University of São Paulo
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Adjustments in timed-artificial insemination protocols without estradiol for *Bos indicus*

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Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

Piracicaba
2024

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

Advisor:
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DEDICATION

To my dad, mom, and brother. My strength and inspiration.

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"We feel that what we are doing is just a drop in the ocean. But the ocean would be less because of that missing drop."

St. Teresa of Calcutta

SUMMARY

RESUMO

Ajustes em protocolos de inseminação artificial em tempo fixo sem estradiol para *Bos indicus*

Foram realizados dois experimentos, visando a melhoria em protocolos de inseminação aritifical em tempo fixo (IATF) sem o uso de ésteres de estradiol (E2) em vacas *Bos indicus*. O primeiro estudo comparou a eficiência reprodutiva de protocolos de IATF ajustados com ou sem E2 e a inclusão ou não de progesterona injetável de longa ação (injP4) prévia aos tratamentos. Para isso, 293 vacas primíparas e 798 multíparas, de primeira inseminação pós-parto, foram submetidas a protocolos de sincronização, seguindo um arranjo fatorial 2 × 2, resultando em 4 tratamentos. injP4 + E2 (n = 279), E2 (n = 266), injP4 + GnRH (n = 267) e GnRH (n=279). Dez dias antes do início do protocolo, as vacas foram distribuídas aleatoriamente para receber ou não 150 mg de injP4. No D0, todas as vacas receberam dispositivo intravaginal de P4 (1 g), concomitante aos tratamentos experimentais, que foram: 2 mg de benzoato E2 (grupos à base de E2) ou 16,8 µg de acetato de buserelina (GnRH; grupos à base de GnRH). No D6, as vacas dos grupos à base de GnRH receberam 0,5 mg de cloprostenol sódico (PGF) e 300 UI de eCG. No D7, todas as vacas receberam 0,5 mg de PGF concomitante à retirada do dispositivo P4, e as vacas dos grupos à base de E2 receberam 300 UI de eCG e 1 mg de cipionato de E2. A IATF foi realizada 48 horas depois, no D9, quando todas as vacas receberam 8,4 µg de GnRH. Exames ultrassonográficos foram realizados nos D0, 7, 9, 39 e 69 para avaliar o diâmetro do folículo dominante, presença de CL, resposta ovulatória após D0 e prenhez por IA (P/AI). O tratamento com injP4 apesar de aumentar o tamanho do folículo no D0, não influenciou a resposta ovulatória ao primeiro GnRH e não teve efeito na fertilidade, independentemente da base hormonal do protocolo. Os protocolos à base de GnRH apresentaram fertilidade inferior aos protocolos à base de E2 (54,0 vs. 62,4%; $P \leq 0,05$), entretanto, não houve influência da base hormonal na perda gestacional. O segundo estudo avaliou a resposta luteolítica de diferentes doses de PGF, utilizando o cloprostenol sódico, em corpos lúteos (CLs) de 6 e/ou 7 dias de idade. Foram usadas 88 vacas Nelore, previamente sincronizadas, que foram divididas em três tratamentos, Simples (n = 30), recebendo uma dose de bula (0,53 mg) no D7, Duas doses (n = 29), recebendo uma dose de bula no D6 e outra no D7 (ambas de 0,53 mg) e Dupla (n = 29), recebendo uma dose dobrada (1,06 mg) no D7. Foram feitas avaliações ultrassonográficas (D6 – D11) e colheitas de sangue para avaliação de P4 circulante (D6 – D10). O tratamento com uma dose simples de PGF não foi eficiente em provocar luteólise completa em todos os animais; até o final das avaliações, apenas 63,3% (19/30) dos animais apresentaram luteólise, enquanto nos demais grupos, 96,9% (28/29) tiveram luteólise.

Palavras-chave: IATF, Estradiol, Nelore, Luteólise

ABSTRACT

Adjustments in timed-artificial insemination protocols without estradiol for *Bos indicus*

Two experiments were conducted to improve fertility in timed-artificial insemination (TAI) protocols without using estradiol esters (E2) in *Bos indicus* cows. The first study compared the reproductive efficiency of adjusted TAI protocols with or without E2 and the inclusion or absence of long-acting injectable progesterone (injP4) prior to treatments. Primiparous (n = 293) and multiparous (n = 798) cows, for the first postpartum insemination, were submitted to synchronization protocols, following a 2 × 2 factorial arrangement, resulting in 4 treatments. injP4 + E2 (n = 279), E2 (n = 266), injP4 + GnRH (n = 267) and GnRH (n = 279). Ten d before the beginning of the protocol, the cows were randomly assigned to receive or not 150 mg of injP4. On D0, all cows received an intravaginal P4 device (1 g), concomitantly with the experimental treatments, which were 2 mg of E2 benzoate (E2-based groups) or 16.8 µg of buserelin acetate (GnRH; groups based on GnRH). On D6, cows in the GnRH-based groups received 0.5 mg of cloprostenol sodium (PGF) and 300 IU of eCG. On D7, all cows received 0.5 mg of PGF concomitant with P4 device withdrawal, and cows in the E2-based groups received 300 IU of eCG and 1 mg of E2 cypionate. The AI was performed 48 h later on D9, where all cows received 8.4 µg of GnRH. Ultrasound evaluations were performed on D0, 7, 9, 39, and 69 to assess the diameter of the dominant follicle, CL, ovulatory response after D0, and pregnancy per AI (P/AI). Treatment with injP4, despite increasing follicle size on D0, did not influence the ovulatory response to the first GnRH and had no effect on fertility, regardless of the hormonal basis of the protocol. GnRH-based protocols resulted in lower fertility than E2-based protocols (54.0 vs. 62.4%; $P \leq 0.05$). However, there was no influence of the hormonal treatments on pregnancy loss. The second study evaluated the luteolytic response to different doses of PGF, using cloprostenol sodium, on 6 and 7 d old corpora lutea (CL). Nelore cows (n = 88) were used, previously synchronized, and were divided into three treatments: Single (n = 30), receiving a regular dose (0.53 mg) on D7; Two doses (n = 29), receiving a regular dose on D6 and another on D7 (both 0.53 mg) and Double (n = 29), receiving a double dose (1.06 mg) on D7. Ultrasound evaluations (D6 – D11) and blood collection were performed to assess circulating P4 (D6 – D10). Treatment with a single dose of PGF was not sufficient to cause complete luteolysis in all cows; by the end of the evaluations, only 63.3% (19/30) of the cows had undergone luteolysis, while in the other groups, 96.9% (28/29) underwent luteolysis.

Keywords: TAI, Estradiol, Nelore, Luteolysis

1. INTRODUCTION

Timed artificial insemination (TAI) programs started in 1995, aiming to facilitate farm management, allowing the insemination of several animals simultaneously without visualization of estrus expression. This biotechnology works by inducing a new synchronized wave and, consequently, synchronized ovulation. There are two strategies to generate a new follicular wave: using exogenous hormones, either by inducing the ovulation [1] or atresia [2] of the follicles of the previous wave. The first strategy occurs when it is used a hormone that induces LH peak, like GnRH, and consequently, ovulation of the largest (or dominant) follicle [3] if there is a follicle with an ovulatory capacity [4,5]; the second strategy induces atresia of the follicles in the current wave by suppressing LH and FSH through the exogenous supply of estradiol (E2) and progesterone (P4) [6]. Both protocols are widely used in cows and heifers. The decision to use each of the protocols is mainly based on the availability of hormones, pregnancy per AI (P/AI), and the animals used; this is especially important when dealing with *Bos indicus* or *Bos taurus*.

In South America, the most common protocol base used is E2. This protocol type has historically presented good efficiency at synchronization and satisfactory P/AI with a low cost [7]. Furthermore, this protocol is effective in Nelore cattle, the central part of beef cattle in this region, which is characterized by a deep anovulatory condition after calving, which may decrease the follicle size [8,9], compromising the ovulatory response to the first GnRH and consequently, synchronization if GnRH-based protocols are used. Despite this protocol's great advantages, E2 is prohibited in many countries, such as the European Union, which initiated restrictions on E2 use in animal production [10]. This fact may affect Brazil even more, as countries near our country, like Uruguay, present prohibitions or restrictions (Paraguay and Argentina) to use E2 in cattle destined for meat for export. Thus, it is necessary to develop a reliable TAI protocol without E2.

Regarding the concern of the ovulatory response to the GnRH in anestrous Nelore cows, the exposition to low concentrations of P4 can be a possibility to circumvent the anestrus effects in follicular size, improving follicular growth [11] and, consequently, ovulatory response. Low circulating P4 concentrations can improve follicular growth, even with an intravaginal progesterone insert, similar to when it is used in dairy cows [12] or injectable progesterone [13] that can improve results due to the pre-exposition to low concentrations of P4.

Moreover, it is interesting to reconcile good results with management. It was proved that GnRH-based protocols require two doses of Prostaglandin F2α (PGF) to guarantee adequate luteolysis and, consequently, good pregnancy results [14–16]. A single dose of PGF may not induce complete luteolysis, resulting in some residual circulating P4 at the time of AI, impairing fertility [17]. While additional PGF doses are essential, this practice means an extra day of work, which may be challenging to be accepted by the technicians and producers, especially in beef cattle farms, which generally have large breeding groups, and the animal handling does not occur daily. In addition, as important as looking for a suitable TAI protocol without E2 with good results, it is necessary to develop one that is as quickly used as usual. One potential strategy to improve results is doubling the dose of PGF, which may result in P/AI similar to the use of two doses at separate moments in dairy cows [18].

Thus, the present study performed two experiments to evaluate if a GnRH-based protocol would result in outcomes similar to E2-based protocols in *Bos indicus* cows. Moreover, a pre-treatment with long-acting injectable P4 was assessed, as well as to test if doubling the dose of PGF on D7 would induce a similar luteolytic response as when cows are treated with two doses of PGF 24 hours apart (on D6 and D7).

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2. TIMED-ARTIFICIAL INSEMINATION PROTOCOLS BASED IN GNRH FOR *Bos indicus* BEEF COWS

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Abstract

Estradiol (E2) prohibition in timed-AI (TAI) protocols can impair fertility in *Bos indicus* cattle, which have a deep and strong postpartum anestrus and historically a lower pregnancy per AI (P/AI) in TAI protocols without E2 esters. The study aimed to evaluate TAI protocols without E2 esters for postpartum *Bos indicus* (Nelore) cows and the inclusion of long-acting injectable progesterone (injP4) before the beginning of the protocols. Primiparous (n=293) and multiparous (n=798) cows (46.7±0.4 d postpartum and body condition score [BCS]=3.1±0.02) were submitted to synchronization protocols, following a 2 × 2 factorial arrangement, resulting in 4 treatments: injP4 + Estradiol (n=279), Estradiol (n=266), injP4 + GnRH (n=267), and GnRH (n=279). On D-10, cows were randomly assigned to receive 150 mg of injP4 or not. On D0, all cows received an intravaginal P4 device (1 g), concomitant with the experimental treatments, which were 2 mg E2 benzoate (EB; E2-based groups) or 16.8 µg buserelin acetate (GnRH; G-based groups). On D6, cows from G-based groups (injP4+G and G) received 0.5 mg cloprostenol sodium (PGF) and 300 IU eCG. On D7, all cows received 0.5 mg PGF concomitant with P4 device withdrawal, and cows from E2-based groups (injP4+E and E) received 300 IU eCG and 1 mg E2 cypionate (EC). TAI was performed on D9, and all cows received 8.4 µg GnRH. Ultrasound examinations were performed on D0, 7, 9, 39, and 69 to evaluate dominant follicle (DF) diameter, CL, ovulatory response after D0, and P/AI. Statistical analyses were performed by PROC GLIMMIX of SAS 9.4 ($P \leq 0.05$). Treatment with injP4 was associated with fewer cows with CL on D0 (19.2 [104/542] vs. 30.2% [163/540]) and larger DF on D0 (13.3±0.2 vs. 12.1±0.1 mm) and D7 (11.8±0.1 vs. 11.3±0.2 mm), with no effect on P/IA (with: 57.9 [316/546] vs. without: 58.5% [319/545]). Moreover, cows from GnRH-based groups had larger DF on D7 (12.3±0.01 vs. 10.8±0.1 mm) and D9 (13.9±0.1 vs. 12.5±0.1 mm) than cows from E2-based groups. Ovulation after D0 was higher in GnRH-based groups (73.1 [350/479] vs. 12.9 % [62/482]), without effect or interaction with injP4. More cows from E2-based groups were detected in estrus (83.1 [453/545] vs. 74.0% [404/546]), and these groups had greater P/AI than cows from GnRH-based groups (62.4 [340/545] vs. 54.0% [295/546]). Expression of estrus affected P/AI only in E2-based groups, in which cows that expressed estrus

had greater fertility (65.3 [296/453] vs. 47.8% [44/92]). Pregnancy loss (PL) between D39 and 69 was similar between groups, but primiparous had higher PL than multiparous cows (12.9 [13/101] vs. 5.2% [21/407]). In conclusion, injP4 did not affect P/AI, and E2-based groups had better fertility.

