



Impact of estradiol cypionate prior to TAI and progesterone supplementation at initial diestrus on ovarian and fertility responses in beef cows



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ABSTRACT

In cattle, early diestrus progesterone (P4) supplementation modulates endometrial function to exert pro- and anti-pregnancy establishment effects; specifically, P4 stimulates conceptus growth, but also induces early onset of luteolysis. This paradoxical effect is frequently related to the inconsistent fertility outcomes that result from P4 supplementation experiments. Aim was to investigate the impact of exogenous estradiol (E2) treatment at the end of timed fixed AI (TAI) on frequency of early luteolysis and pregnancy of beef cows supplemented with P4. Ovulations (D0 of study) of suckled multiparous (n = 643) and primiparous (n = 193) Nelore cows (*Bos indicus*) were synchronized with an E2/P4-based protocol for TAI and assigned to receive 1.0 mg of estradiol cypionate (CP) or nothing (NoCP) on D-2 and 150 mg of injectable long-acting P4 (iP4) or Placebo (NoiP4) on D4 on a 2 × 2 factorial arrangement. On D15, the iP4 supplementation increased (P < 0.05) the frequency of early luteolysis (NoCP + iP4: 26.0%; [13/50] vs. NoCP: 8.0% [4/50]), but CP prevented this effect (CP + iP4: 8.3% [4/48] and CP: 6.4% [3/47]). The CP improved pregnancy/AI (P/AI) of multiparous (CP: 51.6% [165/320] and NoCP: 35.0% [113/323]; P < 0.001) and primiparous cows (CP: 40.4% [40/99] and NoCP: 24.5% [23/94], P < 0.05), regardless of iP4 treatment. The iP4 supplementation affected P/AI of CP and NoCP treated cows according to follicle size at TAI. For the CP treated cows, the iP4 supplementation improved P/AI of sub-populations of cows with follicles <12.35 mm (42.0% [34/81] vs. 53.1% [34/64]), while for NoCP treated cows, the improvements occurred in subpopulations of cows with follicles ≥12.35 mm (46.1% [35/76] vs. 58.7% [37/63]). In conclusion, strategies associating E2 and P4 supplementation decrease the incidence of early onset of luteolysis and improve P/AI of suckled beef cows with smaller follicles.

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1. Introduction

In addition to genetic gains, timed artificial insemination (TAI) programs in beef cattle improves reproductive efficiency because it overcomes challenges associated to long anestrous periods and estrus detection, that delay the time to first service post-partum. Despite of such benefits, cows induced to ovulate dominant follicles smaller than 11.0 mm at TAI present low pregnancy per AI (P/

AI) [1–3]. Such poor results are mainly attributable to the insufficient uterine exposure to estradiol (E2) and progesterone (P4) at the pre-ovulatory (proestrus/estrus) and post-ovulatory (diestrus) periods [4,5], respectively. Indeed, aiming to achieve an adequate sequential uterine exposure to E2 and P4, different strategies to stimulate follicle growth in beef cattle submitted to TAI were proposed [6,7]. For example, consistent fertility gains in TAI protocol have been achieved by extending the proestrus period [8,9] or adding exogenous E2 [5,10,11]. An adequate uterine exposure to E2 affects positively the fertilization process [12], reduces the incidence of early luteolysis [13,14], and provides an uterine

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environment favourable to the establishment of pregnancy [15,16]. Regarding P4 concentrations during diestrus, the relationship between P4 concentrations and fertility are generally positive [3,6,17]. Progesterone is critical for successful maternal recognition and maintenance of pregnancy [18,19]. This steroid stimulates endometrial secretions [20] associated with conceptus growth [21–23] and interferon- τ production [24,25]. Therefore, manipulation of the endocrine environment by the addition of exogenous E2 and/or P4 can potentially improve fertility outcomes in TAI programs.

