tropical countries [1] because of biological and operational constraints. For example, the large size of some herds with thousands of cows under pasturing systems can hamper the use of AI because of an operational perspective. Furthermore, certain physiological traits, such as a short period of estrous behavior (11 hours) and a predominance of nightly manifestations of estrus, can result in failures to detect heat, resulting in low conception rates in the *Bos indicus* breeds [2]. Physiological postpartum anestrus, a condition that can be exacerbated by nutritional imbalances and climate conditions (dry season) and inhibited by suckling, is also problematic in these breeds [3].

Considering these challenges, fixed-timed artificial insemination (FTAI) represents the best solution to increase the use of AI in large *Bos indicus* herds. However, many aspects of FTAI require additional investigation, specifically the cyclicity, postpartum period, and weaning effects. Fixed-timed artificial insemination with progesterone (P4) has been used to induce cyclicity during the breeding season and markedly increasing conception rates [1,4]. The use of eCG and/or temporary calf removal (TCR) in conjunction with progestogen removal has led to conception rates of approximately 50% in noncyclic Nelore cows [5,6].

The use of eCG has been shown to result in an increase in the daily follicular growth rate, an increase in the diameter of the dominant follicle (DF), and higher ovulation rates in noncyclic indicus cows [7]. The controlled interruption of suckling has been used to increase GnRH levels and LH pulsatility, thereby improving ovulation in anestrous cows without hormone treatment [8]. However, the effects of eCG administration and TCR on follicular growth and luteum function in cyclic and anestrous Nelore cows have not been previously investigated.

The objective of this present study was to evaluate the effects of eCG and TCR on the follicular growth, CL size and P4 concentrations in cyclic and anestrous Nelore cows.

2. Materials and methods

2.1. Location, feed management, and animal selection

The experiments were conducted in Parana State, Brazil, 21°18′S, 52°49′W, during the summer in the Southern Hemisphere. This region has a tropical climate with a mean annual temperature of 22 °C and thermal amplitude of 7 °C. The rainy period lasts from November to March, and the annual precipitation ranges from 1000 to 1500 mm.

A total of 66 pluriparous Nelore cows (*Bos taurus indicus*) with average ages of 6.6 ± 2.1 years, 77 ± 12.9 days postpartum (suckling), and body condition score of 3 ± 0.4 (on a scale of 1–5 [9]) were used in this study. The cows were evaluated using transrectal palpation and ultrasonography, and the cyclicity was determined after two ultrasound exams (SSD 500 Aloka, Tokyo, Japan, with a 5.0 MHz linear transducer) of the ovaries performed 10 days apart. Cyclic cows (n = 36) presented a CL, whereas anestrous females (n = 30) had no CL.

During the experimental period, the animals were maintained by continuous grazing of *Brachiaria brizantha* with mineralized salt and water provided ad libitum.

2.2. Experimental design

Cyclic and anestrous cows (n = 66) were divided into six groups according to body condition score and postpartum period. Cyclic cows (n = 36) were allocated into three groups of 12 (cyclic control, cyclic eCG and cyclic TCR), whereas anestrous females (n = 30) were distributed into three groups of 10 (anestrous control, anestrous eCG, and anestrous TCR), as outlined in Figure 1.

In the cyclic control (C-con, n = 12) and anestrous control (A-con, n = 10) groups, animals received an intravaginal device containing 1.9 g of P4 (CIDR; Zoetis, Brazil) and an intramuscular (IM) injection of 2 mg of estradiol benzoate (EB) (Estrogen; Farmavet, Brazil) on Day 0. The device was removed on Day 8, and the cows received an IM injection of 12.5 mg of dinoprost tromethamine (Lutalyse; Zoetis, São Paulo, Brazil). After 24 hours, the animals received 1 mg of EB IM. In the cyclic eCG (C-eCG, n = 12) and anestrous eCG (A-eCG, n = 10) groups, cows received the same treatment as the control group but also received 400 IU of eCG IM (Novormon; Syntex, Buenos Aires, Argentina) at the time of device removal. The cyclic TCR (C-TCR, n = 12) and anestrous TCR (A-TCR, n = 10) groups received the same treatment as the control group, but TCR was also applied for 56 hours starting at the time of P4 device removal. The cows were kept approximately 2 km away from the calves to eliminate the possibility of contact by touch, sight, sound, or smell. The calves were kept on pasture with free access to water.

