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Original Research Article

Lecirelin-cloprostenol: A single-dose compounding formulation for ovulation induction in beef cows

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ABSTRACT

While GnRH induces the preovulatory LH surge, PGF2α triggers luteolysis and uterine contraction. Therefore, Lecirelin-Cloprostenol combination given at the end of the TAI protocol may improve fertility in cows. Eight studies evaluated a novel Lecirelin-Cloprostenol (GPG) fixed-dose combination for ovulation and fertility in Nelore cattle subjected to timed artificial insemination (TAI) protocols. Initial experiments (Exp. 1, n = 28) showed GPG and GnRH induced earlier ovulation than PGF. In Exp. 2 (n = 25) and Exp. 4 (n = 17), the GPG protocol reduced the dispersion of ovulation timing compared to GnRH alone (Bartlett's test, $P < 0.05$). In Exp. 3, the GPG group had lower intrafollicular estradiol ($P = 0.04$) and higher PTGS2 gene expression ($P = 0.05$) compared to the GnRH group. Fertility outcomes varied: GPG tended ($P = 0.1$) to increase pregnancy per artificial insemination (P/AI) in postpartum cows (Exp. 5, n = 326) and improved ovulation rates when estradiol cypionate (EC) was used (Exp. 6, n = 17). While a large field study (Exp. 7, n = 948) showed no overall P/AI difference when the GPG treatment was given following estradiol cypionate. Finally, GPG and GnRH had similar P/AI ($P = 0.3$) in non-estrus cows (Exp. 8, n = 1692) compared to GnRH. In conclusion, GPG upregulated PTGS2 expression and decreased intrafollicular E2 concentrations in the ovulatory follicle relative to GnRH, providing a mechanistic basis for the improved ovulatory synchrony observed in GPG-treated cows. While the GPG formulation consistently reduced the dispersion of ovulation timing across experiments, effects on P/AI were marginal in the present study.

1. Introduction

Although the past two decades have been characterized by remarkable progress in cattle production, with technological and operational innovations driving unprecedented productivity, the pursuit of further optimization remains an ongoing endeavor [1]. In this context, the development and widespread adoption of timed artificial insemination (TAI) protocols, exemplify how technological advances can significantly transform bovine production systems [2]. In Brazil, a prominent tropical country possessing one of the largest cattle herds worldwide, the proportion of breeding-age females subjected to artificial insemination (AI) markedly increased from 7% in 2014 to 23% in 2021 [3]. In South American beef operations, the most employed timed AI (TAI) protocols for synchronizing follicular wave emergence and ovulation typically rely on the administration of estradiol (E2), progesterone (P4), and prostaglandin F2 alpha (PGF2α). In such protocols, ovulation is generally induced with a low dose of estradiol esters concurrently with a P4 insert removal or 24 h later [4–6] or via gonadotropin-releasing hormone (GnRH) [7,8].

Among many factors that affect the fertility of TAI programs in beef

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cows, the timing of AI in relation to ovulation is one of the most critical determinants of pregnancy success [9,10]. In that regard, GnRH injection is deemed to induce the preovulatory peak of LH within 2h [11] and ovulation within 28-30 h [12]. Cedeño et al. [13] demonstrated that administering GnRH 8 h before TAI improved P/AI in cows that did not show estrus compared to cows that received GnRH at TAI. In postpartum *Bos indicus* beef cows, the injection of GnRH ~14 h before TAI increase pregnancy in comparison to nontreated cows [14] and compared to cows treated with GnRH at TAI [15]. Moreover, the administration of GnRH at the onset of behavioral estrus has been demonstrated to positively influence pregnancy rates in dairy cows [16,17]. This effect is primarily attributed to its ability to induce or synchronize ovulation while reducing variability in the interval to ovulation. Consequently, the strategic application of GnRH during the estrous phase enhances P4 concentrations during the subsequent luteal phase [18]. Further investigations have explored the administration of GnRH at the time of AI and reported beneficial effects on reproductive performance in dairy [19,20] and beef cows [21–23].

Although GnRH has been extensively used in TAI protocols, there is continued interest in refining hormonal strategies to maximize pregnancy rates and minimize the need for multiple interventions [24]. In this context, research efforts have increasingly focused on PGF analogs, particularly for its potential to enhance ovulation and fertilization processes [25]. In addition to its established role in luteolysis, PGF exhibits diverse physiological actions for reproduction. These include supporting the ovulatory process and enhancing uterine contractility and sperm transport [25–27]. Different PGF analogs have been described to be associated with periovulatory events in cattle: dinoprost [28,29], and sodium cloprostenol [30,31]; buffaloes: cloprostenol [32,33]; mares: dinoprost [34]; and ewes: D-cloprostenol [35]. The administration of PGF or its synthetic analogs at the time of artificial insemination (AI) has been investigated as a strategy to improve reproductive performance in cattle, providing inconsistent results. Previously, we observed that PGF per se can induce ovulation in prepubertal heifers [36] and can be used as an ovulatory stimulus in cows included in TAI programs [37,38]. López-Gatius et al. [8] observed that intravenous administration of cloprostenol (500 μg) have been associated to enhanced ovulation under heat stress, increased double ovulation, and improved pregnancy rates in specific groups like primiparous repeat breeder cows [8]. Similarly, intramuscular (IM) administration of 10 mg dinoprost tromethamine at AI has been shown to increase pregnancy rates in lactating cows [39]. Conversely, numerous studies have found no improvement in pregnancy rates when cows were treated with 10 mg [40] or 25 mg of dinoprost [28,41], or with 500 μg of cloprostenol [42]. This conflicting evidence highlights the complex and context-dependent nature of PGF2α/analog supplementation at AI.

While GnRH induces the preovulatory peak of LH, PGF is associated with luteolysis, ovulation and uterine contraction. Therefore, the combination of these drugs may positively affect the fertility of cows subjected to TAI programs. In this context, the development of combination therapies presents a promising approach to further increase reproductive outcomes. Based on these considerations, the objective of this study was to determine whether a single-dose lecirelin-cloprostenol formulation improve ovulation synchrony and fertility outcomes compared with lecirelin alone (GnRH) in beef cows subjected to TAI programs. We tested the hypothesis that the combined GnRH-PGF formulation (GPG) would be more effective than GnRH alone in synchronizing ovulation and increasing pregnancy rates in postpartum *Bos indicus* beef cows enrolled in TAI program.

2. Materials and methods

The Committee for Ethics in Animal Experimentation from the Brazilian Agricultural Research Corporation (Embrapa) approved all the procedures performed in the experiments described in this manuscript (Protocol 02/2022).

The Experiments 1, 2, 3, 4, and 6 were performed at Embrapa Rondônia's experimental farm (Brazilian Agricultural Research Corporation, Rondonia, Brazil; 08°48′12″S, 63°50′56″W). The experiments 5, 7, and 8 were performed in commercial farms and the details are specified in the respective experiment.

2.1. Single-dose lecirelin-cloprostenol preparation

Commercial vials of Lecirelin (25 μg/mL, TecRelin®, Agener União, São Paulo, Brazil) and sodium cloprostenol (250 μg/mL, PGF, Estron®, Agener União, Brazil) were used to prepare the single-dose Lecirelin-Cloprostenol formulation. For that purpose, 30 mL of Lecirelin were mixed with 60 mL of sodium cloprostenol, yielding a formulation sufficient for treating 30 cows (3 mL/cow; equivalent to 25 μg Lecirelin + 500 μg sodium cloprostenol).

2.2. Experiment 1. Comparative efficacy of GnRH, PGF, and lecirelin-cloprostenol combination on ovulation in beef cows

Twenty-eight Nelore cows (*B. indicus*), aged 3 to 8 years, weighing 450 to 600 kg, and with a body condition score (BCS) of 2.75 to 3.5 were used. The cows were kept in a grazing system (*Brachiaria brizantha* pasture) with *ad libitum* access to mineral salt and water.

The experimental design and treatment schedule are shown in Fig. 1. In a random day of the estrous cycle, the cows received an intravaginal progesterone-releasing device (IPD, 1g, Sincrogest®, Ourofino, Brazil), 2 mg i. m. of estradiol benzoate (EB, Gonadiol® Zoetis, São Paulo, Brazil) and 500 μg of sodium cloprostenol (PGF, Estron®, Agener União, Brazil) at the beginning of the protocol (Day 0). The IPDs were removed on Day 7, and all cows received 150 μg i. m. of d-cloprostenol (Croniben®, Biogenesis Bago, Buenos Aires, Argentina) and 300 IU i. m. of eCG (Novormon®, Zoetis, São Paulo, Brazil).