Forde et al. [23] demonstrated that insertion of an intravaginal P4-releasing device between days 3 and 7 after estrus advanced expression of endometrial genes related to energy provision and histotroph constituents. Such alterations seems to be associated with greater conceptuses growth during late diestrus, that was also observed by others using a similar approaches [22,23]. Commonly, the benefits of P4 supplementation on fertility are observed when administration occur at early diestrus (i.e., days 3–7 after ovulation) [26,27]. However, in many studies, P4 supplementation at this period causes a greater incidence (30–35%) of early luteolysis (i.e., by day 14), that may impair maternal recognition and maintenance of pregnancy [24,28]. This paradoxical effect is one of the main explanations for the inconsistent fertility results obtained in response to different strategies to supplement P4 at early diestrus. Indeed, studies reported no effect [29–31], a negative effect [29,32,33], or a positive effect [34–36] of P4 supplementation on P/AI of beef and dairy cattle.

One plausible explanation for the incidence of early luteolysis in P4-supplemented cows is that the early increase in circulating P4 is related to advances in the timing of nuclear P4 receptor (PGR) downregulation in the endometrial epithelia [37]. The downregulation of the PGR is closely followed by an increase in epithelial estrogen receptor alpha (ER α) and oxytocin receptors (OXTR), resulting in the pulsatile release of PGF2 α , which induces luteolysis [38,39]. Furthermore, inconsistent fertility results can be a consequence of differences in the timing, duration, source and dose of P4 treatments [26,27].

Recently, a long-acting injectable P4 formulation (iP4) was evaluated by Pugliesi et al. [40] to supplement P4 at early diestrus in a manner that would be more practical than the use of the P4 intravaginal device or multiple P4 injections. By a single administration of 150 or 300 mg iP4 on days 2 or 3 post-ovulation, the authors observed that supplementation efficiently increased the circulating P4 concentrations for ≥ 3 days during early luteal phase in non-suckled Nelore cows, but also increased the frequency of early luteolysis (0% vs. 40.7%). Despite of this apparent negative aspect, in a subsequent fertility trial using an E2/P4-based TAI protocol, Pugliesi et al. [35] verified that 150 mg of iP4 administered 4 days post TAI (~3 days post-ovulation) increased the P/AI of anestrus suckled beef cows by 20%. Thus, our recent findings highlight the somewhat paradoxical effects of P4 supplementation when given in the early diestrus, as shown previously by others [24]; there are both beneficial effects regarding uterine receptivity and conceptus elongation and potentially negative effects associated with reduced CL lifespan. Thus, it is critical to find strategies to minimize the negative, while emphasizing the beneficial effects of P4 supplementation to maximize efficiency of this technology. Here, we propose to test the effects of supplementing E2 concurrent with the withdrawal of the P4-releasing device (i.e., at the beginning of proestrus) as a strategy to support the beneficial effects of exogenous P4 administration to improve fertility in beef cattle.

The exposure of the endometrium to the rising proestrus concentrations of E2 stimulates ER α and PGR [41,42], and this is indirectly responsible for the decrease of OXTR during metaestrus and early diestrus [13]. Thus, it is possible that E2 supplementation

could cause a greater increase of ER α and PGR than the endogenous E2 pre-ovulatory surge. A greater initial rise in PGR could compensate the advanced disappearance of PGR caused by P4 supplementation. This represents a possible alternative to bypass the detrimental aspects of P4 supplementation. In fact, there is evidence for a positive role of proestrus E2 alone on uterine [15] and luteal [8,13,15] functions during diestrus, establishment of pregnancy [5,43,44] and fertility [5,10,11]. However, the associated response to supplemental E2 and P4 is unknown. Therefore, in this study, we aimed to evaluate the role of E2 supplementation at the P4 device withdrawal on the incidence of advanced luteolysis and fertility outcome after iP4 supplementation at early diestrus. Specifically, we tested the hypothesis that the E2 supplementation (1) decreased the incidence of short luteal lifespan and (2) improved the fertility response obtained by diestrus iP4 supplementation.

2. Materials and methods

2.1. Animals

This experiment was carried out during the summer and early fall on a commercial beef operation located in Mato Grosso do Sul, Brazil. Suckled multiparous (n = 643) and primiparous (n = 193) Nelore cows used in this study exhibited an average of days of postpartum of 52.1 and 50.8 (range, 31 to 85) and body condition scores of 3.48 and 3.00 (BCS, range, 2.25 to 5.00; 1 = emaciated to 5 = obese [45], using 0.25 increments), respectively. The cows were kept in grazing conditions (*Brachiaria brizantha*) with *ad libitum* access to water and minerals. The multiparous cows were split into 5 and 2 allotments from farms 1 (n = 524) and 2 (n = 119), respectively, while the primiparous cows were split into 4 allotments on farm 1.