Keywords: Nelore, GnRH, FTAI, injectable progesterone.

2.1. Introduction

The use of reproduction biotechnologies is increasing in the cattle herds. For example, more than 20% of cattle herds in Brazil use timed artificial insemination (TAI) as an animal reproduction technique [1]. There are some essential premises to promote the adequate synchronization of ovulation, which are: 1) to synchronize new wave emergency; 2) to control circulating progesterone (P4) concentrations during follicle development; 3) to induce synchronized ovulation at the end of the protocol. There are hormonal manipulations used to promote the synchronization of ovulation in beef cattle, of which two are the most common: the use of a gonadotropin-releasing hormone (GnRH) and the combination of Estradiol (E2) and P4.

In South America, beef cattle herds' most common hormonal strategy is the combination of E2 e P4 (E2/P4). This hormonal association reduces the release of essential hormones for follicular development, the follicle-stimulating hormone ([FSH)] [2], and the luteinizing hormone (LH), inducing follicular atresia and emergency of a new follicular wave within four days [3]. This type of protocol has good efficiency, achieving an average pregnancy per AI (P/AI) of around 50% [4], reaching 60% when there are some hormonal adjustments [5]. Moreover, it is cost-effective since E2 esters are cheap, sometimes costing 1/10 of the GnRH dose. However, despite the great benefits of E2/P4 protocols, E2 has been prohibited for use in production animals in the European Union since 2006 [6], as reported by [7], by commercial pressure, which claims that exogenous E2 can leave residues in meat and milk, that may have harmful effects on human health, despite having no studies that prove any harmful effects when E2 is used in doses close to physiological, as it is done in TAI protocols. Similar legislation is found in other countries, such as Australia, Canada, the United States, and more recently, Uruguay. Argentina and Paraguay have some restrictions on using E2 in TAI protocols when cattle are used for export.

Although E2 is still allowed in Brazil, where E2/P4 protocols are the most used, there is a concern about trade barriers that make using this hormone unfeasible, considering that

Brazil is currently the largest exporter of beef in the world. So, one alternative treatment for TAI is the use of GnRH-based protocols.

The first GnRH-based protocol, called "Ovsynch," was created for dairy cows, inducing the ovulation of the dominant follicle of the wave in progress and, consequently, a new synchronized wave [8]. After adaptations, GnRH-based protocols have been used in beef cattle, especially *Bos taurus*. Among the variations in GnRH-based protocols in beef cattle, there is an intravaginal P4 device at the beginning of the protocol, concurrent with the first dose of GnRH, providing an exogenous source of P4 [9] since not all animals will ovulate to form a new corpus luteum (CL), which should control the P4 concentration during follicular development in this type of protocol. Therefore, using P4 devices in GnRH-based protocols has yielded promising results in *Bos taurus* cattle. Another improvement in this type of protocol is the use of two doses of prostaglandin F2α (PGF) to induce complete lysis [10,11] of the new CL generated after the first GnRH treatment, which can be refractory to treatment with just one PGF dose. The additional dose can ensure low P4 concentrations at the time of AI, increasing fertility. Although most of the studies using GnRH-based protocols in beef cattle are in *Bos taurus*, the Brazilian herd is composed mainly of *Bos indicus* cattle, which has different endocrine, ovarian, and metabolic physiology [12,13] and some previous studies demonstrated unsatisfactory results in fertility in this type of cattle when GnRH-based protocols are used [14].

Most of the low fertility results in *Bos indicus* cows that receive GnRH-based protocols may happen because of the low ovulatory response to the first GnRH, compromising synchronization of the emergence of the new follicle wave. This low ovulatory response can be related to the postpartum anovulatory condition, which is very common in *Bos indicus* [15]. Postpartum cows usually present small follicles in the ovaries due to low LH pulse frequency [16], contributing to a low ovulation rate after the first GnRH and decreasing the efficiency of the protocol in terms of overall synchronization [17], i.e., follicles do not grow enough to acquire ovulatory capacity [18]. It has been shown that Nelore cattle do not ovulate follicles < 8 mm when challenged with an LH treatment [19].

There were some adjustments to improve fertility in postpartum anestrous cows, such as using P4. Pre-exposition to low circulating P4 concentrations increases LH pulse frequency, resulting in greater follicle development [20]. In dairy cows, a presynchronization strategy is expected to be used to have a responsive follicle at the time of treatment with GnRH. For example, dairy cows that received a P4 implant ten days before the beginning of the protocol had a larger follicle at the time of the first GnRH treatment [21]. An alternative to

producing low, but not zero, circulating P4 concentrations that might increase LH pulse frequency is long-acting progesterone (injP4). A previous study from our group has shown that treatment with injP4 can increase follicle size and the duration of the follicular wave, producing a responsive follicle to GnRH treatment for a more extended period. Moreover, using injP4 as pre-treatment to a TAI protocol can improve follicular size and fertility, especially in primiparous Nelore cows when submitted to the first TAI after calving [22].

Therefore, the objectives of the present study were to evaluate modifications in the Ovsynch protocol in *Bos indicus* beef cows and the effect of treatment with injP4 before TAI protocols with or without E2 esters at the first TAI after calving. Two hypotheses were proposed: 1) cows submitted to a modified Ovsynch protocol will present P/AI similar to cows from the E2/P4 protocol, and 2) pre-treatment with injP4 will increase P/AI both in cows treated with the modified Ovsynch or the E2/P4 protocol.

2.1. Material and Methods

2.1.1. Location

This experiment was conducted at the experimental station "Hildegard Georgina von Pritzelwitz," Figueira Farm, located in Londrina, PR, Brazil, from November 2021 to March 2022. All cows were kept on *Urochloa brizantha* pasture supplemented with mineral salt and access to water *ad libitum*.

2.1.2. Cow management

Suckled Nelore (*Bos indicus*) cows were used for the first AI after calving (n = 1091). Primiparous (n = 293) and multiparous (n = 798) cows (46.7 ± 0.4 d postpartum and body condition score [BCS] = 3.1 ± 0.02) were submitted to four TAI strategies.

The BCS was measured 10 d before the beginning of the protocol (1-5 scale, using 0.25 increments, where 1 is extremely thin and 5 is extremely fat).

2.1.3. Experimental design

The experiment used a 2 x 2 factorial arrangement. The factors were 1) the use or not of the injectable long-acting progesterone (injP4) 10 d before the beginning of TAI protocols (D-10), and 2) the hormonal-based protocol, E2 or GnRH, totalizing four experimental groups (Figure 1).

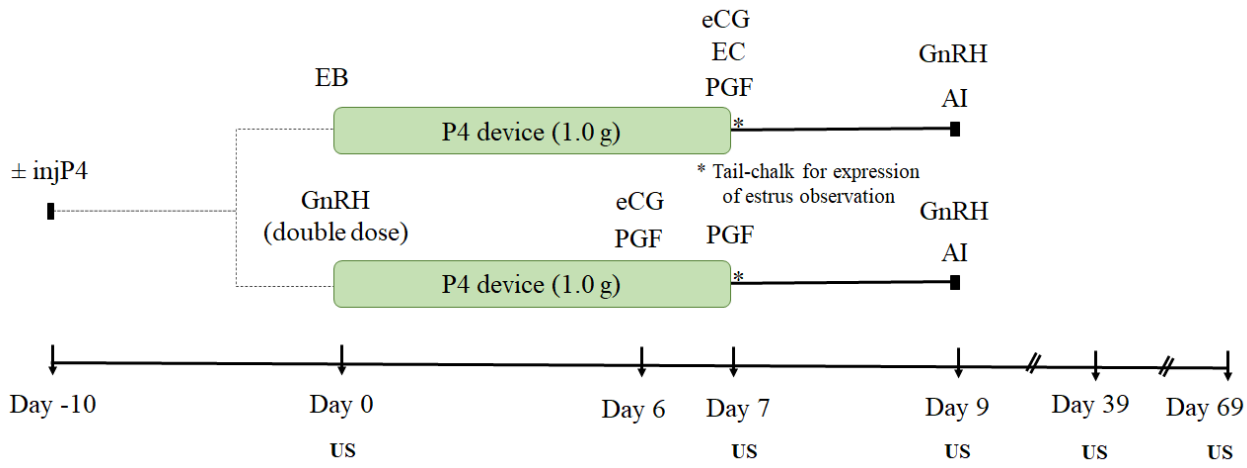

Figure 1. Schematic representation of the experimental design. The design followed a 2 x 2 factorial arrangement, and the factors were: 1) use or not of injP4 10 d before the beginning of the protocols, and 2) the hormonal base of the protocol (E2 or GnRH). On D-10, half of the cows received 150 mg of injectable progesterone (injP4), 10 d later, the half that received injP4 was divided into two new groups, receiving 2.0 mg of estradiol benzoate (EB) or 16.8 µg of buserelin acetate (GnRH), concurrent with P4 device insert; the same treatments were done in cows that did not receive injP4. On D6, cows from the GnRH group received 300 IU of eCG and 0.53 mg of PGF; on D7, the same cows received a new dose (0.53 mg) of PGF, concomitant with P4 device withdrawal; cows from the E2 group received 1.0 mg of estradiol cypionate (EC), 300 IU of eCG and 0.53 mg of PGF; in the same day, P4 insert was removed, and the chalk was painted with tail in all cows On D9, AI was performed, and all animals received 8.4 µg of GnRH. Ultrasound examinations for ovarian dynamics were performed on D0, 7, and 9. The pregnancy diagnosis was made on D39, and the confirmation on D69. injP4 = injectable progesterone; EB = estradiol benzoate; GnRH = gonadotropin-releasing hormone – buserelin acetate; P4 device = intravaginal progesterone device; PGF = Prostaglandin F2α – cloprostenol sodium; eCG = equine chorionic gonadotropin; EC = estradiol cypionate; AI = artificial insemination; US = ultrasound evaluation.

Initially, the cows were randomly assigned to receive or not 150 mg of injP4 (im; Sincrogest injetável, Ouro Fino animal health, Cravinhos, SP, Brazil) 10 d before the beginning of the TAI protocol (D-10). On the first d of the TAI protocol (D0), cows were divided again. The half that received injP4 was split into two new groups, according to the hormonal base of the TAI protocol; thus, cows were randomly divided to receive 2 mg of E2 benzoate im (EB; Syncrogen, GlobalGen vet science, Jaboticabal/SP, Brazil) or a double dose (16.8 µg) of buserelin acetate im (GnRH, Maxrelin, GlobalGen vet science). The GnRH dose

was chosen based on data from other studies, which demonstrated a higher LH peak and better ovulatory response, even if there was high circulating P4 [23,24]. The same treatments were performed in cows that did not receive injP4. On the same day, all cows received a new intravaginal P4 insert (IPI; 1 g; ReproNeo, GlobalGen vet science). Six d later (D6), only cows from the GnRH group received 300 IU equine chorionic gonadotropin im (eCG; eCGen, GlobalGen vet science) and 0.53 mg cloprostenol sodium im (PGF; Induscio, GlobalGen vet science). On D7, only cows of the E2 groups received 300 IU eCG and 1 mg E2 cypionate im (EC; Cipion, GlobalGen vet science). On the same day, all cows received 0.53 mg PGF, concurrent with IPI removal and tail chalk to observe estrus expression. On D9, 48 h after IPI removal, all cows received 8.4 µg GnRH im and were inseminated by the same technician using frozen/thawed semen from proven Nelore bulls. Estrus expression was considered when the cows presented at least 50% of the tail chalk removed.