On Day 8, 34 h after IPD removal, the cows were randomly assigned to one of three treatments to receive: 1) 25 μg i. m. of Lecirelin (TecRelin®, Agener União, São Paulo, Brazil; GnRH, n = 10); 2) 500 μg of sodium cloprostenol (PGF group, n = 9); or 3) 25 μg of Lecirelin + 500 μg of PGF (GPG group, n = 9).

The development of the ovulatory follicle was monitored using transrectal ultrasonography (Mindray® M5 VET® with a 5 MHz linear probe) every 12 h from the time of IPD removal until ovulation. Ovulation was identified when a previously observed follicle measuring ≥ 8 mm in diameter disappeared between consecutive scanning sessions. When ovulation was detected, the time was estimated as the midpoint between the last two ultrasound examinations. Cows that failed to ovulate within 120 h after IPD removal were classified as non-responders to ovulation induction.

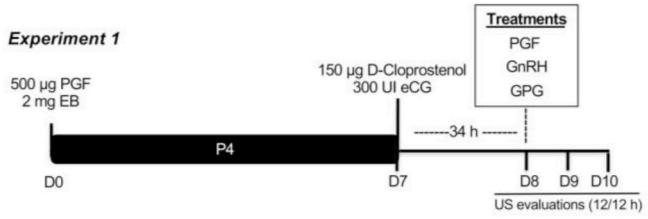

Fig. 1. Experimental design for Experiment 1. Cows received an intravaginal progesterone-releasing device (IPD, 1g), 2 mg i. m. of estradiol benzoate (EB) and 500 μg of sodium cloprostenol (PGF) at the beginning of the protocol (Day 0). The IPDs were removed on Day 7, and all cows received 150 μg i. m. of d-cloprostenol and 300 IU i. m. of equine chorionic gonadotrophin (eCG). On Day 8, 34 h after IPD removal, the cows were randomly assigned to one of three treatments to receive 500 μg of sodium cloprostenol (PGF group, n = 9), 25 μg i. m. of Lecirelin (GnRH group, n = 10), or 25 μg of Lecirelin + 500 μg of PGF (GPG group, n = 9). The development of the ovulatory follicle was monitored using transrectal ultrasonography every 12 h from the time of IPD removal until ovulation.

2.3. Experiment 2. Comparative efficacy of GnRH and lecirelin-cloprostenol combination for ovulation induction on the first follicular wave

For this study, 25 Nelore (*B. indicus*) cows (15 multiparous, and 10 nulliparous) were kept in *Brachiaria brizantha* pasture, with free access to water and mineral supplementation.

The experimental design and treatment schedule are shown in Fig. 2. All females included in the experiment were subjected to a hormonal protocol to pre-synchronize ovulation. Therefore, hormonal treatment started on a random day of the estrous cycle, and all cows received 2.0 mg of EB, and a IPD was inserted. Eight days later, the IPD was removed, and 500 μg of PGF and 1 mg of estradiol cypionate (EC, Cipiotec®, Agener União, São Paulo, Brazil) were administered. Thirty-four hours after IPD removal, 25 μg of lecirelin (TecRelin®, Agener União, Brazil) was administered to all cows. Ultrasonographic examinations were performed 12, 24, and 48 h after GnRH injection to detect ovulation.

Six days after ovulation detection, 500 μg of PGF was administered to all cows. Thirty-four hours after PGF injection, the cows were separated into 2 groups to receive: 25 μg of Lecirelin (TecRelin®, Agener União, Brazil, GnRH) or 25 μg of Lecirelin + 500 μg of PGF (GPG group).

Five days after ovulation was detected, the experiment was repeated in a cross-over design, resulting in 25 animals per group.

To detect ovulation, the ovulatory follicle was monitored by transrectal ultrasonography as in Experiment 1, except that ultrasonographic examinations were performed every 6 h from the treatment until ovulation or up to 3 days after the treatment's injection. Doppler ultrasound was used to evaluate endometrial and mesometrial tissue perfusion on a scale of 1 to 4 according to Ginther [43].

2.4. Experiment 3. GnRH and lecirelin-cloprostenol combination effects on granulosa cell gene expression and intrafollicular hormonal profiles

Nineteen cows that exhibited synchronous ovulation at the end of Experiment 2 were selected for this experiment. The experimental design and treatment schedule are shown in Fig. 3. Six days after ovulation detection, the cows received 500 μg of PGF (Estron®, Agener União, Brazil). Thirty-four hours after PGF injection, the cows were treated in the same way as in Experiment 2: GnRH (n = 9) and GPG (n = 10) Groups.

Cows were subjected to perineal cleaning and epidural anesthesia with 80 mg lidocaine (Lidofarm®, Biofarm, Jaboticabal, SP, Brazil) to obtain follicular samples. Follicular aspiration was performed 20 h after treatments with the aid of a transvaginal ultrasound (SIUI CTS-900, linear probe with 5 MHZ, Guangdong, China) equipped with a 5 MHz microconvex probe, adapted to a conventional ovum pickup (OPU)

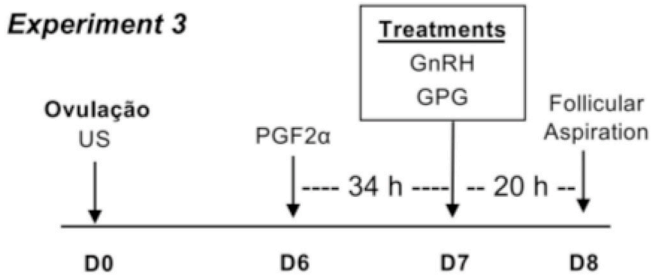

Fig. 3. Experimental design for Experiment 3. Six days after ovulation detection, all cows received 500 μg of PGF. Thirty-four hours later, the cows were separated in two groups to receive GnRH (n = 9) or GPG (n = 10). Follicular aspiration was performed 20 h after treatments. Follicular fluid samples from the preovulatory follicles was used to analyze concentrations of P4 and E2, prostaglandin metabolite (PGFM), and the relative mRNA abundance of *CYP19A1*, *HSD3B1*, *PGR*, *PTGS2,* and *STAR* genes.

system with 16 G catheter attached to a silicon hose and a 3 mL syringe.

Follicular fluid samples from the preovulatory follicles were centrifuged at 1500 x G for 7 min to separate the follicular cells and stored in microtubes at −80 °C. Concentrations of P4 and E2 in the follicular fluid were assessed by chemiluminescence (ADVIA Centaur Systems Progesterone Kit; Siemens; Ref. 01586287; sensitivity of 0.21 ng/mL and Elecsys Estradiol III; Roche; Ref. 06656021119; sensitivity of 5 pg/mL) in a commercial laboratory with intra-assay value lower than 10%. The measurements of the prostaglandin metabolite (PGFM) in the follicular fluid (FF) were performed using EIA commercial kits 13,14-dihydro-15-keto Prostaglandin F2a (Item No. 516671) from Cayman Chemical (Ann Arbor, Michigan, USA). For all the analysis, intra- and inter-assay coefficients of variation were below 10%.

The RNA extraction, reverse transcription, and quantitative PCR (qPCR) was performed as previously described [44]. The qPCR were performed in a CFX384 thermocycler (Bio-Rad; Irvine, CA, USA) using iQ SYBR Green Supermix (Bio-Rad; Irvine, CA, USA) and bovine-specific primers (Table 1; final concentration of 0.5 mM) Samples were analyzed in duplicate, and the results were expressed relative to the average Cq values of the reference genes βactin and GAPDH.

2.5. Experiment 4. Comparative efficacy of GnRH and lecirelin-cloprostenol combination as ovulation inducers in Nelore cows subjected to TAI

Nelore cows (*B. indicus*, n = 17), aged 3 to 8 years, weighing 450 to 600 kg, and with a body condition score (BCS) of 2.75 to 3.5 were used. The cows were kept in an outdoor grazing system (*Brachiaria brizantha* pasture) with *ad libitum* access to mineral salt and water.