2.3. Ultrasonography and follicular dynamics

All animals were evaluated using ultrasonography every 24 hours to measure the DF diameter, CL diameter, and ovulation rate.

The size and location of the follicles and CL were documented for both ovaries and registered in individual maps for further monitoring. Follicles 5 mm or more in diameter were measured using ultrasonography and the diameter was calculated as the average of two cross-sectional linear measurements of the follicular antrum [10].

The DF was defined as that which grew to at least 8 mm and exceeded the diameter of all other follicles. Ovulation was monitored using ultrasonography every 24 hours after FTAI, detected by the absence of the previously identified DF and confirmed by the eventual presence of a CL in the same ovary [11]. At 12 days after ovulation, the CL was measured in the same ovary that previously contained the DF. The diameter was calculated as the average of two linear measurements of the cross-sectional surface of the CL [10]. The follicular growth rate of within each cow was determined by measuring the difference in the follicle size over 24-hour increments of time [11]. Additionally, cyclic animals were evaluated using ultrasonography on Day 8

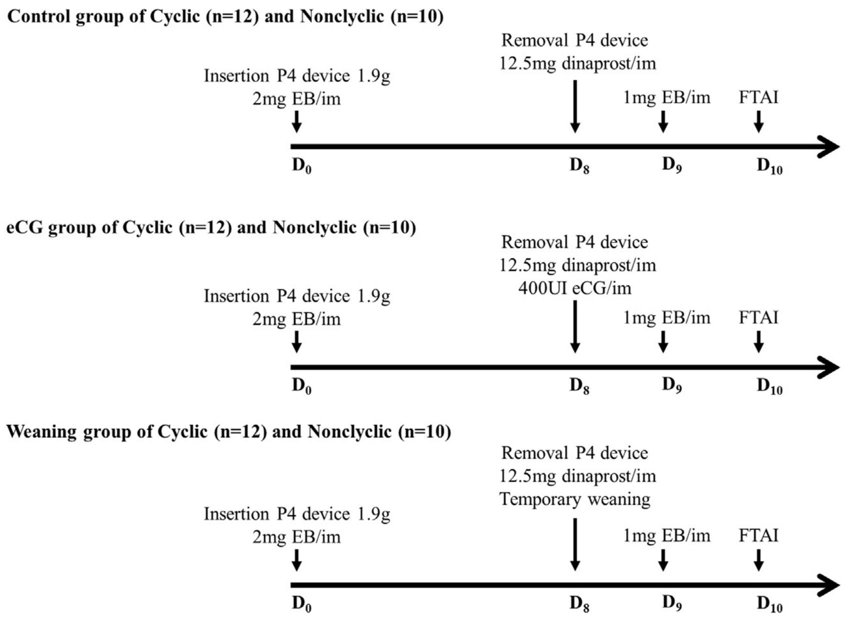

Fig. 1. Schematic representation of the treatment protocols for cyclic and anestrous suckling Nelore cows treated with an intravaginal P4 device, eCG, or TCR. Abbreviations: EB, estradiol benzoate; FTAI, fixed-time artificial insemination; im, intramuscular; P4, progesterone; TCR, temporary calf removal.

(removal P4 device) to compare the follicular diameter between cows with CL versus cows without CL.