2.1.4. Ultrasound evaluations

Transrectal ultrasound ovarian examinations in B-mode with a 7.5 MHz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) were performed on D 0, 7, and 9 of the synchronization protocols to check ovarian structures and on D 39 and 69 after the beginning of the protocol for pregnancy diagnosis.

On D0, ovaries were assessed, and the presence of CL and the side and the diameter of the largest follicle (DF) were checked. The DF diameter was measured by the mean of two measures from follicles. These measures consisted of two maximum distances between two opposite borders. On D7, the ovulatory response after D0 was observed by the presence, side, and type (cavitarium or not cavitarium) of CL. Ovulation was confirmed when the DF present on D0 disappeared, and a new CL was in the same ovary. On the same day, the DF diameter was measured. On D9, the ultrasound evaluation was performed only in a subgroup of animals to assess the DF and measure the diameter to calculate follicular growth (between D7 and D9). Besides, early ovulation was considered when DF disappeared before D9. The same operator performed all ovarian dynamics.

Another operator performed a pregnancy diagnosis on D39 and the confirmation on D69 by transrectal ultrasound to confirm the presence of a viable embryo (with heartbeat).

2.1.5. Statistical analysis

All statistical analyses were performed by Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc, Cary, NC). Data were tested for normality of studentized residuals using the UNIVARIATE procedure, following the Shapiro-Wilk method; homogeneity of variances was evaluated by the Levene test, using Hovtest and Welsh methods. All outliers were removed, and data were transformed into logarithms when necessary.

The study was analyzed as a 2 x 2 factorial arrangement. Binomial variables (CL (on D0 and D7), ovulation after D0, expression of estrus, early ovulation, P/AI (D39 and D69), and pregnancy loss [PL]) were performed using the GLIMMIX procedure. The model included the treatment on D-10 (the use or not of injP4), the treatment on D0 (hormonal base - Estradiol or GnRH), and the interaction between treatments (injP4 and hormonal base). Moreover, the effects of parity (primiparous or multiparous), BCS on D-10 (≤2.75 or >2.75), presence of CL on D0 (presence or absence), ovulation after D0 (presence or absence), DF diameter on D7 (<12 or ≥12), DF diameter on D9 (<14 0r ≥14), and expression of estrus (with or without estrus) were included. The scales of BCS on D-10 (≤2.75 or >2.75), DF diameter on D7 (<12 or ≥12), and DF diameter on D9 (<14 0r ≥14) were defined by the median of the effects. The interactions between treatments and the described variables were also evaluated. The model was selected according to the lowest AICC value. Follicle size on D0, D7, and D9 and follicular growth were analyzed using the MIXED procedure days after calving and breeding groups as random effects.

A LOGISTIC procedure for logistic regression was used to evaluate the probability of pregnancy on D39 according to the diameter of the DF on D7 and D9. Logistic regression curves were created using the coefficients provided by the interactive data analysis from SAS and the formula $Y = \exp(\alpha \times X + \beta) / [1 + \exp(\alpha \times X + \beta)]$, where Y = probability of occurrence; exP = exponential; α =slope of the logistic equation; β = intercept of the logistic equation; and X = analyzed variable. Significant differences were considered when $P \leq 0.05$ and tendency when $0.05 < P \leq 0.10$. All binomial data are presented as percentages (%, n/n) and continuous data as arithmetic means ± standard error of the mean.

2.2. Results

2.2.1. Ovarian dynamics

On D0, 24.7% of cows had CL. Considering the presence of the CL by parity, 28.2 (223/790) multiparous cows had CL, while in primiparous cows, just 15.1% ([44/292]; P = 0.01) had CL at the beginning of the protocol. The treatment with injP4 10 d before the beginning of the TAI protocol was associated with fewer cows with CL on D0, only 19.2% (104/542) cows had CL, while the groups without injP4 had 30.2% ([163/540]; P < 0.01).

InjP4 increased follicular size in two moments, on D0 and D7, in all groups (Table 1). However, injP4 did not influence ovulation after D0 in multiparous cows, but more primiparous cows tended to ovulate after D0 when receiving injP4 (57.5 [42/73] vs. 71.0% [49/69]; P = 0.09), but even with a higher ovulatory response when received injP4, primiparous cows tended (P = 0.09) to ovulate less than multiparous cows.

Table 1. Ovarian dynamics (CL on D0, follicle diameter on D0, D7, and D9) and pregnancy per AI (P/AI) on D39, in suckled Nelore cows (primiparous and multiparous), according to injectable progesterone (injP4) treatment 10 d before the beginning of TAI protocol.

Item	**Treatments**		**P**
	injP4	**No injP4**	
CL on D0, % (n/n)	19.2 (104/542)	30.2 (163/540)	<0.01
Follicle diameter on D0, mm (n)	13.3 ± 0.2 (n=545)	12.1 ± 0.1 (n=540)	<0.01
Follicle diameter on D7, mm (n)	11.8 ± 0.1 (n=179)	11.3 ± 0.2 (n=174)	0.02
Follicle diameter on D9, mm (n)	13.3 ± 0.1 (n=179)	13.1 ± 0.1 (n=174)	0.33
Pregnancy per AI on D39, % (n/n)	57.9 (316/546)	58.5 (319/545)	0.64

As expected, cows from GnRH-based groups had a higher ovulatory response after D0 than E2-based cows (73.1 [350/479] vs. 12.9% [62/482]; P < 0.01). The CL on D0 compromised the ovulatory response in GnRH-based groups; cows with CL on D0 had a lower ovulatory response to the first GnRH than cows without CL (60.9 [53/87] vs. 76.5% [297/388]; P < 0.01).

Cows from GnRH-based protocols presented larger follicles on D7 (12.3 ± 0.01 vs. 10.8 ± 0.1 mm) and D9 (13.9 ± 0.1 vs. 12.5 ± 0.1 mm) than cows that initiated TAI protocols with E2 (Table 2).

Table 2. Ovarian dynamics (CL on D0, follicle diameter on D0, D7, and D9), ovulatory response after the treatment performed on D0, and early ovulation (ovulation occurred between D7 and D9) in suckled Nelore cows (primiparous and multiparous) submitted to four TAI strategies: with or without pre-treatment with injectable progesterone (injP4) 10 d before the beginning of the protocol and two different hormonal bases, Estradiol (E2) or gonadotropin-releasing hormone (GnRH).

Item	Treatments				P		
	E2		GnRH				
	injP4	No injP4	injP4	No injP4	Hormonal base	injP4	Base*injP4
CL on D0, % (n/n)	20.6 (57/277)	29.2 (77/264)	17.7 (47/265)	31.2 (86/276)	0.73	<0.01	0.27
Follicle diameter on D0, mm (n)	13.1 ± 0.2 (279)	12.1 ± 0.2 (264)	13.5 ± 0.2 (266)	12.2 ± 0.2 (276)	0.44	<0.01	0.66
Follicle diameter on D7, mm (n)	11.0 ± 0.2 (87)	10.5 ± 0.2 (87)	12.5 ± 0.2 (92)	12.1 ± 0.2 (87)	<0.01	0.03	0.81
Follicle diameter on D9, mm (n)	12.6 ± 0.2 (87)	12.4 ± 0.2 (87)	14.0 ± 0.2 (92)	13.9 ± 0.2 (87)	<0.01	0.33	0.73
Ovulatory response after D0, % (n/n)	12.6 (31/246)	13.1 (31/236)	75.9 (186/245)	70.1 (164/234)	<0.01	0.52	0.30
Early ovulation on D9, % (n/n)	7.4 (7/95)	4.3 (4/93)	2.1 (2/94)	6.4 (6/93)	0.50	0.40	0.11

2.2.2. Expression of estrus

Overall, 78.6% (857/1091) of cows were detected in estrus. More cows from E2-based groups were detected in estrus than GnRH-based groups (83.1 [453/545] vs. 74.0% [404/546]; $P < 0.01$).

More cows got pregnant when expressed estrus (59.9 [513/857] vs. 52.1% [122/234]; $P = 0.03$). Although, expression of estrus affected P/AI only in E2-based groups, in which cows that expressed estrus had greater fertility (65.3 [296/453] vs. 47.8% [44/92]; $P = 0.03$).

When cows did not show estrus, there was no difference between treatments ($P = 0.37$), but when the cows showed estrus, P/AI was better in E2-based groups ($P < 0.01$).

2.2.3. Pregnancy per AI

Pregnancy per AI was not affected by injP4 treatment (Table 3), but the hormonal base influenced fertility without interaction with injP4. In this case, cows from the E2-based protocol had greater fertility than GnRH-based (Figure 2).

Table 3. Expression of estrus, pregnancy per AI on D39, pregnancy on D69, and pregnancy loss between D39 and D69 in suckled Nelore cows (primiparous and multiparous) submitted to four TAI strategies with or without pre-treatment with injectable progesterone (injP4) 10 d before the beginning of the protocol and two hormonal bases, Estradiol (E2) or GnRH.

Item	**Treatments**				***P***		
	E2		**GnRH**				
	injP4	**No injP4**	**injP4**	**No injP4**	**Hormonal base**	**injP4**	**Base*injP4**
Expression of estrus, % (n/n)	83.1 (232/279)	83.1 (221/266)	70.8 (189/267)	77.1 (215/279)	<0.01	0.37	0.24
Pregnancy per AI on D39, % (n/n)	63.1 (176/279)	61.6 (164/266)	52.4 (140/267)	55.6 (155/279)	<0.01	0.64	0.52
Pregnancy per AI on D69, % (n/n)	59.1 (165/279)	56.8 (151/266)	49.1 (131/267)	52.3 (146/279)	0.01	0.76	0.50
Pregnancy loss, % (n/n)	6.1 (10/165)	8.6 (13/151)	6.8 (9/131)	6.2 (9/146)	0.74	0.68	0.82

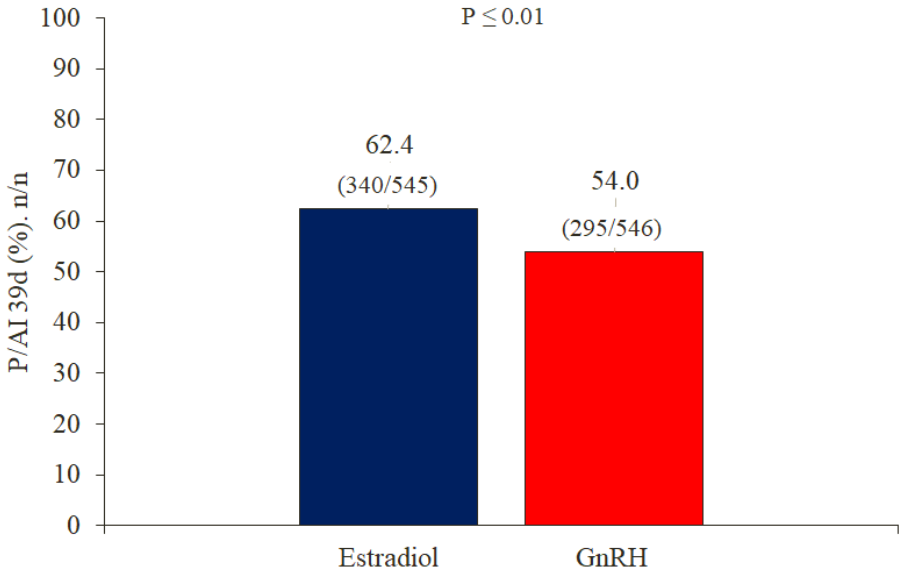

Figure 2. Pregnancy per AI (P/AI) on D39 (30 d after AI) in suckled Nelore cows (primiparous and multiparous) submitted to two TAI protocols, an estradiol-based or GnRH-based protocol.