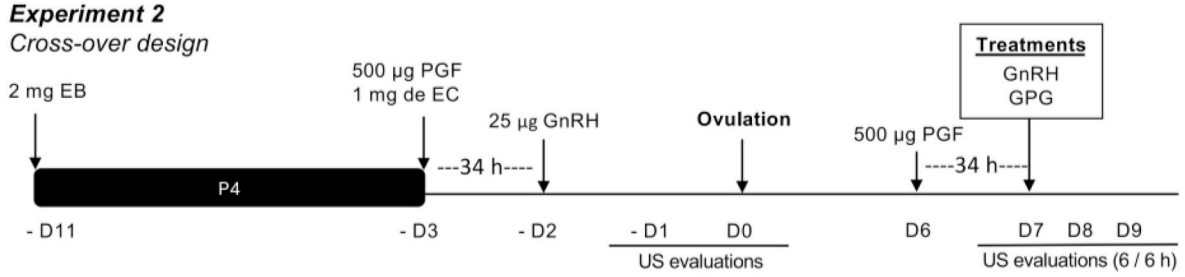

Fig. 2. Experimental design for Experiment 2. Cows included in the experiment were subjected to a hormonal protocol to pre-synchronize ovulation. All females received 2.0 mg of EB, and a IPD. Eight days later, the IPD was removed, and 500 μg of PGF and 1 mg of estradiol cypionate (EC) were administered. Thirty-four hours after IPD removal, 25 μg of lecirelin (GnRH) was administered to all cows. Ultrasonographic examinations were performed 12, 24, and 48 h after GnRH injection to detect ovulation. Six days after ovulation detection, 500 μg of PGF was administered to all cows. Thirty-four hours later the cows were separated into 2 groups to receive 25 μg of Lecirelin (GnRH group) or 25 μg of Lecirelin + 500 μg of PGF (GPG group). The experiment was repeated in a cross-over design. To detect ovulation, the ovulatory follicle was monitored by transrectal ultrasonography every 6 h from the treatment until ovulation or up to 3 days after the treatment's injection.

Table 1
Primers sequency of the genes studied in the Experiment 3.

Gene		Primer Sequence	REFERENCE
CYP19A1	Forward	GTGTCCGAAGTTGTGCCTATT	Moraes et al. (2021)
	Reverse	GGAACCTGCAGTGGGAAATGA	
HSD3B1	Forward	TCTCTGCAGTACTGGCTTGC	
	Reverse	GTCACTAGGTGGCGGTTGAA	
PGR	Forward	GCCGCAGGTCTACCAGCCCTA	
	Reverse	GTTATGCTGTCCTTCCATTGCCCTT	
PTGS2	Forward	CATCCAGAATGGGGCGATGA	
	Reverse	AGCAGCAATACGGTTCTGGT	
STAR	Forward	CCCAGCAGAAGGGTGTCATC	
	Reverse	TGCGAGAGGACCTGGTTGAT	
GAPDH	Forward	ACACTGAGGACCAGGTTG	
	Reverse	TGGTCGTTGAGGGCAATG	
βactin	Forward	AGGCATCCTGACCCTCAAGTA	
	Reverse	GCTCGTTGTAGAAGGTGTGGT	

The experimental design and treatment schedule are shown in Fig. 4. The cows received an IPD (1g, Sincrogest®, Ourofino, Brazil), 2 mg i. m. of estradiol benzoate (EB; Gonadiol® Zoetis, São Paulo, Brazil) at the beginning of the protocol (Day 0). The IPDs were removed on Day 8, and all cows received 150 μg i. m. of d-cloprostenol (PGF analog, Croniben®, Biogenesis Bago, Buenos Aires, Argentina) and 300 IU i. m. of eCG (Novormon®, Zoetis, São Paulo, Brazil). On Day 9, the cows were randomly assigned to two treatments and received: 25 μg i. m. of lecirelin (TecRelin®; GnRH, n = 17) or 25 μg of Lecirelin + 500 μg of PGF (GPG group, n = 17), 34 h after IPD removal. On Day 8, all cows were painted with a marker stick in the sacrocaudal region to identify animals that showed estrus. On Day 10, estrus was evaluated, and cows that had significant paint loss (>75% loss) in the region were considered in estrus.

This study was performed in a cross-over design, in such a way that all cows received all treatments.

To detect ovulation, the ovulatory follicle was monitored by transrectal ultrasonography as in Experiment 1.

2.6. Experiment 5. Comparative impact of GnRH and lecirelin-cloprostenol combination on fertility in beef cows subjected to TAI protocols

This study was conducted on two commercial farms in the Amazon biome. For this experiment, 326 postpartum Nelore (*B. indicus*) cows aged 3 to 9 years, weighing 450 to 650 kg, and with a body condition score (BCS) of 2.75 to 3.5 were used. The cows were kept in an outdoor grazing system (*Brachiaria brizantha* pasture) with *ad libitum* access to mineral salt and water. The experimental design and treatment schedule are shown in Fig. 4. All cows were treated as in the Experiment 4, except that on Day 8, a subgroup of cows (n = 178) were painted with a chalk marker on the sacrocaudal region to identify animals that displayed estrus. Cows were randomly separated into 2 groups to receive: 25 μg i. m. of lecirelin (TecRelin®; GnRH, n = 167) or 25 μg of Lecirelin + 500 μg of PGF (GPG group, n = 159). At TAI, estrus was evaluated in the subgroup of cows and deemed to have occurred in cows without a tail-head chalk mark (>75% paint loss). Ultrasonographic examinations were performed on the day of TAI to measure the preovulatory follicle (POF) in a subgroup of 77 animals, and 30 days after TAI to assess pregnancy status in all cows.

2.7. Experiment 6. Comparison between GnRH and lecirelin-cloprostenol combination following estradiol cypionate treatment in Nelore cows subjected to TAI protocol

This study was conducted at Embrapa Rondônia's experimental farm in Porto Velho, RO, Brazil. Seventeen Nelore (*B. indicus*) cows, aged 3 to 8 years, weighing 460 to 620 kg, and with a body condition score (BCS) of 2.75 to 3.5 were used. The experimental design and treatment schedule are shown in Fig. 5. The cows in this study received the same treatments as in Experiment 4, with the exception that, on the day of IPD removal, all cows received 1 mg of estradiol cypionate (Cipiotec®, Agener União, São Paulo, Brazil). This study was not conducted using a cross-over design. Therefore, the experimental groups were: 25 μg i. m. of lecirelin (TecRelin®; GnRH, n = 9) or 25 μg of Lecirelin + 500 μg of PGF (GPG group, n = 8). The estrus detection was performed as described in the Experiment 5.

To detect ovulation, the ovulatory follicle was monitored by transrectal ultrasonography in the same way as Experiment 1. Color Doppler ultrasonography (Mindray® M5 VET equipped with a 5 MHz linear probe) was used to evaluate the blood flow of the preovulatory follicle every 12 h from the moment of treatment until ovulation, as described by Castro et al. [45]. The evaluation of the CL was performed by B-mode and Doppler ultrasonography (Mindray® M5 VET® equipped with 5 MHZ linear probe) was performed eight days after IPD removal according to Siqueira et al. [46] to evaluate the area and vascularized area of the CL.

2.8. Experiment 7. Fertility outcomes of GnRH and lecirelin-cloprostenol combination following estradiol cypionate treatment in Nelore cows subjected to EC-GnRH34 TAI protocol

The study was performed on three farms located in Ariquemes, RO, Brazil, and one farm located in Rio Verde, MS, Brazil. For this experiment, 948 Nelore (*B. indicus*) cows with 30 – 100 days postpartum, and BCS ranging from 2.5 to 4.0 were used. All cows were treated as in Experiment 6. The experimental design and treatment schedule are

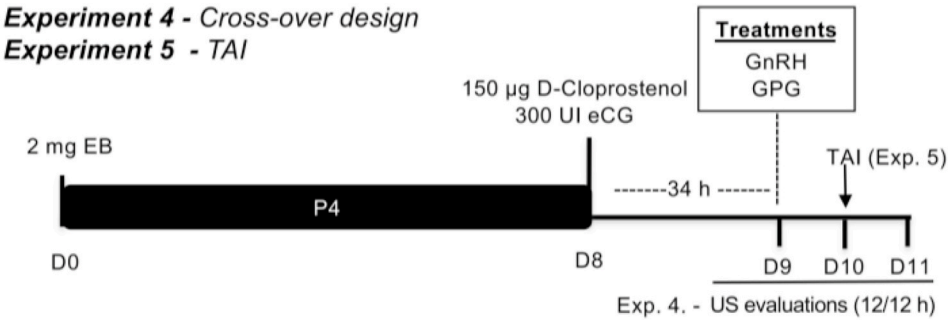

Fig. 4. Experimental design for Experiment 4 and 5. The cows received an IPD (1g), and 2 mg i. m. of estradiol benzoate (EB) at the beginning of the protocol (D0). The IPDs were removed on Day 8, and all cows received 150 μg i. m. of d-cloprostenol (PGF analog) and 300 IU i. m. of equine chorionic gonadotrophin (eCG). On Day 9, the cows were randomly assigned to two treatments to receive 25 μg i. m. of lecirelin (GnRH group) or 25 μg of Lecirelin + 500 μg of PGF (GPG group), 34 h after IPD removal. This study was performed in a cross-over design. The ovulatory follicle was monitored every 12 h from D9 to ovulation by transrectal ultrasonography in Experiment 4 only. Cows in Experiment 5 were treated as in Experiment 4, with exception that timed AI (TAI) was performed on D10.