2.4. Blood sample collection and P4 plasma levels

Twelve days after ovulation and immediately after measuring the CL, blood samples were collected by venipuncture using 5 mL tubes containing EDTA (Beckton & Dickson, Juiz de Fora, Brazil). Immediately after collection, the tubes were kept at 4 °C for 5 to 10 minutes and then centrifuged at 500× g for 20 minutes. Subsequently, the plasma was transferred into 1.5 mL polypropylene tubes, which were frozen at −20 °C until the time of analysis. Plasma P4 concentrations were determined using a commercial solid-phase radioimmunoassay kit (Coat-a-Count DPC; Diagnostic Products Corporation, Los Angeles, California, USA) with 100 μL samples. The sensitivity of the test was 0.009 ng/mL, and the intratrial variance was 3.31%.

2.5. Statistical analyses

The groups were evaluated for the follicular growth rate average (mm/day), DF diameter (mm), ovulation rate (%), average CL diameter (mm), and P4 concentration (ng/mL). The normality of the data was verified using the Kolmogorov–Smirnov test.

On Day 8, the influence of CL (present or absent) on the follicular diameter of cyclic animals was compared using ANOVA followed by Tukey's test between cows with versus without CL.

Effects of cyclicity (cyclic or anestrous cows), treatments (control, eCG, and TCR) and interactions between cyclicity and the different treatments were evaluated by factorial analysis (2 × 3). When the effect was significant, the variable was subjected to ANOVA followed by Tukey's test to determine differences between groups.

All data were analyzed using Minitab® statistical software 15.1.3 [12]. The significance level for rejecting the null hypothesis was 5%, and therefore, a significance level of 0.05 or less was considered to be indicative of the effects of categorical variables and their interactions. The data are presented as the mean ± one standard error of the mean.

3. Results

Ultrasonography examinations performed at the time of device removal (Day 8) showed that 72% (26/36) of the cyclic cows and 73% (22/30) of the anestrous cows had follicles of 6 mm or more in diameter. On the basis of these findings, the cyclic groups remained with C-con (n = 8), C-eCG (n = 9), and C-TCR (n = 9) and anestrous groups remained with A-con (n = 7), A-eCG (n = 8), and A-TCR (n = 8).

The cyclicity was the most influent factor on the variables of this study ($P \leq 0.05$; Table 1). Despite the lower influence of the treatment, the interaction between cyclicity and treatment showed a positive effect ($P \leq 0.05$) on follicular diameter on Day 10, the follicular growth rate, and P4 concentration 12 days after ovulation.

In cyclic cows, the presence of the CL at the time of device insertion (Day 0) resulted in a smaller follicular diameter at the time when the device was removed (Day 8) (7.4 ± 0.3 mm in cows with CL vs. 8.9 ± 0.4 mm in cows without CL; $P = 0.03$). However, at Day 8 there were no differences in the follicular diameters between cyclic cows C-con (8.5 ± 0.6 mm), C-eCG (8.2 ± 0.5 mm), and C-TCR (8.5 ± 0.6 mm) or compared with anestrous cows A-con (7.1 ± 0.5 mm), A-eCG (7.2 ± 0.5 mm), and A-TCR (7.6 ± 0.3 mm;

Table 1
Effect of cyclicity (cyclic or anestrous cows), treatments (control, eCG, and TCR), and interactions (cyclicity vs. treatments) on DF diameter on Days 8, 9, and 10, follicular growth rate, CL size, and P4 concentration from Nelore cows synchronized to fixed-timed artificial insemination.

Variable	Cyclicity			Treatment				Cyclicity versus treatment	
	Anestrous	Cyclic	P-value	Control	eCG	TCR	P-value	Mean	Interaction
DF diameter on Day 8 (mm)	7.3 ± 0.3	8.4 ± 0.3	0.015[a]	7.8 ± 0.4	7.7 ± 0.4	8.0 ± 0.3	0.785	7.8 ± 0.5	0.898
DF diameter on Day 9 (mm)	8.9 ± 0.2	9.6 ± 0.2	0.041[a]	8.8 ± 0.2	9.2 ± 0.2	9.8 ± 0.2	0.014[a]	9.2 ± 0.4	0.153
DF diameter on Day 10 (mm)	10.1 ± 0.3	11.2 ± 0.2	0.004[a]	10.2 ± 0.3	10.7 ± 0.3	11 ± 0.3	0.138	10.7 ± 0.5	0.046[a]
Follicular growth (mm/day)	1.1 ± 0.1	1.7 ± 0.1	0.004[a]	1.2 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	0.328	1.4 ± 0.3	0.025[a]
CL size (mm)	18.5 ± 1.1	16.1 ± 1.0	0.303	16.2 ± 1.2	18.6 ± 1.2	18.5 ± 1.2	0.295	17.8 ± 1.5	0.464
P4 concentration (ng/mL)	7.3 ± 0.7	5.2 ± 0.6	0.030[a]	5.0 ± 0.8	6.7 ± 0.8	6.9 ± 0.7	0.179	6.2 ± 1.7	0.006[a]