Multiparous cows had greater fertility than primiparous cows without treatment interaction (Figure 3). Furthermore, BCS also affected P/AI (Figure 4); cows with higher BCS (> 2.75) had greater fertility than cows with BCS ≤ 2.75 (61.0 [399/654] vs. 54.0% [236/437]; P = 0.04).

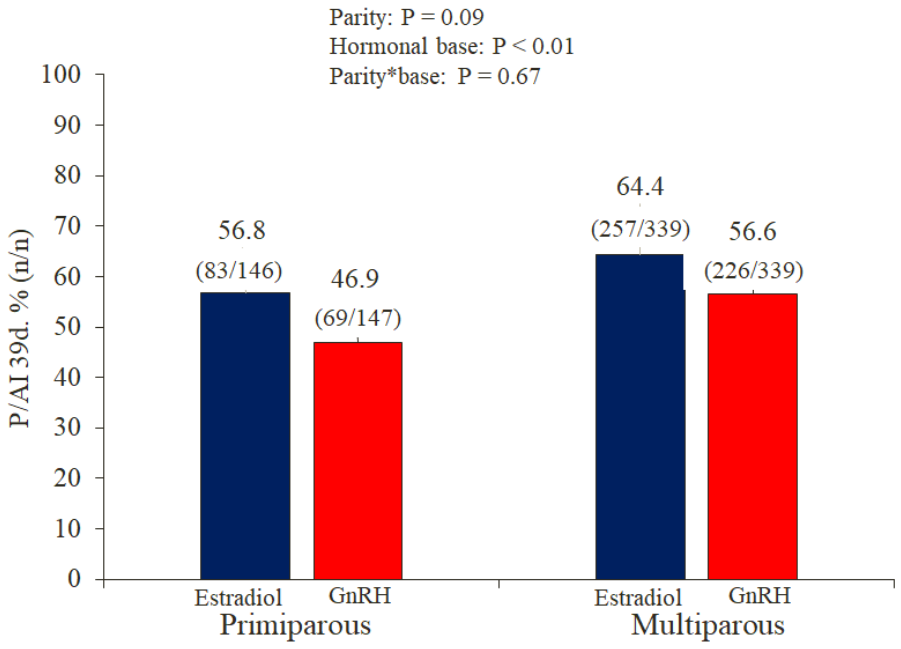

Figure 3. Pregnancy per AI (P/AI) on D39 (30 d after AI) in suckled Nelore cows in both hormonal bases used in the study (estradiol or GnRH), according to parity (primiparous and multiparous).

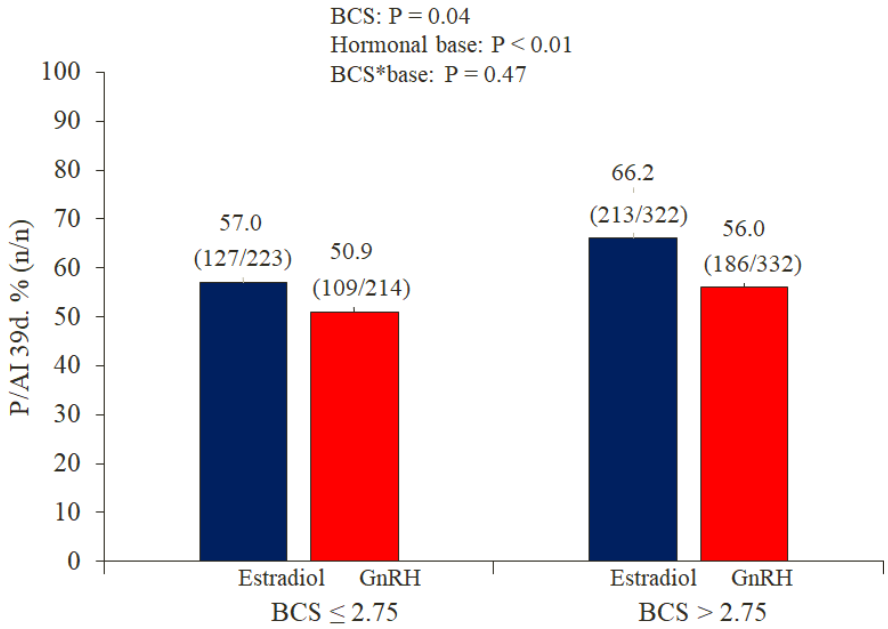

Figure 4. Pregnancy per AI (P/AI) on D39 (30 d after AI) in suckled Nelore cows (primiparous and multiparous), according to the body condition score (BCS), measured 10 d before the beginning of TAI protocol, classified in two levels, BCS ≤ 2.75 or BCS > 2.75 (1-5 scale) in both hormonal bases used in the study, estradiol or GnRH.

In GnRH-based groups, the probability of pregnancy was explained by a linear regression with follicle size on D7 (Figure 5) and D9 (Figure 6), evidencing a positive relationship between P/AI and the size of the follicles. In other words, the larger the follicle size, the greater the likelihood of pregnancy. However, in E2-based groups, the probability of pregnancy had a quadratic regression, showing that cows with smaller follicles and those with larger follicles on D7 and D9 were less likely to become pregnant.

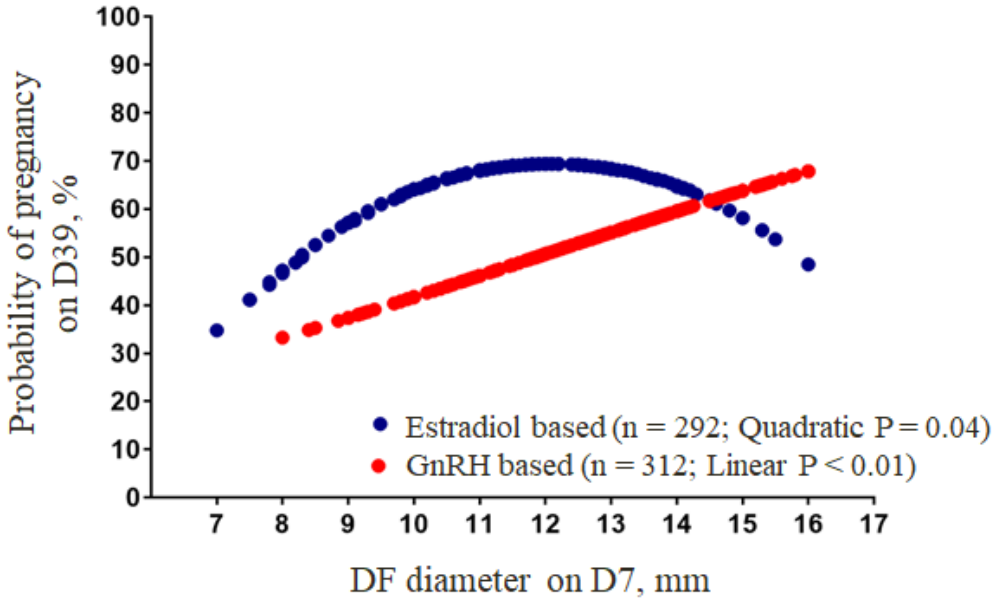

Figure 5. Probability of pregnancy on D39 in suckled Nelore cows (primiparous and multiparous), submitted to TAI protocols, estradiol or GnRH based, according to the larger dominant follicle (DF) diameter at the time of P4 device removal (D7).

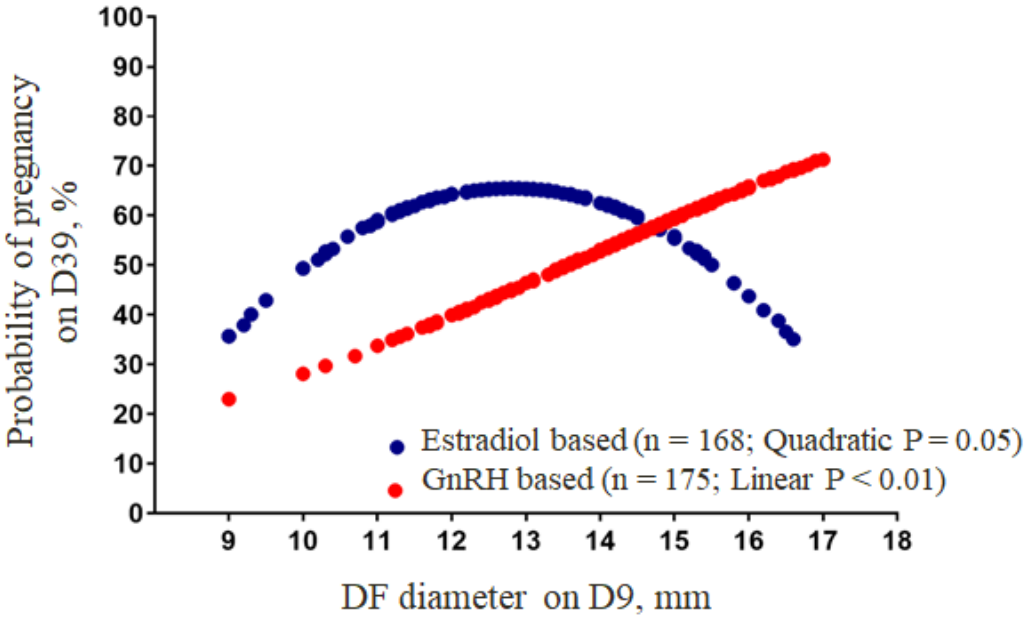

Figure 6. Probability of pregnancy on D39 in suckled Nelore cows (primiparous and multiparous), submitted to TAI protocols, estradiol or GnRH-based, according to the larger dominant follicle (DF) diameter at the time of artificial insemination (D9).

In GnRH-based groups, cows with a follicle ≥ 12.0 mm at the time of P4 device removal had greater P/AI than cows with follicles < 12.0 mm (P < 0.01; Figure 7). At the time

of AI, cows with dominant follicles > 14.0 mm tended to have greater P/AI (P = 0.06; Figure 8).

Pregnancy loss was only influenced by parity. Primiparous cows had greater PL than multiparous (12.2 [17/152] vs. 5.2% [25/483]; P = 0.01). There was no treatment effect on PL (Table 3).

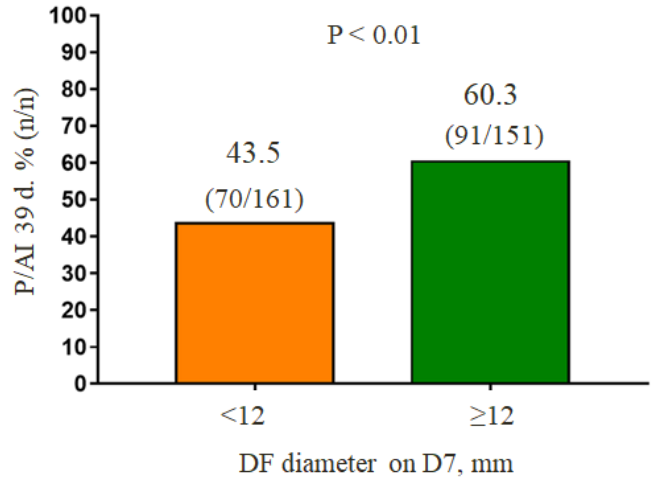

Figure 7. Pregnancy per AI (P/AI) on D39 in suckled Nelore cows (primiparous and multiparous), submitted to GnRH-based protocol, according to the larger dominant follicle (DF) diameter at the time of P4 device removal (D7).