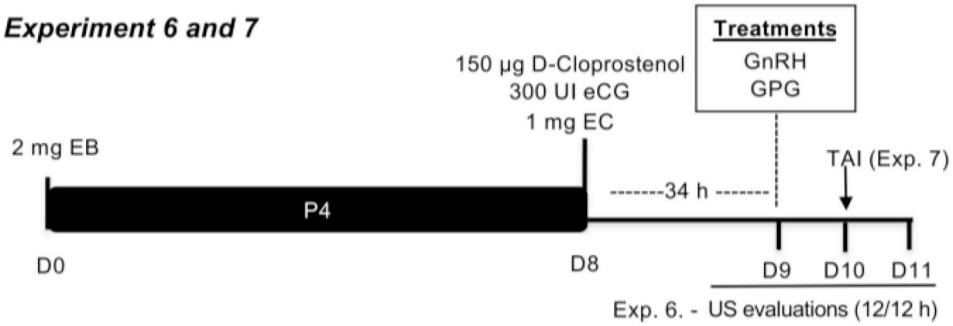

Fig. 5. Experimental design for Experiment 6 and 7. The cows received an IPD (1g), and 2 mg i. m. of estradiol benzoate (EB) at the beginning of the protocol (D0). The IPDs were removed on Day 8, and all cows received 150 µg i. m. of d-cloprostenol (PGF analog), 1 mg of estradiol cypionate (EC), and 300 IU i. m. of equine chorionic gonadotrophin (eCG). On Day 9, the cows were randomly assigned to two treatments to receive 25 µg i. m. of lecirelin (GnRH group) or 25 µg of Lecirelin + 500 µg of PGF (GPG group), 34 h after IPD removal. The ovulatory follicle was monitored every 12 h from D9 to ovulation by transrectal ultrasonography in Experiment 6 only. Cows in Experiment 7 were treated as in Experiment 6, with exception that timed AI (TAI) was performed on D10.

shown in Fig. 5. The cows were divided into two groups to receive the treatments: 25 µg i. m. of lecirelin (TecRelin®; GnRH, n = 462) or 25 µg of Lecirelin + 500 µg of PGF (GPG group, n = 486). All cows were inseminated 48 h after IPD removal. Semen from 9 bulls was used for TAI procedures, and they were equally distributed among the treatments. Ultrasonographic examinations were performed 30 days after IATF to evaluate pregnancy.

2.9. Experiment 8. Impact of GnRH and lecirelin-cloprostenol combination on fertility in TAI-treated cows not exhibiting estrus

This study was conducted on two commercial farms in the Amazon biome. For this experiment, 1692 postpartum Nelore cows aged 3 to 12 years, weighing 435 to 670 kg, and with a body condition score (BCS) of 2.5 to 3.75 were used. The cows were kept in an outdoor grazing system (*Brachiaria brizantha* pasture) with *ad libitum* access to mineral salt and water.

Initially, all cows were treated as in Experiment 6 with the exception that the cows did not receive any treatment on Day 9. The experimental design and treatment schedule are shown in Fig. 6. The estrus detection was performed as described in the Experiment 5. On Day 10, at the time of TAI, cows that showed estrus were considered as the Estrus group (Estrus, n = 1096) and cows that did not express estrus were randomly separated to receive 25 µg i. m. of Lecirelin (TecRelin®, Agener União, São Paulo, Brazil; GnRH group, n = 293) or 25 µg of Lecirelin + 500 µg of SC (GPG, n = 303).

Ultrasonographic examinations were performed 30 days after TAI to assess pregnancy status.

2.10. Statistical analysis

All statistical analyses were performed using the SAS 9.0 software (2006; SAS Institute Inc., Cary, NC, USA). In Experiments 1, 2, 3, 4, and 6, single-point outcome variables (e.g., time of ovulation, follicle growth, the diameter of dominant follicle, LTA, CLBF, and adjusted CLBF) were analyzed by ANOVA (PROC GLIMMIX; SAS Inst. Inc., Cary, NC). After assessing normality via the Shapiro-Wilk test, the homogeneity of variances for the time of ovulation was evaluated using Bartlett's test. When the assumption of normality was not met, Levene's test was used instead. Follicular and uterine vascularization from the P4 insert removal to the ovulation was compared between treatments using the MIXED procedure for repeated measures to evaluate the main effects of treatment and time and their interaction. Ovulation occurrence was analyzed using the Chi-square test.

The pregnancy per AI (binary outcome: pregnant/not pregnant) in experiments 5, 7 and 8, was analyzed using a Generalized Linear Mixed Model (GLMM) with a binomial distribution and a logit link function, implemented via the GLIMMIX procedure. Farm was included as a random effect. The initial full model considered treatment, bull, inseminator, BCS, parity, and days postpartum as fixed effects. A backward elimination procedure was employed for fixed effects, with variables retained in the model if they were statistically significant at $P < 0.05$. The variables bull, inseminator, BCS, parity, and days postpartum had no significant effect and were therefore excluded from the model. Therefore, only treatment was maintained in the final model. Statistical difference was considered if the P-value was ≤ 0.05, and tendency was considered if the P-value was between 0.05 and 0.1.

3. Results

3.1. Experiment 1

The ovarian response of cows according to the treatment are described in Table 2 and presented in Fig. 7A.

Cows treated with GnRH and GPG ovulated earlier and more synchronously than cows treated with PGF ($P < 0.05$). Cows treated with

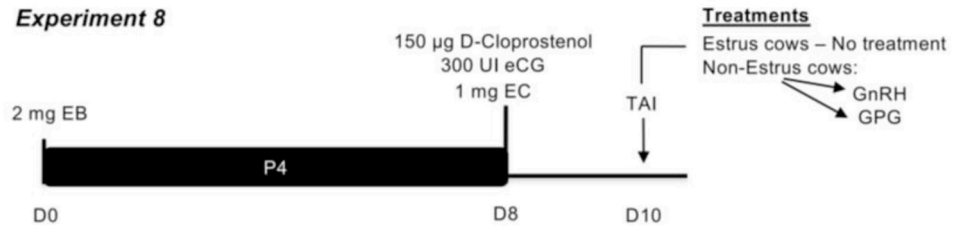

Fig. 6. Experimental design for Experiment 8. The cows received an IPD (1g), and 2 mg i. m. of estradiol benzoate (EB) at the beginning of the protocol (D0). The IPDs were removed on Day 8, and all cows received 150 µg i. m. of d-cloprostenol (PGF analog), 1 mg of estradiol cypionate (EC), and 300 IU i. m. of equine chorionic gonadotrophin (eCG). On Day 10, at TAI, cows that showed estrus were considered as the Estrus group (Estrus, n = 1096) and cows that did not express estrus were randomly separated to receive 25 µg i. m. of Lecirelin (GnRH group, n = 293) or 25 µg of Lecirelin + 500 µg of SC (GPG, n = 303).

Table 2
Ovarian response in Nelore cows treated with GnRH, GPG or PGF for ovulation induction in Nelore cows subjected to TAI protocol (Experiment 1).

	Groups			P-Value
	GnRH	GPG	$PGF_2\alpha$	
Diameter of POF[a], mm	12.8 ± 0.5^{A}	11.9 ± 0.5^{A}	14.9 ± 0.5^{B}	0.001
Proportion of cows ovulating, %	80 (8/10)	77.8 (7/9)	66.7 (6/9)	0.77
Time of ovulation, h	66.0 ± 0.0^{A}	66.0 ± 0.0^{A}	92.0 ± 3.7^{B}	0.0001

[AB]Values followed by different letters were different ($P \leq 0.05$).
[a] POF, preovulatory follicle.