Abbreviations: DF, dominant follicle; eCG, equine chorionic gonadotropin; P4, progesterone; SEM, standard error of the mean; TCR, temporary calf removal.
[a] Means ± SEM significantly different ($P \leq 0.05$).

$P = 0.248$). Neither eCG nor TCR appeared to affect the follicular growth in cyclic Nelore females. The A-con group showed smaller follicular diameter on Days 9 (7.5 ± 0.2 mm) and 10 (8.5 ± 0.2 mm) and lower follicular growth rate (0.7 ± 0.1 mm/day) compared with cyclic cows ($P \leq 0.05$; Table 2). However, numerically the use of eCG or TCR in anestrous cows increased the follicular diameter and the follicular growth rate (Days 9 and 10) compared with A-con group.

All cows (cyclic and anestrous) with follicles of 6 mm or greater in diameter at the time of P4 device removal ovulated within 72 hours, and the time of ovulation did not differ between the experimental groups. No differences were observed in the CL size between the groups ($P = 0.368$). However, the P4 concentrations were higher in the A-TCR group than A-con and C-TCR 12 days after ovulation ($P = 0.01$), as shown in Table 2.

4. Discussion

Despite the differences in the cyclicity of the animals in this study, there were no clear differences in the follicular dynamics of the cyclic and anestrous groups treated with TCR or eCG. However, anestrous cows treated with eCG or TCR showed follicular diameter, follicular growth rate, CL size, and P4 concentration similar cyclic cows. Moreover, TCR was found to produce results similar to the eCG treatment.

Considering only cyclic cows, the presence of the CL at the beginning of treatment (Day 0) resulted in a smaller follicular diameter at the time of device removal (Day 8), with follicular diameters of 7.4 ± 0.33 mm in cows with CL and 8.9 ± 0.43 mm in cows without CL ($P < 0.05$). It is possible that a higher P4 concentration (P4 device and CL) reduced LH pulses and consequently the development of DF after divergence. The use of intravaginal P4 device has been shown to reduce the LH pulse frequency in *Bos taurus* cows with CLs [13]. Similar data were obtained for Nelore heifers that received PGF2α at the beginning of treatment; in these animals, the P4 concentrations were the highest after implantation of the device and decreased over time (3.9 ± 0.2 and 6.8 ± 0.5 ng/mL to Day 0 and Day 8, respectively; $P < 0.05$), and the follicular diameters were greater on the day of device removal (8.2 ± 0.4 vs. 7.3 ± 0.3 mm; $P < 0.05$) [14].

The follicular diameters of cyclic and anestrous cows were similar among the treatment groups (7.1–8.5 mm) at the time of device removal (Day 8; $P = 0.24$). Despite the difference in the follicular diameter at FTAI (C-con, C-eCG, and C-TRC vs. A-con; $P = 0.001$), the application of eCG or TCR did not improve the diameter of dominant follicle in cyclic (C-con, 11.7 ± 0.6; C-eCG, 11.0 ± 0.6; and C-TCR, 12.0 ± 0.5 mm) or in anestrous cows (A-con, 8.5 ± 0.2; A-eCG, 10.2 ± 0.3; and A-TCR, 10.3 ± 0.5 mm). This situation would be unexpected to anestrous cows. However, we emphasize the viability of the treatment, because anestrous cows showed follicular diameter similar to cyclic animals.