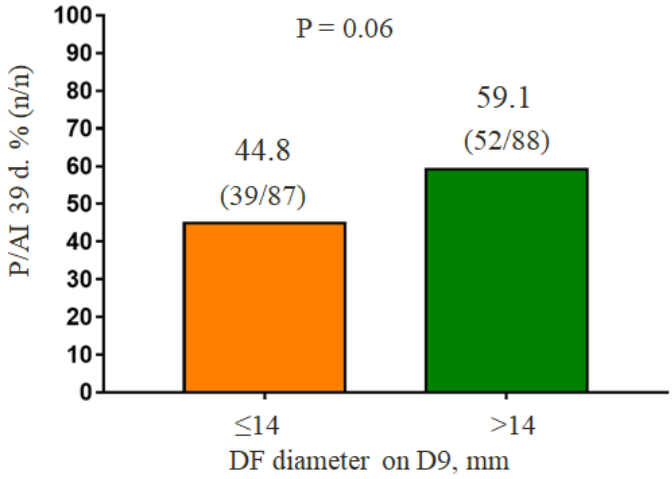

Figure 8. Pregnancy per AI (P/AI) on D39 in suckled Nelore cows (primiparous and multiparous), submitted to GnRH-based protocol, according to the larger dominant follicle (DF) diameter at the time of artificial insemination (D9).

2.3. Discussion

Concerning treatments with injP4, cows that received injP4 had larger follicles on D0 and D7 in both TAI strategies. Previous data from our laboratory showed larger follicles in anestrous primiparous cows treated with injP4 (Silva et al., unpublished). The increase in follicular size occurs because low concentrations of P4 may increase LH pulsatility, stimulating follicular growth. Similar results were reported by Simões et al., 2018, who found an increase in follicle size at the beginning of the protocol and at the time of implant removal in groups that initiated TAI protocols with E2, at the same time, cows that received GnRH at the beginning of the protocol, had an increase in follicular growth just at the beginning of the protocol, apparently, in this study, the presence of the CL induced by the first GnRH treatment, impaired follicular growth. There was no difference in follicle size on D9 in cows that were or were not previously exposed to injP4, probably because on D6/D7, all cows received eCG, an exogenous hormone that acts similarly to LH and FSH, reducing the difference in follicular size between treatments. Fewer cows from injP4 treatment had CL on D0. This can not be interpreted as a reduction in cyclicity; instead, low but constant P4 concentrations prevented cows from ovulating before D0 [22,25].

Furthermore, the pre-exposition to P4 did not influence P/AI differently than what was reported by previous studies that had shown benefits in fertility when anestrous cows, especially with low BCS (~2.7), received injP4 10 d before the beginning of the protocol [22]. In the present study, the average BCS was 3.1, and there were few cows with low BCS. This factor probably contributed to better fertility with no effect of injP4.

Cows from GnRH-based groups had larger follicles in all moments after treatment (D7 and D9). In this protocol type, the new follicular wave usually emerges 24 h after the first GnRH in cows that ovulated [18,19], so these animals had more days for follicular growth. Thus, when protocol starts with GnRH, the follicles are larger than in cows treated with E2, especially those that ovulated to the first GnRH [26].

In the present study, 75% of the cows ovulated to the first GnRH, considering a high ovulatory response, but without interaction with injP4, even though cows that were previously exposed to P4 had larger follicles at the moment of the first GnRH treatment. That happened because in all groups (with or without injP4), cows had adequate follicle sizes responsible for GnRH. This ovulatory response was unexpected to all groups because historically, *Bos indicus* did not have a good reaction to GnRH after calving; otherwise, Pinheiro et al., 2013 found a good hypothalamic response 2 weeks after calving when the cows were supplemented

with ground corn and cottonseed meal and received GnRH treatment. However, the cows of the present study did not receive any supplementation, and the BCS at the moment of injP4 treatment was relatively high, which may have contributed to the high GnRH response, regardless of the injP4 treatment. The only injP4 benefit in the ovulatory response was found in primiparous cows. These animals ovulated more when previously exposed to P4, and more primiparous were in deep anestrus; thus, when exposed to P4, they had the sensibilization of the hypothalamus to respond to an LH peak and ovulate.

CL at the beginning of the protocol decreased the ovulatory response to GnRH, and cows with CL on D0 ovulated less than cows without CL. This probably happened because cows with CL have higher circulating P4 concentrations, which can impair LH release, resulting in a lower LH peak and decreasing the ovulatory response after GnRH treatment [28–30]. This effect can be reduced using a double dose of GnRH at the beginning of the protocol to induce a higher peak of LH [23], which can be potentiated according to the GnRH analogs [24,31]. However, even with a double dose of buserelin acetate, the high P4 concentrations from the CL could have impaired the ovulatory response. There was no difference in premature ovulation between treatments, different from what was reported by Abreu et al., 2022, who found a higher occurrence of premature ovulation in cows that initiated a TAI protocol with GnRH.

GnRH-based groups resulted in 54% P/AI; considering the 50% mean P/AI in South American cattle when E2-based protocols are used [4], this type of protocol had good fertility, but when it was compared in the same system, the GnRH-based protocol resulted in fewer cows pregnant than the E2-based protocol, presenting 13.5% lower fertility; thus, results did not support our first hypothesis, regarding P/AI being similar between treatments.

In GnRH-based protocols, we observed that the larger the follicle, the higher the probability of cows becoming pregnant (linear regression). These results demonstrate the importance of circulating E2 to pregnancy. This type of protocol does not have an exogenous source of E2. Thus, all circulating E2 was from the follicles; larger follicles can provide more circulating E2, increasing fertility. There is a positive correlation between follicle size and endogenous circulating E2, so smaller follicles possibly did not have ideal concentrations of E2, compromising fertility in beef [33], dairy [34], and buffalo [35] cows. This result emphasizes the importance of follicle size in this protocol type, not only for the ovulatory response to GnRH but also for the endogenous supply of E2. Jinks et al., 2013 showed the importance of follicle size and circulating E2 on P/AI, not only for fertilization but especially for embryo development, where cows with smaller follicles to have good P/AI should receive

an exogenous E2 treatment. Also, cows with higher circulating E2 should have better endometrial thickness [37], uterine quality, and embryo viability [38]. Thus, in GnRH-based protocol, it is essential to have a larger follicle at the time of AI, which can be achieved by increasing the proestrus period, increasing growth time, expression of estrus, and, consequently, fertility [39]. GnRH-based protocols with a more extended proestrus period can produce similar results in Bos indicus cows compared to protocols based on E2 [40]. This is because the high levels of circulating E2, combined with low levels of progesterone, interact strongly with LH and FSH peaks and the endometrial area [41], contributing to a better quality of the uterus.

On the other hand, E2-based protocols have a different outcome regarding the likelihood of cows becoming pregnant. The probability of pregnancy occurring depends on the size of the follicle. Specifically, the possibility of cows becoming pregnant presented a quadratic regression when the follicle was around 13mm at the time of AI. This indicates that the probability of successful pregnancy was greater when the follicle measures approximately 13mm compared to smaller or larger. Other authors found similar results when using an E2-based protocol [42,43]. This phenomenon may occur when initiating the protocol with E2/P4. In this protocol, the hormone combination regresses the current follicular wave and starts a new one 3-5 days after initiation.

Regarding the importance of high circulating E2 during TAI protocol, an earlier study from our group compared the fertility levels in protocols initiated with GnRH or E2/P4 and found no significant differences in fertility levels between the two, different than found in the present study[44]. However, the current study used an E2 ester (EC) as an ovulation inducer, emphasizing the importance of maintaining high levels of circulating E2 at the protocol's end, even with a successful ovulatory response and good synchronization. Therefore, low E2 concentrations before AI could potentially compromise fertility.

Parity and BCS affected P/AI without treatment interaction. Primiparous cows had lower P/AI than multiparous, as reported in several studies in *Bos indicus* [5,44,45] cattle. This result is supported by the differentiated physiology in primiparous cows, smaller follicles and metabolism, and higher energetic demand (factors that can reduce fertility). Regarding the BCS effect, cows with higher BCS ($\geq$ 2.75) had greater P/AI. The BCS at first AI after calving can impact the fertility and success of the breeding season; cows with a higher BCS usually have better fertility [5,46] and may have better ovarian activity [42], which impacts P/AI.

Despite other studies showing a negative relationship between circulating E2 before TAI and PL, the present study showed no difference in PL between treatments, with or without exogenous E2 treatment at the end. This is intriguing because it has been demonstrated that circulating E2 concentrations have an essential interaction with uterine quality, P/AI, and embryo development. Although there was no difference between treatments, there was a parity effect, in which primiparous cows had greater PL than multiparous; the same was described by Consentini et al., 2023. This result can be associated with higher nutritional demands in primiparous. It is important to point out that, on this farm, all cows received the same dietary program without supplementation. Thus, the energy demand of primiparous cows may have impacted pregnancy maintenance, culminating in greater PL. Besides, primiparous cows, which usually have smaller follicles, longer postpartum anestrus, and lower fertility, are challenging, even when some adjustments are made [45].

2.4. Conclusion

In conclusion, treatment with injP4 before the beginning of the TAI protocol did not affect P/AI in this study. Furthermore, the GnRH-based treatment produced a high ovulatory response after D0, adequate ovulatory follicle size, and good P/AI (54%). Despite that, this protocol could not produce reproductive outcomes similar to the E2-based protocol, which resulted in better expression of estrus and P/AI on D39 and D69.

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3. STRATEGIES TO LYSE A YOUNG CORPUS LUTEUM USING CLOPROSTENOL SODIUM IN *Bos indicus* COWS

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Abstract

A new corpus luteum (CL) can be refractory to a single dose of luteolytic drugs, impairing luteolysis and, consequently, compromising ovulation and fertility at the end of the timed-AI (TAI) protocol. This study evaluated the luteolytic effect of cloprostenol sodium (PGF) treatments in a 6- or 7-day-old CL. On day -9, 109 Nelore (*Bos indicus*) cows (2.8±0.1 body condition score) underwent a synchronization protocol. On day 0, cows received 8.4 µg buserelin acetate for ovulation induction and formation of a new CL. Ovulation was confirmed on day 6, and only cows with a CL (n=88) were utilized. On the same day, cows were randomly assigned to PGF treatments: Single: a conventional dose (0.53 mg) on day 7 (n=30); Double: a double dose (1.06 mg) on day 7 (n=29); or Two doses: one dose (0.53 mg) on day 6 followed by the same dose on day 7 (n=29). Ovarian structures were assessed by ultrasound, using B-mode and Doppler from day 6 to 11 to analyze CL and dominant follicle [DF] size, CL blood perfusion, and ovulation. Blood samples were collected from days 6 to 10 to analyze circulating progesterone (P4) concentrations. The CL volume was calculated by the formula $V=4/3\times\pi\times R^3$. Luteolysis was considered when P4 concentration was ≤0.5ng/mL, or <0.6 ng/mL, in addition to a reduction in CL volume (>80% of the original), without blood perfusion, expression of estrus and/or ovulation. Statistical analyses were performed by PROC GLIMMIX of SAS 9.4 (P≤0.05). In response to PGF treatments, CL volume was reduced by day 11 in all groups, but with a greater decrease in Two doses than in Single and Double (91[a] *vs*. 73b vs. 80%[b] reduction from the initial volume). Circulating P4 (ng/mL) was lower in Two doses than Single and Double on d7 but did not differ from the Double group on d9 (0.36±0.1[b] *vs*. 0.53±0.1[a] *vs* 0.48±0.1[ab]) and d10 (0.33±0.1[b] *vs*. 0.69±0.1[a] *vs*. 0.39±0.1[ab]). Luteolysis was greater on d8 in Two doses than Single and Double (48.3[a] [14/29] *vs*. 26.7[b] [8/30] *vs*. 20.7%[b] [6/29]).