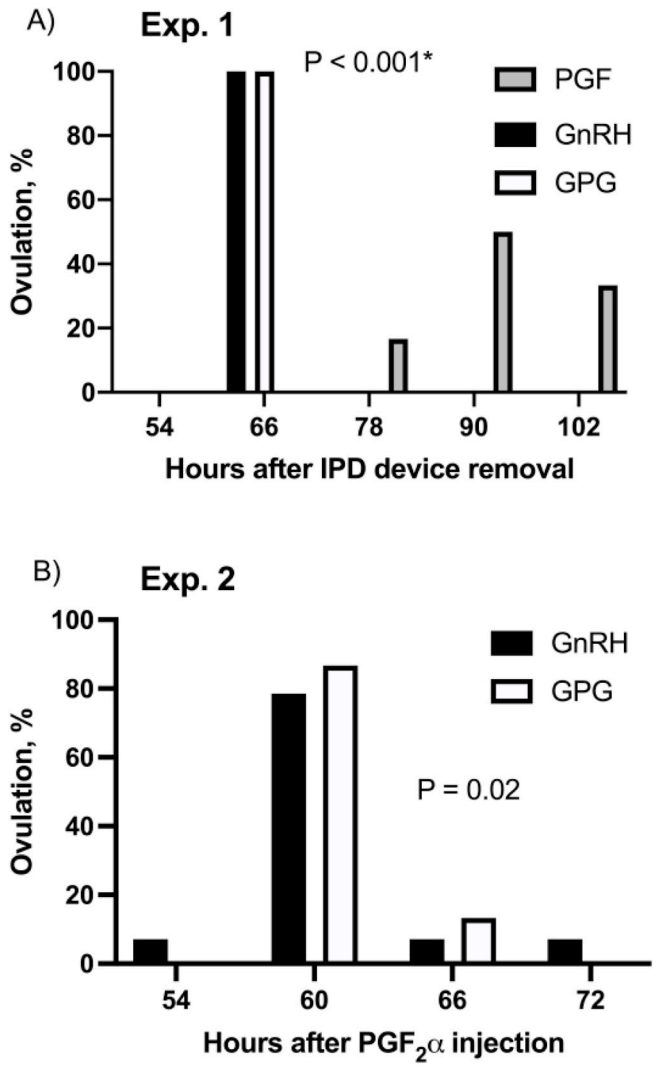

Fig. 7. A) Distribution and percentage of ovulation in beef cows treated with GnRH, PGF or GPG, 34 h after IPD removal, in Experiment 1. B) Distribution and percentage of ovulation in beef cows treated with GnRH or GPG, 34 h after luteolysis induction, Experiment 2.
*GnRH, and GPG groups were different from PGF group.

PGF had larger ($P < 0.01$) POF than cows treated with GnRH and GPG. No differences ($P > 0.05$) on the ovarian response were detected between cows treated with GnRH and GPG.

3.2. Experiment 2

Two cows did not ovulate in the pre-synchronization treatment and were, therefore, excluded from the study. Additionally, in replicate 1, 4 cows from the GPG group and 2 cows from the GnRH group did not ovulate and were removed from replicate 2. Therefore, the total number of cows considered for each group was 19 in the GnRH and 21 in the GPG groups, according to Table 3.

The distribution of ovulation in cows treated with GnRH and GPG is shown in Fig. 7B. Cows treated with GPG reduced ($P = 0.02$) the dispersion of ovulation timing compared to GnRH alone (Fig. 7B). No differences ($P > 0.05$) on the proportion of cows ovulating, diameter of the POF, and time of ovulation were detected between groups. The uterine vascularization after treatment was also not different ($P > 0.05$) between the groups (Fig. 8).

3.3. Experiment 3

Cows treated with GPG had an increase ($P = 0.05$) in the relative mRNA abundance of the *PTGS2* gene. The relative mRNA abundance of *CYP19A1*, *HSD3B1*, *PGR*, and *STAR* did not differ ($P > 0.05$) between the groups (Fig. 9).

The intrafollicular concentrations of E2, P4, and PGFM are shown in Fig. 10. Cows treated with GnRH had higher ($P = 0.04$) intrafollicular E2 concentration than cows treated with GPG. No differences ($P > 0.05$) were detected in the intrafollicular concentration of P4 and PGFM between groups.

3.4. Experiment 4

In the first replicate of the experiment, three cows did not have follicles $\geq$ 8 mm at the time of treatments and, therefore, were excluded from the rest of the experiment. Therefore, both experimental groups had 14 animals.

Cows treated with GPG reduced ($P = 0.01$) the dispersion of ovulation timing compared to GnRH alone (Fig. 11A). The ovarian responses of cows according to the treatment are shown in Table 4.

3.5. Experiment 5

The data on the proportion of females in estrus and pregnant according to the treatment are presented in Table 5. No differences were observed on the diameter of POF ($P = 0.79$) and proportion of cows in estrus ($P = 0.45$) between cows treated with GnRH and GPG. However, cows treated with GPG tended ($P = 0.1$) to have greater P/AI than cows treated with GnRH.

3.6. Experiment 6

The distribution of ovulation in cows treated with EC-GnRH and EC-GPG is shown in Fig. 11B. The ovarian responses of cows according to the treatment are shown in Table 6. The treatment with GPG increased ($P < 0.01$) the proportion of cows ovulating in comparison with cows treated with GnRH.

The vascularization of the POF in cows treated with GnRH and GPG is shown in Fig. 12. There was no effect ($P > 0.05$) of treatment and day*treatment interaction on follicular vascularization. However, there was a day effect ($P < 0.001$). No effect ($P > 0.05$) of treatments was observed on the luteal tissue area, CL blood flow, and percentage of

Table 3
Ovarian response in cows treated with GnRH or GPG for ovulation induction on the first follicular wave of the estrus cycle (Experiment 2).

	Groups		P-Value
	GnRH	GPG	
Diameter of POF[a], mm	11.6 ± 0.5	12.2 ± 0.4	0.42
Proportion of cows ovulating, %	73.7 (14/19)	71.4 (15/21)	0.87
Time of ovulation, h	60.9 ± 0.8	60.8 ± 0.8	0.96

[a] POF, preovulatory follicle.

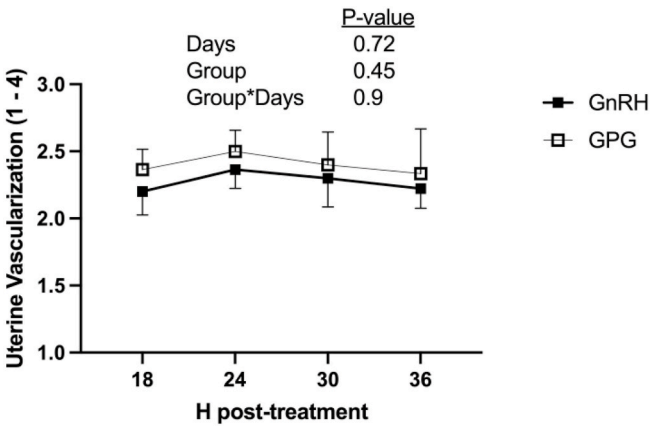

Fig. 8. Uterine vascularization (1 – 4 scale) of Nelore cows treated with GnRH or GPG, 34 h after luteolysis induction, in Experiment 2.

vascularized area of the CL (adjusted CLBF; Fig. 13).

3.7. Experiment 7

The proportion of cows in estrus and pregnant cows according to the treatments are presented in Table 7. No differences ($P > 0.05$) were detected in estrus expression and P/AI between the groups.

3.8. Experiment 8

Cows that displayed estrus had higher ($P < 0.001$) P/AI (59.8%) than cows that did not show estrus treated with GnRH (43.0%) and GPG (46.5%; Fig. 14). Among cows that did not show estrus, no difference in the P/AI was detected between the GnRH and GPG groups ($P = 0.3$).

4. Discussion

Our study investigated the effects of GnRH versus a single-dose GnRH-cloprostenol combination (GPG) on ovulation synchrony and subsequent fertility in *Bos indicus* beef cows subjected to timed artificial insemination (TAI) programs. Cows treated with the GPG single-dose formulation reduced the dispersion of ovulation timing compared to GnRH alone. Moreover, our findings revealed distinct physiological responses within the follicular environment. Notably, cows treated with the GPG combination exhibited a greater mRNA abundance of the *PTGS2* gene and lower intrafollicular estradiol concentrations. Furthermore, the GPG combination tended to increase P/AI, particularly in cows that were not treated with estradiol cypionate (EC) within the TAI protocol. To our knowledge, this represents the first investigation of a combined GnRH and sodium cloprostenol treatment as an ovulation inducer at the end of the TAI protocol.