Neither eCG injection nor TCR improved the follicular growth rate compared with the control group in cyclic cows (C-eCG, 1.4 ± 0.2; C-TCR, 1.6 ± 0.2; and C-con, 1.7 ± 0.1 mm/

Table 2
Mean ± SEM of the dominant follicle diameter on Days 8, 9, and 10, follicular growth rate, CL size, and P4 concentration 12 days after ovulation in cyclic and anestrous suckling Nelore cows treated with intravaginal P4 device, eCG, or TCR.

Group	Dominant follicle diameter (mm)				Follicular growth (mm/day)	CL size (mm)	P4 concentration (ng/mL)
	N	Day 8	Day 9	Day 10			
Cyclic-control	8	8.5 ± 0.6	9.8 ± 0.6[a]	11.7 ± 0.6[a]	1.7 ± 0.1[a]	16.5 ± 0.8	6.0 ± 0.9[a,b]
Cyclic-eCG	9	8.2 ± 0.5	9.3 ± 0.4[a,b]	11.0 ± 0.6[a]	1.4 ± 0.2[a,b]	17.3 ± 0.8	6.4 ± 0.8[a,b]
Cyclic-TCR	9	8.5 ± 0.6	10.6 ± 0.6[a]	12.0 ± 0.5[a]	1.6 ± 0.2[a,b]	16.7 ± 1.4	4.4 ± 1.0[b]
Anestrous-control	7	7.1 ± 0.5	7.5 ± 0.2[b]	8.5 ± 0.2[b]	0.7 ± 0.1[b]	15.8 ± 1.6	4.0 ± 1.0[b]
Anestrous-eCG	7	7.2 ± 0.5	8.7 ± 0.4[a,b]	10.2 ± 0.3[a,b]	1.5 ± 0.2[a,b]	20.0 ± 2.5	6.5 ± 1.6[a,b]
Anestrous-TCR	8	7.6 ± 0.3	9.2 ± 0.3[a,b]	10.3 ± 0.5[a,b]	1.3 ± 0.1[a,b]	20.1 ± 2.4	9.6 ± 1.4[a]
P-value		0.248	0.005	0.001	0.050	0.368	0.017

For analysis were used only cows with dominant follicle greater than 6 mm in diameter on Day 8, 26/36 of the cyclic cows and 22/30 of the anestrous cows.
Abbreviations: eCG, equine chorionic gonadotropin; CL, corpus luteum; P4, progesterone; SEM, standard error of the mean; TCR, temporary calf removal.
[a,b] Means ± SEM followed by different letters within the same column are significantly different ($P \leq 0.05$).

day) or in anestrous cows (A-eCG, 1.5 ± 0.2; A-TCR, 1.3 ± 0.1; and A-con, 0.7 ± 0.1 mm/day; P = 0.05). Comparing our data with other studies with FTAI in *Bos indicus* heifers [7,15], we found similar follicular diameters, demonstrating that anestrous cows treated with eCG or TCR presented regular follicular growth. Our results show that eCG treatment and TCR are not necessary in cyclic Nelore cows and therefore the costs associated with hormonal treatment and animal management could be reduced. However, for anestrous cows, the use of eCG or TCR seems to have a positive effect on follicular growth, as recently demonstrated by Tortorella, et al. [13].

In our study, a TCR duration of 56 hours in anestrous or cyclic cows showed similar effects to eCG treatment. Both eCG and TCR have frequently been used in FTAI protocols to improve the follicular growth of cattle, resulting in larger, more responsive follicles for ovulation [16] and increased pregnancy rates [17]. Meneghetti, et al. [18] also found that a TCR duration of 48 hours increased the DF diameter in anestrous Nelore cows. In this present study, eCG treatment and TCR in anestrous cows showed follicular dynamics similar to cyclic Nelore cows, indicating that the protocols used (eCG treatment or TCR) were equally effective in cyclic and anestrous cows, in agreement with previous studies [7,16,17]. Our results showed that anestrous cows treated with eCG or TCR presented follicular diameter and follicular growth rate similar to cyclic cows.