Nevertheless, on d10, luteolysis was similar in Two doses and Double, and both were greater than Single (96.9[a] [28/29] *vs.* 96.9[a] [28/29] *vs.* 63.3%[b] [19/30]). There was no effect of treatment on the expression of estrus, although more cows from Two doses ovulated vs. Single, without a difference between both and Double (68.9[a] [20/29] *vs*. 30%[b] [9/30] *vs*. 37.9%[ab] [11/29]). In conclusion, the treatment with a conventional dose of cloprostenol on day 7 of the estrous cycle was inefficient in inducing complete luteolysis in several cows. In contrast, treatment with a double dose on day 7 or 2 doses, 24 h apart, starting on day 6, resulted in a greater incidence of luteolysis within 72 h.

Keywords: FTAI, luteolysis, Nelore, progesterone.

3.1. Introduction

Concerning timed-artificial insemination (TAI) protocols and the importance of the premises for its proper functioning, we can highlight the control of progesterone (P4) concentration during follicular development and ovulation. For follicular development, some concentration of P4 is essential to guarantee adequate growth, to control luteinizing hormone (LH) pulse frequency [1], and to prevent premature estrus and ovulation. However, at the time of AI, the ideal endocrine set should be high circulating E2 and no P4. Residual circulating P4 can impair ovulation, compromising pregnancy per AI (P/AI) [2,3]. All these endocrine factors can influence ovulation and uterine development [4,5].

The P4 used during the follicular development can be from an endogenous source, such as the corpus luteum (CL) generated at the beginning of the TAI protocol, when it starts with GnRH, or exogenous, by intravaginal P4 device (IPI) or long-acting injectable progesterone (injP4). When using IPI, controlling the drop in circulating P4 is more accurate by removing the insert at the end of the TAI protocol. When the protocol uses P4 as an endogenous source, it is necessary to cause the lysis of the CL, a transient reproductive gland and the biggest endogenous producer of P4 in ruminants. Luteolysis occurs by the prostaglandin F2α (PGF) liberation by the endometrium, which will induce the luteolytic process [6]. First, functional regression occurs, marked by a decline in P4 production, followed by structural regression, the death of CL cells, until *corpus albicans*, the structure formed after complete luteolysis [7]. Physiologically, PGF release occurs in pulses during the generation of the luteolytic cascade [8]. This process is expected to naturally occur around day 17-18 of the bovine estrous cycle [9].

In the TAI protocol, luteolysis occurs by administration of an exogenous PGF. Two types of PGF are most used: a natural molecule, dinoprost tromethamine, which is quickly metabolized, or a PGF analog, cloprostenol sodium, which has a longer half-life. There are many studies about the use of these molecules, demonstrating excellent luteolytic performance; however, some factors can affect luteolytic efficiency, such as the dose used and the age of the CL.

The age of the CL is critical to luteolysis achievement because the younger the CL, the more resistant it is to PGF. When a TAI protocol begins with GnRH, such as with the Ovsynch protocol, it is expected to ovulate in response to the GnRH-induced LH pulse and the emergence of a new synchronized wave [10]. This process results in the formation of a new CL. At the end of this protocol, this CL is young and, consequently, more refractory to PGF. The refractoryness of CL to PGF is not entirely elucidated; it is known that the CL has PGF receptors since its formation [11]. However, younger CL presents higher concentrations of 15-hydroxy-prostaglandin dehydrogenase [12], a PGF-catalyzing enzyme, which may hinder the response to PGF at this stage.

Studies have evaluated the increase in PGF dose to improve the lysis of young CL. When the PGF dose was increased by 50%, more dairy cows had undergone luteolysis in an Ovsynch protocol [13]. The use of two doses of PGF can increase luteolysis in cows submitted to TAI protocols [14], using both dinoprost trometamine [15] or cloprostenol sodium [16]. The use of two doses of PGF 24 h apart is commonly used in GnRH-based TAI protocols in *Bos taurus*, especially dairy cows. In beef cows, as reviewed by Monteiro et al., 2023), two doses of PGF promote an increase of 19.2% in P/AI when the protocol started with GnRH. There are few studies of induced luteolysis in young CL in *Bos indicus* cows, mainly because the chosen TAI protocol in this genetic group is usually estradiol-based [18]. However, Ferraz Júnior., 2016 demonstrated that even using the recommended dose of PGF in a 5 or 7-day-old CL, it did not induce complete luteolysis in several animals, confirming the importance of the use of additional doses of PGF in both genetic groups.

Although treatment with an additional dose of PGF is well established, especially on dairy farms, the extra day of management can hinder adherence to the technique on beef farms, where the management is not usually daily. A possible alternative would be doubling the dose of PGF without any extra management. When a double dose of PGF is used, results demonstrate that the luteolysis is better than with a single dose, considering luteolysis when P4 is < 1.0 ng/mL, but not different when luteolysis was considered if P4 was < 0.5 ng/mL in dinoprost trometamine treatments [19]. In contrast, when cloprostenol sodium was used, no

difference in P/AI was observed, but animals that received a double dose had less pregnancy loss than the dairy cows treated with a single dose [20].

Studies evaluating alternative treatments with PGF in young CL in *Bos indicus* cattle are scarce. Therefore, the objectives of this study were to assess luteolysis in *Bos indicus* cows using different doses of cloprostenol sodium and evaluate if a double dose on the same day had an equal luteolytic effect as two doses 24 h apart. Two hypotheses were proposed: 1) cows treated with a double dose of cloprostenol sodium will present similar luteolysis as cows treated with two doses 24 h apart; 2) a single dose of PGF will not be able to induce complete luteolysis in young CL.

3.2. Material and Methods

3.2.1. Location

This experiment was conducted in two farms: 1) an experimental station, "Hildegard Georgina von Pritzelwitz", Figueira Farm, located in Londrina, PR, Brazil, and 2) a commercial farm, Morro do Brumado farm, located in Itatinga, SP, Brazil, from March to April 2023. All cows were kept on *Urochloa brizantha* pasture supplemented with mineral salt and access to water *ad libitum*.

3.2.2. Cow management

Nelore cows (n = 109) were submitted to a pre-synchronization protocol to produce a new CL; only cows that responded to the pre-synchronization and had a 6-day-old CL were used in the study (n = 88). These cows with body condition score [BCS] = 2.8 ± 0.01 underwent one of three luteolytic strategies using cloprostenol sodium. The BCS was measured on the first day of evaluation, on D6, using a 1-5 scale, with 0.25 increments (where 1 is extremely thin and 5 is extremely fat).

3.2.3. Experimental design

Before the beginning of the study, all cows underwent synchronization (Figure 1). On day -9, 109 Nelore (*Bos indicus*) received 2 mg of estradiol benzoate intramuscularly (im; Syncrogen, GlobalGen vet science, Jaboticabal, SP, Brazil), concomitant with 0.53 mg of

cloprostenol sodium im (PGF; Induscio, GlobalGen vet science) and a new intravaginal insert containing 1.0 g of P4 (IPI; ReproNeo, GlobalGen vet science). Seven d later (day -2), the IPI was removed, and all cows received 0.53 mg im of PGF, 1.0 mg of estradiol cipionate im (Cipion, GlobalGen vet science), and 300 IU of equine chorionic gonadotropin im (eCGen, GlobalGen vet science). On day 0 (48 h after IPI removal), all cows received 8.4 µg buserelin acetate im (GnRH; Maxrelin, GlobalGen vet science). Six d after the end of synchronization (day 6), all cows were submitted to an ultrasound evaluation to check the presence of a new CL; if the cow did not have a new CL at this moment (n = 21), it was removed from the study. Therefore, 88 cows were randomly assigned to three PGF treatments: Single (n = 30): all cows received 0.53 mg of PFG on d7; Two doses (n = 29): all cows received one dose (0.53 mg) of PGF on d6 followed by the same dose on day 7; and Double: all cows received a double dose (1.06 mg) of PGF on day 7. After treatment distribution, only cows from Two doses received 0.53 mg of PGF on day 6. On the following day (day 7), cows from the Single and Two doses groups received 0.53 mg of PGF, and cows from the Double group received 1.06 mg of PGF (Figure 1).

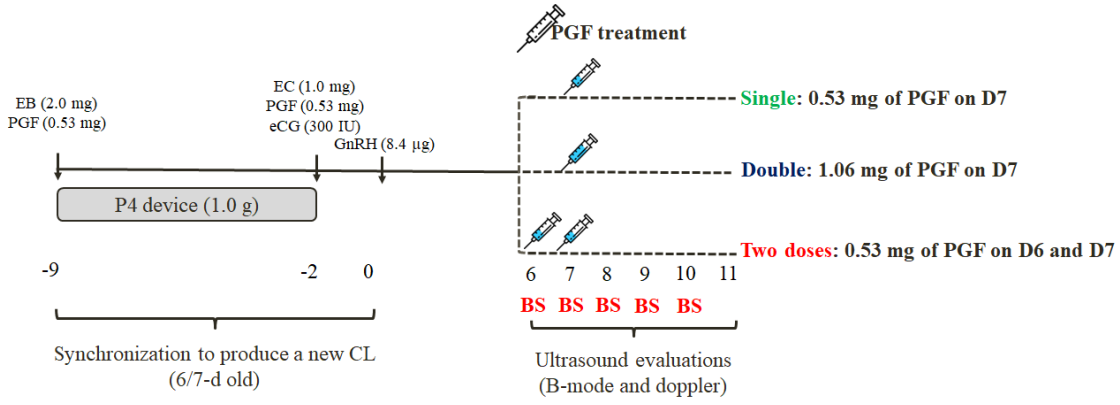


Figure 1. Schematic representation of the experimental design. On D-9, synchronization began to produce a new CL; all cows received a P4 device, concomitant with 2.0 mg of estradiol benzoate (EB) and 0.53 mg of PGF; 7 d later, the device was removed, and all cows received 1.0 mg of estradiol cypionate (EC), 0.53 mg of PGF and 300 IU of equine chorionic gonadotropin (eCG); on D0 all cows received 8.4 µg of buserelin acetate (GnRH). Six d later, an ultrasound evaluation was performed to check the presence of a new CL. Only cows with a new CL were used in the study and were randomly assigned to 1 of 3 treatments. Single: received a single dose (0.53 mg) of PGF on D7; Double: received a single dose (1.06 mg) of PGF on D7; and Two doses: received two doses (0.53 mg) of PGF on D6 and D7. Blood samples were collected from day 6 to day 10 to measure circulating P4, and ultrasound evaluations were performed from day 6 to day 11 to evaluate ovarian dynamics. EB = estradiol benzoate; PGF = cloprostenol sodium; P4 device = intravaginal progesterone device; EC = estradiol cypionate; eCG = equine chorionic gonadotropin; GnRH = buserelin acetate; BS = blood sampling.

3.2.4. Ultrasound evaluations

Transrectal ultrasound examinations of the ovaries in B-mode and color Doppler with a 7.5 MHz linear transducer (E2v SonoScape) were performed on days 6, 7, 8, 9, 10, and 11 after the synchronization to evaluate ovarian structures.

As mentioned before, on day 6, all cows were submitted to an ultrasound evaluation to check if ovulation occurred. The ovulation was defined by an active new CL with blood perfusion (>25%). Only cows with new CL were selected for the study.

Ultrasound evaluation was performed on days 6, 7, 8, 9, 10, and 11 in all cows selected for the study to evaluate the CL, blood perfusion, and volume. Additionally, the diameter of the largest dominant follicle (DF) was measured in each cow.

The volume of the CL was measured by the sphere formula $V = 4/3*\pi*R^3$ as described by Sartori et al. [21]. The DF diameter was estimated using two measures from each follicle.

The color Doppler mode measured blood perfusion (BP); in percentage, 0 is a total absence of blood perfusion, and 100 is extremely high blood perfusion. An active CL was considered when BP was > 25% [22].

3.2.5. Blood sampling and hormone assay

Blood sampling was done by jugular venipuncture on days 6, 7, 8, 9, and 10. All blood samples were collected into 9 mL heparinized evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and immediately placed on ice until the moment of

centrifugation. The tubes were centrifugated for 15 min at 1,800xg, and the plasma was stored in 2 mL microtubes at -20ºC until hormone assays for circulating P4. The assays were performed by Immulite 1000 Progesterone (LKPW1, Diagnostic Product Corporation, Caernarfon, UK), a competitive solid-phase immunoassay with enzyme-labeled chemiluminescent technology validated for bovine plasma (Silva et al., 2021 – unpublished), with a sensitivity of 0.2 ng/mL and inter-assay CV 2.3%.