Eight studies were performed to compare the efficacy of GnRH or a GPG combination for ovulation induction in TAI programs. In five of these studies (1, 4, 5, 6, and 7), the GnRH34 protocol (with or without EC) was used, as previous research indicated its superior fertility outcomes in *Bos indicus* cattle [14,15]. Consequently, the present experiments aimed to compare GnRH and GPG formulations within this previously identified highly fertile TAI program for beef cows. Of the experiments performed, three (Experiments 5, 7, and 8) specifically aimed to compare P/AI between GnRH and GPG formulations. In Experiment 7, in which postpartum *Bos indicus* beef cows received 1 mg of estradiol cypionate (EC) at IPD removal, followed by either GnRH or GPG treatment 34 h later, no significant difference in P/AI was detected between the treatment groups. Previous research has demonstrated that cows receiving EC in combination with GnRH treatment exhibit greater P/AI compared to those treated with EC alone at IPD removal [13,14]. Furthermore, GnRH administration either 8 h before TAI [13] or at the time of TAI [21,22] has been shown to improve P/AI in EC-pretreated cows that did not exhibit estrus. Additionally, Silva et al. [15] reported that, despite similar ovulation times, postpartum *Bos indicus* cows

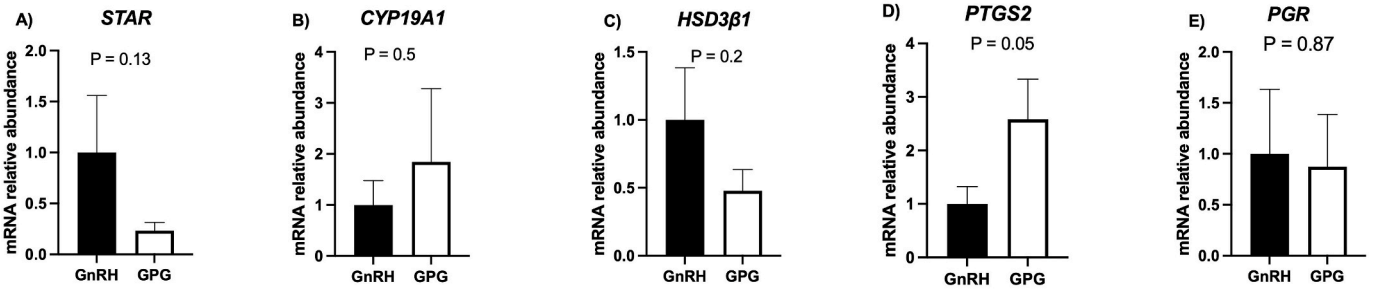

Fig. 9. Relative abundance of mRNA of genes associated with steroidogenesis and ovulation in cows treated with GnRH or GPG, 34 h after luteolysis induction, in Experiment 3. Follicular aspiration was performed approximately 20 h after the injection of ovulation inducers.

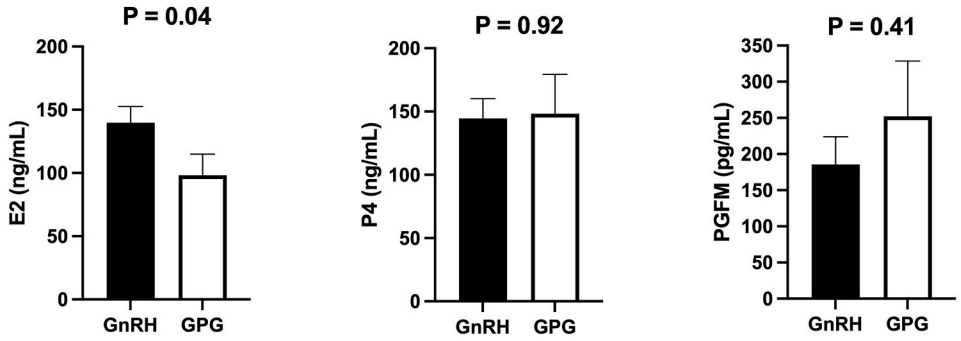

Fig. 10. Intrafollicular concentrations of E2, P4, and PGFM in preovulatory follicles of cows treated with GnRH or GPG, 34 h after luteolysis induction, in Experiment 3. Follicular aspiration was performed approximately 20 h after the injection of ovulation inducers.

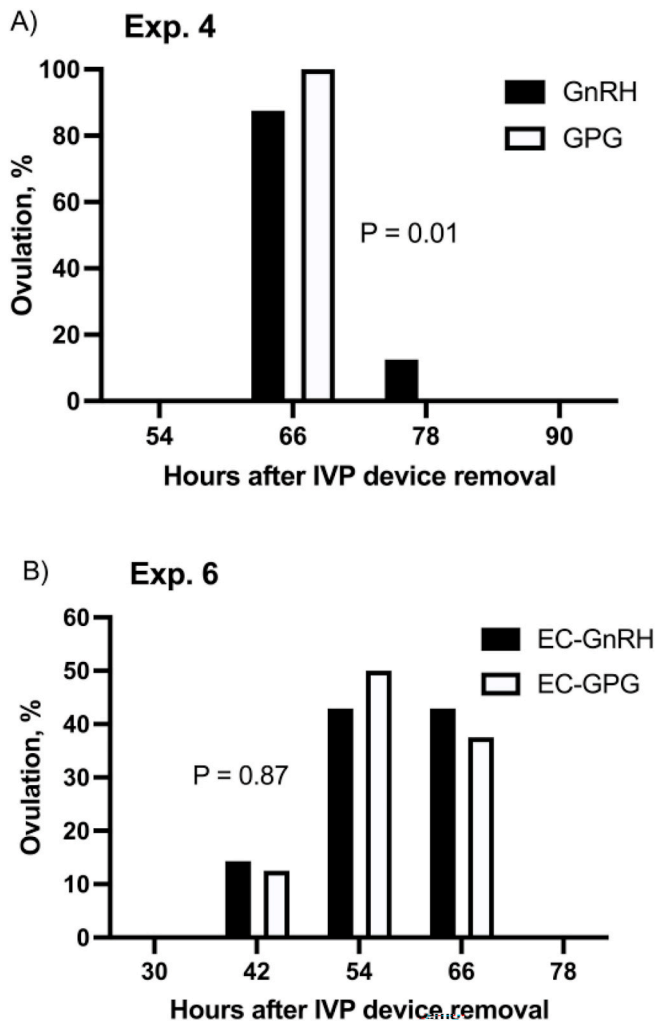

Fig. 11. A) Distribution and percentage of ovulation in beef cows treated with GnRH or GPG in Experiment 4. B) Distribution and percentage of ovulation in beef females treated with EC-GnRH or EC-GPG in Experiment 6. The ovulatory treatments were performed 34 h after IPD removal in both experiments.

Table 4
Ovarian response, and proportion of estrus in cows treated with GnRH or GPG for ovulation induction in Nelore cows subjected to TAI protocol (Experiment 4).

	Groups		P-Value
	GnRH	GPG	
Diameter of POF[a], mm	10.2 ± 0.5	9.3 ± 0.4	0.16
Proportion of cows detected in estrus, %	7 (1/14)	7 (1/14)	1.0
Proportion of cows ovulating, %	57.1 (8/14)	71.4 (10/14)	0.43
Time of ovulation, h	67.5 ± 1.0	66.0 ± 0.9	0.27

[a] POF, preovulatory follicle.

treated with GnRH 14 h before TAI achieved greater P/AI when previously treated with EC, in contrast to cows treated solely with GnRH. The administration of 0.5 mg or 1 mg of EC at IPD removal results in elevated serum estradiol (E2) concentrations 24 h later [47]. This preovulatory increase in circulating E2, derived from both the exogenous treatment and follicular activity, is considered a prerequisite for estrus expression. Additionally, this increase in the E2 has been implicated in supporting postovulatory uterine function [47–49]. Although Experiment 6, which employed a protocol identical to Experiment 7, demonstrated an increased ovulation rate in cows receiving the GPG formulation compared to those treated with GnRH, this did not translate into a

Table 5
Diameter of the POF, proportion of estrus, and P/AI in cows treated with GnRH or GPG for ovulation induction in Nelore cows subjected to TAI protocol (Experiment 5).

	Groups		P-Value
	GnRH	GPG	
Diameter of POF[a],[b], mm	13.3 ± 0.4	13.4 ± 0.4	0.79
Proportion of cows detected in estrus[b], %	43.6 (51/117)	48.6 (53/109)	0.45
Pregnancy per AI, %	51.5[a] (86/167)	59.7[b] (95/159)	0.1

[ab]Different letters indicate tendency between treatment (P ≤ 0.1).
[a] POF, preovulatory follicle.
[b] Estrus and Diameter of POF were measured in a subgroup of cows.

Table 6
Ovarian response, and proportion of estrus in cows treated with GnRH or GPG combination following estradiol cypionate treatment in Nelore cows subjected to TAI protocol (Experiment 6).

	Groups		P-Value
	EC-GnRH	EC-GPG	
Diameter of POF[a], mm	12.1 ± 0.9	12.5 ± 0.8	0.73
Proportion of cows detected in estrus, %	77.8 (7/9)	75 (6/8)	0.89
Proportion of cows ovulating, %	77.8 (7/9)	100 (8/8)	<0.01
Time of ovulation, h	57.4 ± 3.3	57.0 ± 3.1	0.92

[a] POF, preovulatory follicle.