All animal procedures were approved by the Ethics and Animal Handling Committee of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo under the protocol number CEUA-6236220316.

2.2. Experimental design

Within each allotment, cows were subjected to an estrus synchronization protocol based on a single administration of 2 mg of estradiol benzoate (2.0 mL, i.m., Sincrodiol[®] Ourofino Saúde Animal) and insertion of an intravaginal P4-releasing device (1.0 g, Sincrogest[®] Ourofino Saúde Animal) followed by visual evaluation of BCS, on day –10. On this day, a transrectal ultrasonography exam was performed to exclude any cows with abnormalities of the reproductive tract and to establish the ovarian status. The ovarian status was determined based in three predefined categories, presence of CL, absence of CL and presence of follicles <8.0 mm and absence of CL and presence of follicles ≥ 8.0 mm.

On day –2, P4 devices were removed, and cows received 0.53 mg of sodium cloprostenol (2.0 mL, i.m., Sincrocio[®] Ourofino Saúde Animal) followed by administration of 300 IU of equine chorionic gonadotropin (1.5 mL, i.m., SincroCG[®] Ourofino Saúde Animal). At the time of device removal, EstroTECT[™] patches (Western Point Inc., Apple Valley, MN) were applied halfway between the hip and tail head to determine the occurrence of mounting behavior associated with estrus. Concurrent with TAI (day 0), all cows received 10 μ g of gonadotropin releasing hormone analogue (buserelin acetate, 2.5 mL, i.m., Sincroforte[®] Ourofino Saúde Animal).

Cows were blocked based on the BCS (low: L-BCS, 2.00 to 2.50, moderate: M-BCS, 2.75 to 3.50 or high: H-BCS, 3.75 to 5.00) to receive one of four treatments: injection of 1.0 mg of estradiol cypionate (CP, 1.0 mL, i.m, SincroCP[®] Ourofino Saúde Animal) or

none (NoCP) on day –2 and supplementation with 150 mg of long-acting P4 (iP4, 1.0 mL, i.m., Sincrogest[®] injectable Ourofino Saúde Animal) or placebo (NoiP4, Sincrogest[®] injectable vehicle) on day 4, on a 2 by 2 factorial arrangement of treatments (CP, CP + iP4, NoCP and NoCP + iP4). Administration of CP at the time of P4 device withdrawal was based on previous publications [46,47]. Compared to estradiol benzoate, CP releases estradiol more slowly. Thus, it may be injected earlier in the protocol, at the same moment as the P4-releasing device withdrawal. This is advantageous for management because it reduces one handling of the animals and allows easy supplementation of estradiol.

On the day of TAI (day 0), cows were assigned randomly within treatments to receive a single insemination by one of four experienced operators using frozen-thawed commercial semen from 12 and three sires on farm 1 and farm 2, respectively. At the time of TAI, the cows with approximately 50% or more of the silver rub-off coating removed from the Estrotect device were considered to have been in estrus between days –2 and 0.

2.3. Ultrasound examinations

Ultrasonography exams were performed in B-mode with a 7.5 MHz linear-array transrectal transducer by the same operator on days –10, 0 and 4.

On day 0, the diameter of the largest ovarian follicle observed in the B-mode still image was determined by taking the average between measurements of its two perpendicular axes. These measurements were taken only in cows with follicles ≥ 6.5 mm ($n = 561$ and $n = 154$ for multiparous and primiparous cows, respectively). On day 4, the ovaries were evaluated for detection of a newly formed CL, and its maximum area was determined by B-mode still image and the tracing function. For CL with an anechoic fluid-filled cavity, the area of the cavity was subtracted from the total area [48]. For primiparous cows, the evaluations on day 4 were performed in only 94 out of 193 animals due to operational constraints.