Luteolysis was defined when P4 concentration was < 0.5 ng/mL or < 0.6 ng/mL when cows expressed estrus and/or ovulated.

3.2.6. Statistical analysis

All statistical analyses were performed by Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc, Cary, NC). Data were tested for normality of studentized residuals using the UNIVARIATE procedure, following the Shapiro-Wilk method; homogeneity of variances was evaluated by the Levene test, using Hovtest and Welsh methods. All outliers were removed, and data were transformed into logarithms when necessary.

Binomial variables (luteolysis, expression of estrus, and ovulation) were performed using the GLIMMIX procedure. Continuous variables (follicle size, CL size, blood perfusion, mean circulating P4 were evaluated using the GLIMMIX procedure. To assess the effect of treatment on P4 concentration, the model included the treatment of PGF on d6 and/or d7 as a fixed effect and parity and BCS as random effects.

Significant differences were considered when $P \leq 0.05$. All binomial data are presented as percentages (%, n/n) and continuous data as arithmetic means ± standard error of the mean.

3.3. Results

3.3.1. Ovarian dynamics

Follicular dynamics evaluation started on day 6. At that moment, the largest follicle of each cow was measured. Cows from the Single group tended d6 to have a greater follicle (10.8 ± 0.03 mm) than Two doses (9.8 ± 0.3 mm), and cows from the Double dose had a 10.4 ± 0.4 mm follicle (P = 0.09). However, there was no difference in the maximum diameter of

the largest follicle among groups of Single, Double, and Two doses, respectively (13.5 ± 0.3; 13.6 ± 0.6; 13.5 ± 0.4).

In all groups, the CL volume was reduced by day 11 in response to PGF treatments (Table 1). This decrease in volume occurred after PGF treatment. As expected, on day 7, only cows from the Two-dose group experienced a decrease in CL volume. On day 8, cows from the Double and Single groups also underwent a decrease in CL volume. Cows that received two doses of PGF had a higher reduction of total volume of CL, followed by Single and Double dose (Table 1).

Cows from Two doses group had experienced a higher decrease in CL volume from PGF administration by day 11 (the last day of ultrasound evaluation); cows from Double dose and Single had a similar CL reduction (91.0[a], 80.0[b], and 73.0%[b]) in volume, respectively.

Table 1. CL volume, in cm^3, of Nelore cows submittted to different strategies, using cloprostenol sodium, to lysis young CL. The measurements are from the first day of evaluation (D6), before treatment, until D10 in all cows, during ultrasound evaluation per day in all treatments.

	Treatments (n)			
Item	**Single (30)**	**Double (29)**	**Two doses (29)**	**P**
CL volume on D6, cm^3	2.7 ± 0.2	2.6 ± 0.2	2.4 ± 0.2	0.49
CL volume on D7, cm^3	3.7 ± 0.3[a]	3.3 ± 0.2[a]	1.5 ± 0.1[b]	< 0.01
CL volume on D8, cm^3	2.0 ± 0.2[a]	1.7 ± 0.1[a]	0.9 ± 0.1[b]	< 0.01
CL volume on D9, cm^3	1.3 ± 0.2[a]	1.0 ± 0.1[a]	0.5 ± 0.1[b]	< 0.01
CL volume on D10, cm^3	1.0 ± 0.2[a]	0.7 ± 0.1[a]	0.3 ± 0.1[b]	< 0.01

3.3.2. P4 concentration

In all groups, the plasma P4 concentration was reduced until day 10 in response to PGF treatments (Figure 2). Cows from the Two doses group had a higher decrease in P4

concentrations on day 7, continuing with lower P4 concentrations on day 8, compared to the other groups. On day 9, although the P4 concentrations remained lower in the Two doses group compared to the Single group, there was no difference in the Double group between both treatments. The same profile was found on day 10 (Table 2).

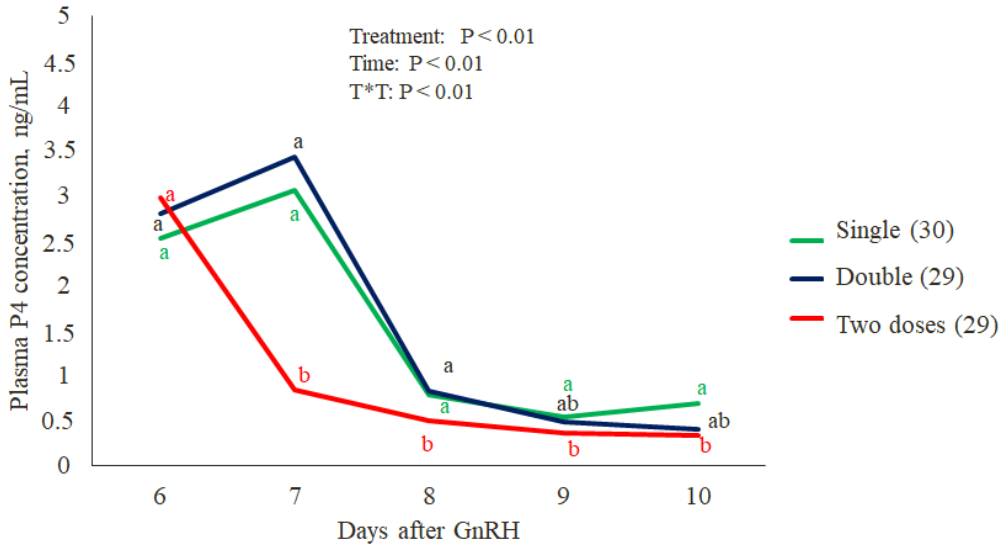


Figure 2. Plasma progesterone (P4) concentration in all cows, during 5 d, from day 6 after ovulation (before treatment) until day 10. Cows from the Two doses group received the first PGF dose on day 6 and day 7; Single and Double dose groups received PGF treatment only on day 7.

Table 2. Plasma progesterone concentration [P4] of Nelore cows submittted to different strategies, using cloprostenol sodium, to lysis young CL. The concentrations are from the first day of avaliation, 6 d, until day 10 in all cows.

	Treatments (n)			
Item	**Single (30)**	**Double (29)**	**Two doses (29)**	**P**
[P4] on D6, ng/mL	2.5 ± 0.3	2.8 ± 0.2	3.0 ± 0.2	0.40
[P4] on D7, ng/mL	3.1 ± 0.3^{a}	3.4 ± 0.3^{a}	0.8 ± 0.1^{b}	< 0.01
[P4] on D8, ng/mL	0.8 ± 0.1^{a}	0.8 ± 0.1^{a}	0.5 ± 0.1^{b}	< 0.01
[P4] on D9, ng/mL	0.5 ± 0.07^{a}	0.5 ± 0.05^{ab}	0.4 ± 0.02^{b}	0.02
[P4] on D10, ng/mL	0.7 ± 0.1^{a}	0.4 ± 0.1^{ab}	0.3 ± 0.0^{b}	0.01

Cows from the Two doses and Double group had a higher decrease in P4 concentration from the day of PGF administration by day 10 (the last day of blood sampling); cows from Single had a lower decrease (87.0^{a}, 86.0^{a}, and 72%^{b}) of reduction in P4 concentration, respectively.

3.3.3. Luteolysis

Luteolysis was initiated first in animals that received two doses (Figure 3). Partial luteolysis, or incomplete luteolysis, was determined when an initial reduction in CL volume and P4 concentration occurred, followed by an increase in P4 concentration (Figure 4), whether concurrent or not with an increase in CL volume.

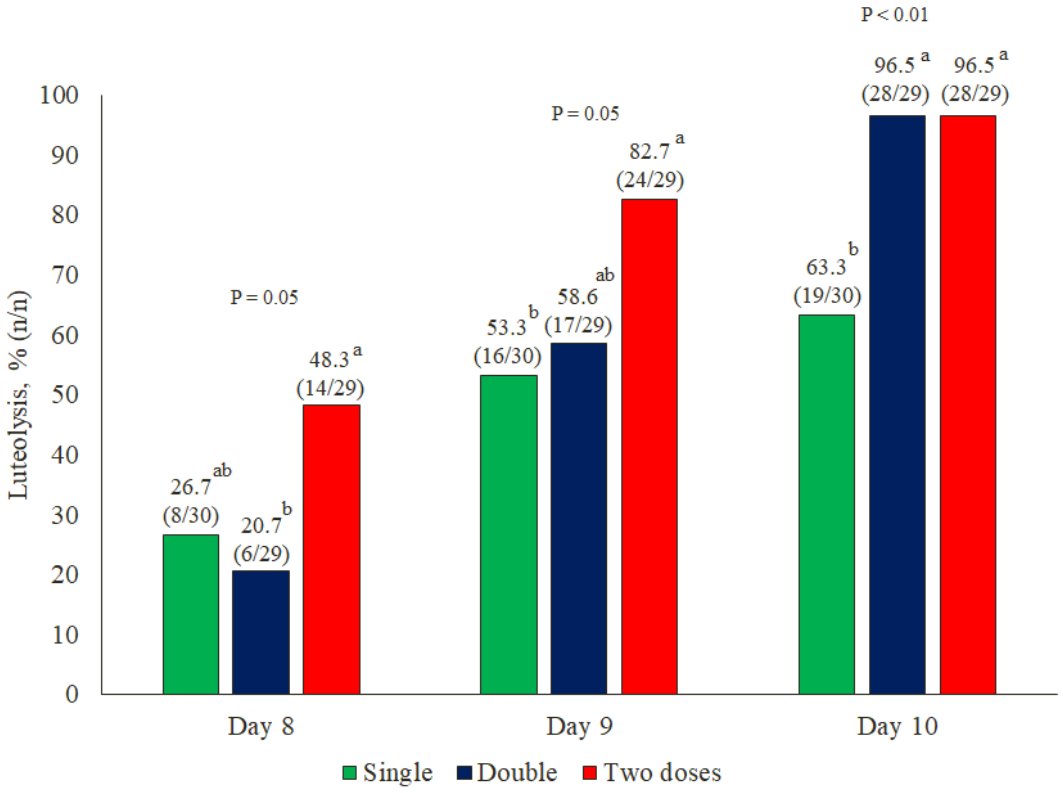
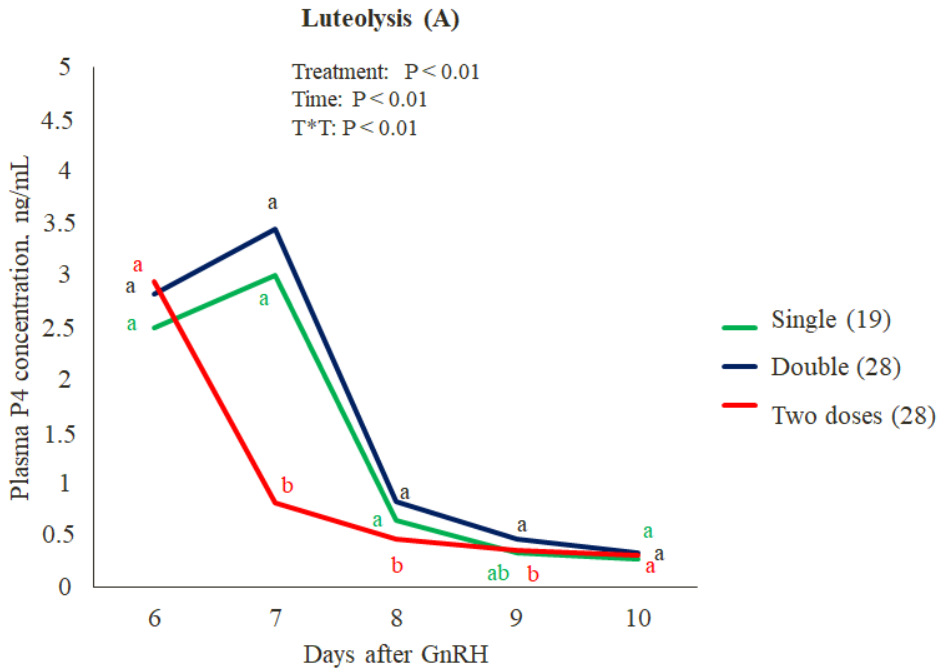


Figure 3. Accumulated luteolysis on days 8, 9, and 10 in all cows by treatment.