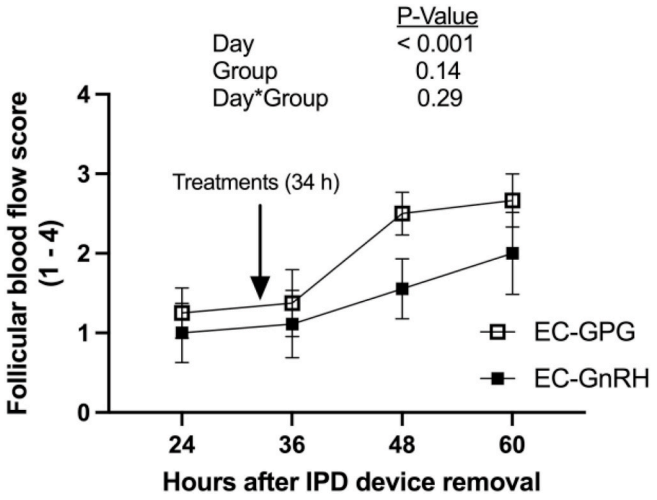

Fig. 12. Vascularization score of the POF in Nelore cows treated with EC-GnRH and EC-GPG in Experiment 6. The ovulatory treatments were performed 34 h after IPD removal.

concomitant enhancement of fertility. This outcome suggests that the expected fertility benefits associated with PGF component of the GPG formulation were not evident in EC-pretreated cows, possibly because these animals were already into the periovulatory period, thereby limiting the efficacy of PGF in influencing overall fertility. A similar effect was observed in Experiment 8. In this experiment, no difference in P/AI was detected between GnRH and GPG treatment among cows that did not express estrus at TAI following EC treatment.

In contrast, in Experiment 5, the GnRH and GPG compounded formulation were compared in cows that did not receive EC at IPD removal. Cows treated with GPG formulation tended to have increased P/AI. One may note that this field study (Experiment 5) was not designed as a multi-location trial with herd-level replication; therefore, the P/AI data should be interpreted with caution. A larger, randomized

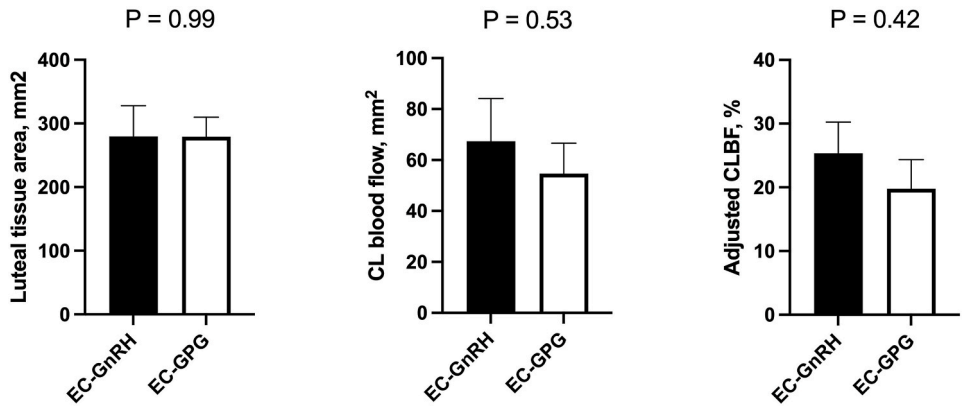

Fig. 13. Characteristics of the CL 8 days after the treatments in Nelore cows in Experiment 6. The ovulatory treatments were performed 34 h after IPD removal in both experiments. CLBF, CL blood flow; Adjusted CLBF, CLBF:Luteal tissue area.

Table 7
Diameter of the POF, proportion of estrus and P/AI in cows treated with GnRH or GPG combination following estradiol cypionate treatment in Nelore cows subjected to TAI protocol (Experiment 7).

	Groups		P-Value
	GnRH	GPG	
Proportion of cows detected in estrus[a], %	53.2 (131/246)	51.7 (140/271)	0,73
Pregnancy per AI, %	64.9 (300/462)	65.2 (317/486)	0,76

[a] Estrus was measured in a subgroup of cows.

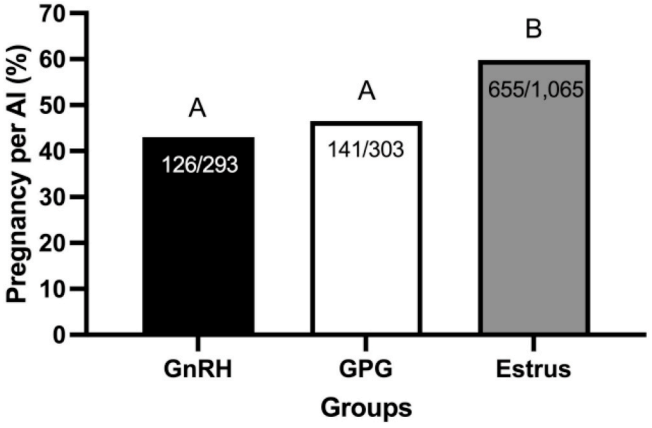

Fig. 14. Pregnancy per AI in cows that presented estrus at TAI (Estrus), and in cows that were not detected in estrus and were treated with GnRH or GPG.
[AB]Values followed by different letters were different ($P \leq 0.05$)

controlled trial across multiple herds is warranted to confirm these findings.

Previous research indicates that the manifestation of estrus prior to TAI is associated with an increased P/AI in both *Bos indicus* [50–52] and *Bos taurus* females [53–55]. Accordingly, in Experiment 8, cows that displayed estrus at TAI exhibited a greater P/AI than GnRH, and GPG-treated cows that did not display estrus at TAI.

To our knowledge, no prior research has investigated the specific compound formulation of GnRH and PGF employed in the present study. However, two studies have reported the concomitant administration of PGF and GnRH at TAI in dairy cows [41,56]. These previous studies differed from the present one, not only regarding the animal model but also in the specific GnRH and PGF analogs utilized. Furthermore, Archbald et al. [41]; reported that dairy cows not observed in estrus within 72 h following PGF treatment were subsequently treated with 100 μg gonadorelin, 25 mg PGF, or a combination of both GnRH and PGF. Despite the overall low P/AI observed, the group treated with GnRH (26%) exhibited greater P/AI compared to cows treated with PGF (18%) or both hormones (21%). In contrast, Mohammadi et al. [56] did not specify whether the cows in their study were enrolled in a standardized TAI protocol (e.g., Ovsynch or estradiol/progesterone-based programs). In that study, no significant difference in P/AI was detected among dairy cows treated with 150 μg d-cloprostenol, 10 μg buserelin acetate, or a combination of both. Given the notable differences identified and the innovative character of the GPG treatment as a single-dose compound formulation, a patent application was submitted to the Brazilian National Institute of Industrial Property (INPI) under process number BR 10 2025 014717 3.

GnRH analogs are extensively employed in beef cattle breeding programs to induce the LH surge for ovulation, especially within TAI protocols [57,58]. The use of GnRH at the beginning of a protocol can synchronize the emergence of a new follicular wave after ovulation [59,60]. Conversely, GnRH at the end of TAI protocol is utilized to optimize ovulation and improve fertility outcomes [21,23]. While GnRH has been widely employed in TAI programs since the 1990s, there is a continued need for research to elucidate the effects of GnRH analogs and their respective doses on LH release and ovulatory response in *Bos indicus* beef females. Silva et al. [61] reported that buserelin consistently induced a greater LH surge and enhanced ovulatory response when compared to gonadorelin treatments. Similarly, Picard-hagen et al. [62] demonstrated that 100 μg of gonadorelin resulted in lower LH concentrations than either 10 μg of buserelin or 25 μg of lecirelin. Unlike previous studies, which were typically conducted during diestrus, when progesterone concentrations are high, the present study used the label-recommended dose of 25 μg of lecirelin per animal during the periovulatory period (34 h after progesterone device removal or at TAI). However, in the present study, the efficacy of alternative GnRH dosages for ovulation induction in TAI protocols was not evaluated. Therefore, optimizing the dosage of the GnRH analog used in the GPG formulation represents a critical area for future experiments.