For determining early onset of luteolysis after P4 supplementation, a subgroup of ovulated multiparous cows ($n = 195$) were evaluated by Colour Doppler ultrasonography on day 15. The CL scanning was performed using an ultrasound equipped with pulse-wave colour Doppler function and a multi-frequency linear transducer. The evaluation and proportion of colour signals of luteal blood flow were performed and determined as described previously in cattle [49]. A scale from 0 to 100% with 5% interval points was used for visually determining the proportion of the luteal area with blood flow signals. All scans were performed at a constant colour-gain setting and a velocity setting of 5.4 cm/s by an operator unaware of the treatment allocation of animals. The criteria for describing a cow that underwent early structural luteolysis were: (1) the presence of a CL area < 2.0 cm² and (2) CL blood flow signals that covered $\leq 25\%$ of the total luteal area on day 15, as reported previously [34,40,49].

Pregnancy diagnosis was conducted by transrectal ultrasonography 30–35 days after TAI. Conception rate was calculated as the proportion of ovulated cows (i.e. with CL on day 4) that became pregnant due to TAI, and P/AI was calculated as the proportion of total cows inseminated that were pregnant.

2.4. Blood sampling for analysis of serum P₄ concentrations

Blood sampling for determination of circulating concentrations of P4 was taken from the subgroup of cows that were submitted to ovarian ultrasonography on day 15 ($n = 195$). The serum P4 concentrations were used for identification of functional luteolysis, defined as P4 concentrations < 1.0 ng/mL [40]. Blood samples were collected from coccygeal vessels, centrifuged at 1500g for 10 min at

room temperature; serum was stored at -20 °C until hormonal assays were performed.

Serum P4 was assayed by solid-phase radioimmunoassay using an Immuchem[™] Double Antibody Progesterone Kit (Cat. 07–170105, MP Biomedicals, NY, USA) according to manufacturer's instructions. The detection limit (sensitivity) of the assay was 0.1 ng/mL. The intra-assay coefficients of variation (CV), were 0.17% (low) and 7.39% (high), respectively. The inter-assay CVs were 14.48% (low) and 9.95% (high), respectively.

2.5. Statistical analyses

All statistical analyses were carried out using SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). Cows were the experimental units in all models. The analyses of datasets containing primiparous and multiparous cows were ran separately.

For continuous dependent variables (follicle diameter, CL area, and P4 concentrations), the assumptions of normality of residues and homogeneity of variance were checked by histograms, q-q plots, and formal statistical tests as part of GLM and the UNIVARI-ATE procedure of SAS. The natural logarithmic transformation and square root were respectively used to normalize the data distribution of the CL area and P4 concentrations. Non-transformed data were shown for clarity. These variables were analyzed by ANOVA using the MIXED procedure of SAS. Follicle diameter on day 0 and CL area on day 4 were measured before P4 supplementation. For these variables, only the effect of CP was included into the model as a fixed effect. Area of the CL on day 15 was analyzed according to the fixed effects of CP, iP4 supplementation and their interaction. On additional analyses, the variable estrous behavior and the appropriate interactions were included as fixed effects into the model, but only for multiparous cows, due the limited number of primiparous cows detected in estrus.

Binomial, dependent variables were analyzed by the GLIMMIX procedure of SAS, using binomial distribution. For rates of ovulation and estrus, only the fixed effect of CP it was included in the model. For rates of conception and pregnancy, the fixed effect of CP, iP4 supplementation and their interaction were included in the model. For primiparous cows, the conception rate analysis was not performed because the ovulation was checked only in a limited number of animals on day 4 ($n = 94$). For further analyses of pregnancy, the variables estrous behavior (only for multiparous) or ovarian status at day –10 (i.e., presence or absence of CL), and the appropriate interactions were included as fixed effects in the model.

For continuous and binomial dependent variables, the effects of BCS block, farm, and allotment nested within farm were included as random effects in the model. For primiparous cows, the farm effect was not included because they belonged to a single farm. To determine the denominator degrees of freedom for tests of fixed effect, the options Kenward-Roger and Between-Within degrees was used into the models of continuous and binomial dependent variables, respectively.