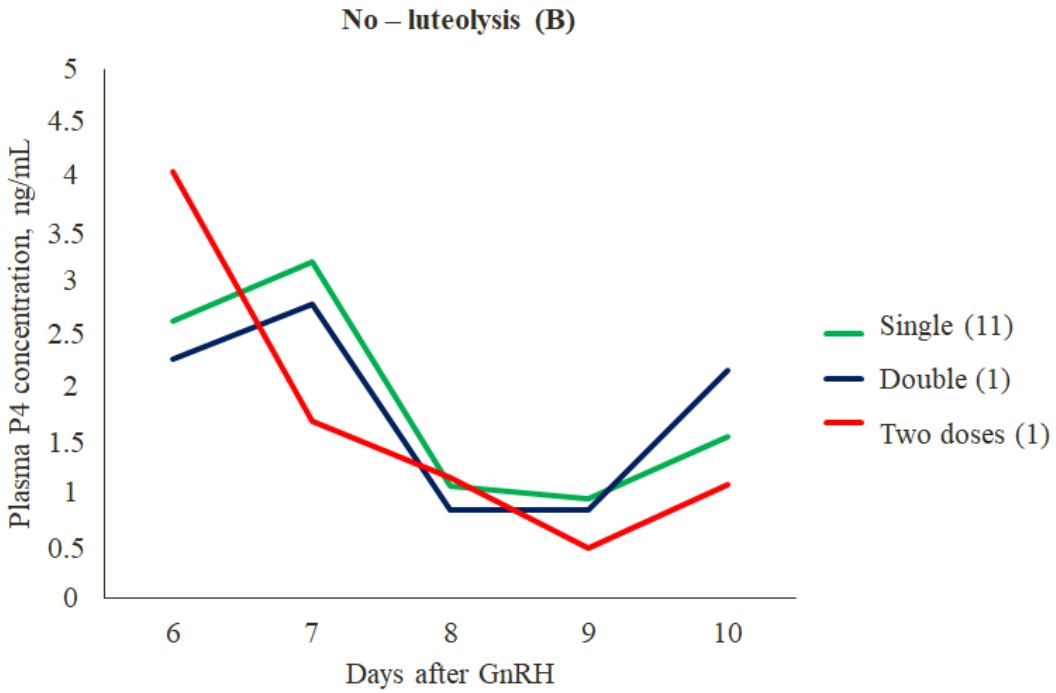


Figure 4. Plasma progesterone (P4) concentration, for 5 d, from day 6 (before treatment) until day 10 in cows with complete luteolysis (A – above) and cows without complete luteolysis (B - below) in all treatments.

3.3.4. Ovulation and expression of estrus

There was no difference in the expression of estrus between treatments (Figure 5). However, cows that received two doses of PGF ovulated more than the Single group, with no difference between both and the Double dose group (Figure 5).

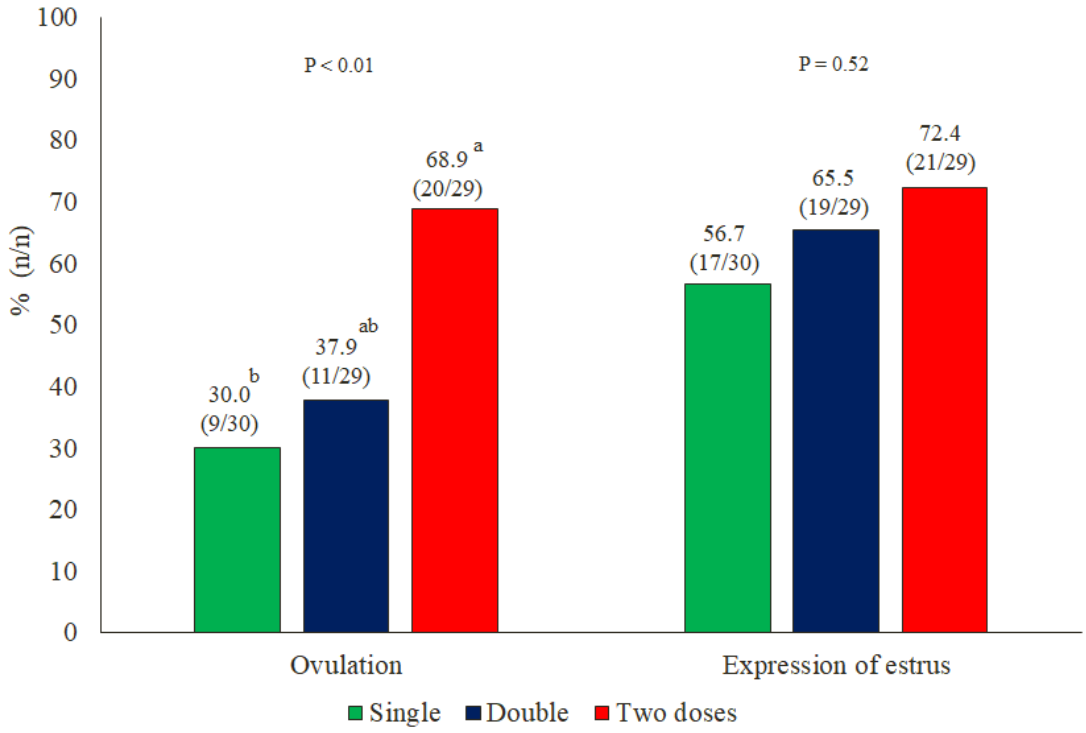


Figure 5. Accumulated ovulation and estrus expression until day 11 in all treatments.

There was no difference in circulating P4 concentrations on day 10 among treatments in cows that ovulated; however, considering just the cows without ovulation, the Single group had higher P4 concentration than the Two doses group and no difference in the Double group between both (Table 3).

Table 3. Plasma progesterone concentration [P4] on day 10 in cows with or without ovulation until day 11 in all treatments.

	Treatments (n)			
Item	**Single**	**Double**	**Two doses**	**P**
[P4] With ovulation, ng/mL	0.24 ± 0.02 (9)	0.34 ± 0.04 (11)	0.33 ± 0.03 (19) *	0.20
[P4] without ovulation, ng/mL	0.88 ± 0.2[a] (21)	0.43 ± 0.1[ab] (18)	0.25 ± 0.02[b] (9)	< 0.01

*One outlier was removed.

3.4. Discussion

CL volume and circulating P4 concentration reductions occurred first in the Two doses group because the animals received the first PGF on day 6. The other treatments started only on day 7, presenting the reductions from day 8. These results emphasize the action of PGF in CL function, inducing a dramatic decrease in CL in the first hours after treatment [23], followed by a decline in the next few days until the absence of P4.

The present study considered luteolysis when P4 was < 0.5 ng/mL or cows with P4 < 0.6 ng/mL, but with expression of estrus, without blood perfusion and CL volume regression > 80%. Studies support the idea that functional luteolysis occurs when P4 < 1.0 ng/mL; however, it was reported that cows with P4 concentrations around 1.0 ng/mL at the time of TAI presented a low probability of pregnancy[2]esides, in the present study, some cows had P4 < 1.0 ng/mL, but 1 d later, circulating P4 increased, characterizing partial luteolysis. Cows from the Single group had a lower incidence of luteolysis. In contrast, cows from the double- and Two-dose groups had similar and higher incidences of luteolysis, different from when dinoprost was used in a double dose [19]. The difference between treatments in the reported study appears only when luteolysis was considered when P4 < 1.0 ng/mL; this may have

happened due to the different molecules used in the studies. The present study used a long-action molecule, while the reported study used a molecule metabolized more quickly.

Cows from the Double and Two doses groups had a similar decrease in circulating P4 concentration, higher than Single. In contrast, in CL volume, the Double dose did not differ from the Single, and the Two dose group had a higher decrease in volume, although the final result for luteolysis was the same in the Two doses and the Double group. This probably occurred because functional luteolysis occurs before structural luteolysis [7]. Therefore, the CL of the Double and Single groups had one less day to decrease in volume after PGF treatment. So, even though luteolysis was greater in the Double group, in the Single on the last day of evaluation, there was no difference in the size of CL, emphasizing that functional luteolysis occurs first. Still, structural may take a few days for complete cell apoptosis until it transforms into a *corpus albicans* [6].

Considering days after PGF treatment, numerically, all treatments presented similar P4 concentrations and CL volume until 2 d after treatment, showing the luteolytic effect of PGF, regardless of the chosen dosage; however, there was detected a difference among groups 3 d after treatment, in which circulating P4 concentration increased only in Single group. This increase was caused by a failure of complete luteolysis in several cows that received just 0.53 mg of PGF on D7.

Several cows from the Single group underwent partial luteolysis, with a transitory decline in CL volume and circulating P4, but with a rebound later [24] can happen when the PGF treatment is not potent enough to lyse the CL completely, reducing P4 production and CL volume, followed by an increase in both or just in P4 production. The PGF induces some damage in the CL structure but not enough to induce complete lysis. This phenomenon can be pronounced using lower doses of PGF [25] or in younger CL [19,26], even using two doses [27].

There was no difference in the accumulated expression of estrus (until day 11) nor in maximum DF diameter. However, more cows from the Two-dose group ovulated compared to the Single. Still, no difference was detected in the Double group, although the numeric difference between these groups seems substantial. These results probably occurred because, in the Single and Double groups, P4 reduction occurs 1 d later; in other words, in the Two doses group, the proestrus started first, followed by estrus and ovulation, while in the other groups, the ultrasound evaluations were concluded before ovulation.

Circulating P4 concentration on D10 did not differ between groups when cows ovulated (P4 $\leq$ 0.4 ng/mL); however, considering just the cows without ovulation, P4

concentration on D10 was higher in Single than Two doses group, evidencing incomplete luteolysis observed, especially in cows submitted to this treatment, and consequently, impairing ovulation. In the Double dose group, circulating P4 concentration did not differ between treatments, presenting an intermediary concentration, possibly due to complete luteolysis failure in some cows.

3.5. Conclusion

Treatment with a conventional dose of cloprostenol sodium on day 7 of the estrous cycle was inefficient in inducing complete luteolysis in several Nelore cows. However, treating cows with a double dose on day 7 or 2 doses, 24 h apart, starting on day 6, resulted in a greater incidence of luteolysis within 72 h. This study confirmed the need to use an additional dose of cloprostenol sodium in young CL to guarantee complete luteolysis. Further studies are needed to verify if the double dose is as efficient as two doses in consecutive days.

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4. FINAL CONSIDERATIONS

The present study showed that GnRH-based TAI protocols still do not perform comparable to E2-based in *Bos indicus* cows, even when adjustments occur. Despite the many problems usually described in this genetic group when GnRH-based protocols were used, we achieved good ovulatory response after D0, with good follicle size at the time of AI, signaling adequate response to synchronization, nonetheless with lower fertility, possibly due to lower circulating E2 before AI. Moreover, the second study confirmed the importance of an additional dose of PGF in cows bearing younger CL, reaffirming that the use of a single dose (indicated on the leaflet) may not be enough to cause complete luteolysis, impairing the functioning of protocols that start with GnRH. However, further studies are still needed to prove whether the double dose on the same day is as effective as two doses 24 hours apart. Finally, this study provided exciting results involving TAI protocols without E2, showing the critical points for the proper functioning of this protocol and guiding us to the next steps in free-exogenous E2 protocol adjustments for *Bos indicus* cows.