While Cloprostenol injection has been associated with influencing ovulation timing in dairy [28,29,63] and beef cows [24,37,38], our

results from Experiment 1 indicated a delayed ovulation in PGF-treated cows compared to those receiving GnRH or a GPG combination. Previously, Pfeifer et al. [37] reported that PGF-treated cows ovulated between 72 and 84 h after IPD removal. However, our findings from Experiments 4 and 6, utilizing an estradiol/progesterone-based protocol, demonstrated a more synchronous ovulatory pattern in cows receiving GnRH and GPG treatments. Conversely, in Experiment 4, cows treated with GnRH or GPG formulation as the sole ovulation inducer ovulated approximately 66 h after IPD removal. This timing was notably advanced in Experiment 6, in which cows receiving concurrent EC treatment at IPD removal ovulated approximately 9 h earlier (57 h). Moreover, In Experiment 6, in which cows were pretreated with EC, we observed an increase in the ovulation rate of cows treated with GPG formulation in comparison with cows that received GnRH. These data collectively demonstrate that EC administration at IPD removal significantly modulates the timing of ovulation induced by GnRH or GPG treatments. These findings may elucidate the lack of difference in P/AI observed in Experiment 7, where all cows received EC prior to GnRH or GPG treatments. Unlike GnRH, which acutely induces the LH surge within 2 h [11] and ovulation within 28-30 h [12], PGF mediates a wide array of physiological processes within the mammalian female reproductive tract [25,26]. Within the preovulatory follicle, granulosa cells produce both PGF and prostaglandin E (PGE [64]). These prostaglandins are recognized as initiator of ovulation, and play a crucial role in mediating the steroidogenic actions of LH essential for oocyte maturation and release [25–27]. The precise mechanisms by which PGF contributes to ovulation induction have been a subject of extensive research [65–67]. For example, previous reports indicate that administering a PGF analog to anestrous cows leads to an increase in LH pulse frequency approximately 6 h post-treatment [65]. Furthermore, it has been proposed that PGF directly influences the anterior pituitary gland [25], thereby potentiating its responsiveness to GnRH and consequently enhancing LH secretion [65]. Moreover, PGF treatment concurrent with AI may increase pregnancy rates through both the support of ovulation and the induction of LH release [26,27]. However, when spayed cows were treated with 300 mg D-cloprostenol (PGF analog), 10.5 μg Buserelin Acetate (GnRH analog), or 0.9% saline, only the GnRH-treated animals had increased plasma LH concentration [45]. A detailed understanding of the complex processes and signaling pathways by which prostaglandins mediate ovulation was reviewed by Weems et al. [25]. While no difference in the timing of ovulation was detected between GnRH and GPG treatments, cows treated with the GPG single-dose exhibited a more synchronous pattern of ovulation, specifically in Experiments 2 and 4. Moreover, GPG treatment increased *PTGS2* mRNA abundance in granulosa cells and intrafollicular estradiol concentrations than cows treated with GnRH in the Experiment 3. These findings suggest that this formulation may accelerate the ovulatory process compared to GnRH administered alone. In cows, the GnRH-mediated LH surge induces a characteristic shift in follicular fluid steroid concentrations [68,69], marked by an increase in progesterone (P4) and a decrease in estradiol (E2), which characterizes preovulatory luteinization [68]. In Experiment 3, the observed decrease in intrafollicular E2 concentration did not occur concurrently with the increase in P4, a finding that contrasts with established literature [68]. Specifically, previous research indicates that an elevation in P4 and progesterone receptor (PGR) expression typically precedes the increase in prostaglandins within GnRH-induced ovulation models [67]. Moreover, an increase in P4 secretion by internal theca cells (*in vitro*), isolated from follicles 24 h post-GnRH administration was described previously [64]. These inconsistencies suggest that the 20-h post-treatment collection time for intrafollicular fluid in Experiment 3 may have been premature to capture the full cascade of hormonal shifts. We therefore hypothesize that a delayed collection, at 24 h post-treatment, would more accurately reflect the temporal dynamics of these events. Addressing this temporal aspect is crucial for a comprehensive understanding of GPG-induced follicular changes, and our research group is currently undertaking further experiments to elucidate these unanswered questions.

Because serum P4 concentrations were not measured in the present study, we could not evaluate whether PGF2α in the GPG formulation enhanced luteal regression relative to that in cows that received GnRH alone. Accordingly, the present experiments do not allow us to definitively conclude whether the GPG combination exerted its effects directly on the preovulatory follicle or indirectly through decreased P4 concentrations in cows with incomplete luteal regression. In this regard, delayed or incomplete luteal regression following a single administration of PGF2α has been observed in cows subjected to TAI protocols and can result in reduced fertility [70–72]. To determine whether the effect of PGF on ovulation is mediated by luteolysis, Pfeifer et al. [73] evaluated progesterone (P4) concentrations in dairy cows receiving one or two injections of PGF. Ovulation occurred earlier in cows treated with two PGF injections, despite no differences in P4 concentrations between treatment groups. Based on these findings, the authors suggested that preovulatory administration of PGF may improve ovulation synchronization in dairy cows through mechanisms that extend beyond its direct luteolytic effects. Conversely, among dairy cows treated with Ovsynch-type protocols, circulating P4 concentrations at GnRH administration were greater in cows that received one PGF injection than in those that received two, although P4 concentrations were below 0.5 ng/mL in both groups 16 h before TAI in that study [74]. Taken together, an important limitation of the present study is that the mechanism through which the additional PGF included in the GPG treatment exerted its effects could not be fully elucidated, even though the findings from Experiments 2 and 3 indicate that the PGF administered in the GPG treatments exerted a direct effect on the ovarian follicle. Consequently, the widespread adoption of this protocol under field conditions should be approached with caution until the underlying mechanism is better understood.

Although the benefits of compounded therapies for animals are well-established [75], in animal reproduction area this practice is scarce. The scarcity of approved drugs for the wide range of species and conditions in veterinary medicine creates significant therapeutic gaps. Pharmaceutical compounding is therefore essential for providing necessary treatment options where no commercial products exist [76]. In that regard, previously, the combination of E2 and P4 in a single injection (compounded formulation) on the beginning of the TAI programs was tested in beef cattle [77]. Despite the established efficacy of initial co-administration of injectable P4 and E2 in reducing premature ovulation and enhancing the synchrony of follicular wave emergence during reproductive protocols [78], its widespread application has diminished. Within the Brazilian market, only a single commercial compounding formulation, comprising both E2 and P4, is currently available (BetaActive®, Virbac). In contrast, the present study investigates a single-dose compounding formulation incorporating a GnRH analog (Lecirelin) and a PGF analog (Cloprostenol Sodic). While one might question the novelty of this approach, given that both active pharmaceutical ingredients are commercially available and can be administered separately to induce ovulation in cattle, the GPG compounding formulation offers distinct advantages. Notably, this single-injection strategy mitigates the detrimental consequences associated with multiple injections. Indeed, multiple injections constitute a substantial challenge to animal welfare and productive efficiency, leading to potential complications such as stress, inflammation, tissue irritation, and pain [79]. Therefore, the compounding formulation developed in this study represents a significant strategy to enhance reproductive outcomes by minimizing animal handling and stress compared to the separate administration of its individual components.

5. Conclusions

In the present study, we performed eight experiments comparing ovarian features and pregnancy rates of *Bos indicus* beef cows treated with GPG to those receiving a standard GnRH-treatment. While the

mean interval to ovulation did not differ between GPG and GnRH groups, the GPG formulation reduced the dispersion of ovulation timing relative to GnRH alone. In Experiment 3, cows treated with GPG had lower intrafollicular estradiol concentrations than GnRH-treated cows. This reduction in estradiol is consistent with closer proximity to ovulation at the time of sampling. Additionally, there was an increase in *PTGS2* gene expression, which is responsible for the synthesis of prostaglandins enrolled in follicular rupture and the inflammatory process that culminates in ovulation, in granulosa cells of cows that received GPG formulation. Although cows treated with the GPG formulation ovulated with greater synchrony, effects on P/AI were marginal and observed only in cows not treated with EC; these findings require validation in a multi-location trial designed specifically for that endpoint.

CRediT authorship contribution statement

Luiz Francisco Machado Pfeifer: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Cintia Batista Hurtado:** Data curation, Investigation, Methodology. **Vanessa Lemos de Souza:** Data curation, Methodology. **Ingrid Pedraça Barbosa:** Data curation, Investigation, Methodology. **Samira Alves de Souza Silva:** Data curation, Methodology. **Lígia Margareth Cantarelli Pegoraro:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Renata Reis da Silva:** Formal analysis. **Fabiane Pereira de Moraes:** Formal analysis. **Natalia Avila de Castro:** Formal analysis. **André Cascalho Andrade:** Conceptualization, Data curation. **Monique Tomazele Rovani:** Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Bernardo Garziera Gasperin:** Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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