To evaluate the effect of follicle diameter (day 0) and CL area (day 4) on pregnancy rate in the model described previously, each variable was manually included as a covariate, and the appropriate interactions were considered. According to the covariate effects, the GLM procedure of SAS was used to establish whether the effect was linear, quadratic, or cubic. The significant, more complex model was selected. Then, the logistical regression models were designed using the intercept and slope value generated by the LOGISTIC DESC procedure of SAS for the following equation: Probability = $(e^{\text{logistic equation}})/(1 + e^{\text{logistic equation}})$. Subsequently, the same analysis was conducted, but each continuous variable was divided in two classes according to the median ($< m$ or $\geq m$) to be included as fixed

effects in the models. None of these latter analyses were performed for primiparous cows due the limited number of animals.

The effects of treatments were determined by F-tests using Type III sums of squares. When necessary, means across treatments were compared using Fisher's protected least significant difference (LSD, i.e., the DIFF option of the LSMEANS statement). Results of continuous variables were reported as LSMEANS \pm S.E.M and from binomial variables as means. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $0.05 > P \leq 0.10$ indicated that significance was approached.

The proportion of the luteal area containing blood flow signals was analyzed using the non-parametrical Kruskal-Wallis test of the NPAR1WAY procedure of SAS. The comparisons of frequency of luteolysis between treatments were performed with the FREQ procedure using the Chi-squared distribution of SAS.

3. Results

Effects of supplementation of CP prior to TAI and iP4 at initial diestrus on fertility of suckled beef cows.

An effect of CP treatment on the conception rate was detected for multiparous cows (CP: 59.1% [165/279] vs. NoCP: 41.2% [113/274]; $P < 0.001$, Table 1). The P/AI was also influenced positively by CP treatment both in multiparous (CP: 51.6% [165/320] vs. NoCP: 35.0% [113/323]; $P < 0.001$) and primiparous (CP: 40.4% [40/99] vs. NoCP: 24.5% [23/94]; $P = 0.03$) cows. There was no main effect of iP4 nor a CP by iP4 interaction ($P > 0.10$; Table 1).

The CP increased the proportion of cows showing estrus at TAI (Table 2), and this was associated positively with P/AI. In fact, multiparous cows detected in estrus presented greater ($P < 0.001$) conception (67.4% [145/215] vs. 39.3% [133/338]) and P/AI (65.0% [145/223] vs. 31.2% [133/420]), regardless of treatment. Multiparous cows in estrus presented larger follicle diameters at TAI (Estrus: 13.55 ± 0.29 vs. Noestrus: 11.62 ± 0.27 mm; $P < 0.001$), regardless of treatment.

There was an interaction ($P = 0.04$) between the covariate follicle diameter at TAI and the variables CP and iP4 on pregnancy rate of multiparous cows. The relationship that better explained the effect of this covariate on pregnancy rate according to treatment was linear for NoCP and NoCP + iP4 treatments and quadratic for CP and CP + iP4 treatments (Fig. 1). The iP4 supplementation appears to improve probability of pregnancy when follicle diameters were greater in cows not treated with CP (NoCP + iP4 group), while for cows treated with CP (CP + iP4 group) the apparent positive effect occurred when cows presented smaller follicle diameter.

For clarity, when follicles were categorized according to the median (12.35 mm) in the classes < 12.35 and ≥ 12.35 mm, the treatment by follicle size interaction approached significance ($P = 0.06$; Fig. 2). For follicles that were ≥ 12.35 mm, the mean group comparisons demonstrated that pregnancy rate of NoCP + iP4 group was intermediate between the NoCP and CP groups. This indicated an increase in fertility promoted by iP4 supplementation, compatible with the observations of results from Fig. 1 for this group. For each group, the comparisons between

Table 1

Effect of CP supplementation 2 days prior to TAI and/or iP4 supplementation 4 days post-TAI on fertility of suckled beef cows.