All cows with follicles 6 mm or greater on the day of P4 device removal ovulated within 72 hours, and the rates were similar between the experimental groups. These data are interesting because most of the experiments conducted with Nelore cows have demonstrated ovulation rates of approximately 70% to 95% [16,19] with variations in the ovulatory response in postpartum cows treated with EB as a function of the follicle age (2 or 4 days after the emergency phase). A variation according to the follicle size on Day 8 was not observed here, although it is important to note that gonadotropic support provided by eCG treatment or TCR can enhance the ovulatory capability of the DF. In some studies, eCG treatment resulted in a 20% to 30% increase in the ovulation rate of suckled and noncyclic Nelore cows [7,20] and a 34% increase relative to TCR associated with GnRH treatment [18].

Many previous studies have investigated the relationship between ovulatory follicle diameter, CL size, and P4 concentrations, and the ovulation of small follicles in *Bos indicus* heifers has been shown to reduce the CL size and plasmatic concentrations of P4 [14,21]. However, variation in the induction of ovulation during the follicular phase can alter the subsequent P4 concentrations [22,23].

Despite cyclic or anestrous cows treated with eCG and TCR had different numbers regarding the size of CL, it was not statistically significant. The P4 concentrations determined here were similar among the cyclic groups and closely agreed with the results of Marques, et al. [24] in Brangus cows. In anestrous cows, the follicular diameter at FTAI did not affect the size of the CL; however, the P4 concentrations varied among cows with similarly sized CLs at 12 days after ovulation. Starbuck, et al. [25] reported that variations in the P4 concentrations during the luteal phase of the estrous cycle may be related to variations in the timing of ovulation or in the delay of the luteinization process in *Bos indicus* cows. In the present study, however, no differences were observed in the timing of ovulation, indicating that the eCG treatment and TCR produced a luteotropic effect in anestrous Nelore cows.

Higher P4 concentrations were found in cows of the A-TCR group compared with those of the C-TCR and A-con groups at 12 days after ovulation (9.6 ± 1.4, 4.4 ± 1.0, and 4.0 ± 1.0 ng/mL in A-TCR, C-TCR, and A-con, respectively; P = 0.01). However, Marques, et al. [24] demonstrated that eCG treatment increased the P4 concentration of animals with similarly sized CLs at the time of device removal (8.6 ± 0.4 ng/mL in eCG-treated vs. 6.4 ± 0.4 ng/mL in untreated animals, $P < 0.05$). Similar increases in the P4 plasma concentrations 12 days after FTAI were also reported by Baruselli, et al. [1], when eCG treatment was included in the FTAI protocol.

Our results indicate that the application of eCG or TCR did not have a gonadotropic or luteotropic effect with respect to increasing diameter in the DF or CL size in cyclic cows. In previous studies, we observed an increase in the conception rates of cyclic Nelore cows after the use of eCG or TCR, in agreement with the findings of Gimenes, et al. [15]. However, eCG injection or TCR induced similar follicular growth rates and increased the P4 concentrations in anestrous cows compared with cyclic cows. Additionally, TCR has increased the conception in 15% to 20%, providing approximately 50% in the pregnancy rates of noncyclic Nelore cows [5,6,17]. Similarly, Baruselli, et al. [1] observed an increase of 15% in the conception rate of suckled and noncyclic Nelore cows treated with eCG.

4.1. Conclusions

The eCG treatment and TCR associated with an estradiol- or P4-based FTAI protocol influenced the follicular dynamics and P4 concentrations of animals. The treatment did not improve the results of cyclic cows however, animals in anestrous produced similar follicular growth rates, DF diameter at FTAI, CL size, and P4 concentrations compared with cyclic animals, improving the fertility of anestrous cows. Furthermore, the TCR was shown to increase the P4 concentrations in cows with similarly sized CLs.

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