Variables/Category	CP		NoCP		P value		
	NoiP4	iP4	NoiP4	iP4	CP	iP4	CP*iP4
Multiparous							
Conception/AI, % (n/n) ^a	60.1 (83/138)	58.2 (82/141)	38.3 (54/141)	44.4 (59/133)	<0.001	0.62	0.36
P/AI, % (n/n) ^b	51.2 (83/162)	51.9 (82/158)	32.9 (54/164)	37.1 (59/159)	<0.001	0.51	0.65
Primiparous^c							
P/AI, % (n/n) ^b	48.0 (24/50)	32.7 (16/49)	25.5 (12/47)	23.4 (11/47)	0.03	0.23	0.41

Suckled primiparous (n = 193) and multiparous (n = 643) beef cows were assigned to receive an intravaginal P4 implant device plus 2 mg of EB at initiation of synchronization protocol for TAI (D–10). Devices were removed 8 days later (D–2) and animals received 300 IU of eCG and 0.53 mg of PGF and were selected to receive nothing or 1 mg of CP. On D0, all cows were TAI and received 100 μ g of a GnRH analogue. On D4, animals were further sub-divided to receive placebo or 150 mg of iP4, on a 2 by 2 factorial arrangement of treatments. Pregnancy diagnosis was performed by transrectal ultrasonography between days 30 and 35.

^a Conception/AI = number of pregnant cows divided by the number of cows detected with a CL 4 days after TAI.

^b P/AI = number of pregnant cows divided by the total number of cows that received TAI.

^c Conception analysis was not performed for primiparous because ovulation was checked only in a sub-sample of animals on D4 (n = 94).

Table 2

Effect of CP supplementation prior TAI on ovarian characteristics and estrus behavior.

Variables	Multiparous		P value	Primiparous		P value
	CP	NoCP		CP	NoCP	
FD on D0 (mm)	12.32 \pm 0.33	12.37 \pm 0.33	0.83	11.06 \pm 0.29	11.16 \pm 0.30	0.75
Estrus on D0, % (n/n)	53.4 (171/320)	16.1 (52/323)	<0.001	31.3 (31/99)	3.2 (3/94)	<0.001
CL area D4 (cm ²)	1.31 \pm 0.13	1.22 \pm 0.13	0.006	1.07 \pm 0.06	0.95 \pm 0.07	0.13
Ovulation, % (n/n) ^a	87.2 (279/320)	84.3 (274/323)	0.38	85.7 (42/49)	82.2 (37/45)	0.59
Double ovulation, % (n/n)	2.7 (8/279)	1.4 (4/274)	0.27	2.2 (1/46)	5.3 (2/38)	0.46

Abbreviations: FD on D0 = diameter of the largest ovarian follicle at TAI.

Suckled primiparous (n = 193) and multiparous (n = 643) beef cows underwent to TAI (D0) were treated 2 days prior TAI with 1 mg of CP or nothing (NoCP) and received an estroject device. On D0, all cows received 100 μ g of a GnRH analogue. On D0, the diameter of the largest ovarian follicle at TAI was determined with ultrasound and cows with estroject device activated were considered to have been in estrus. On D4, the ovaries were evaluated with ultrasound for detection of a newly formed CL, and determination of its maximum area. Dataset of primiparous and multiparous cows were analyzed separately.

^a On D4, the ultrasound exam was performed only in 94 out of 193 primiparous cows due to operational constraints.

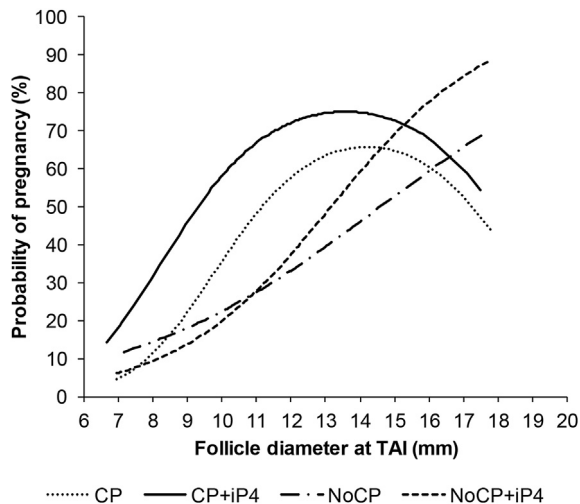


Fig. 1. Relationships between follicle diameter at TAI (D0) and probability of pregnancy of suckled multiparous beef cows ($n = 561$) synchronized with E2/P4 based protocol and assigned to receive nothing or 1 mg of CP on D-2 and placebo or 150 mg of iP4 on D4 on a 2 by 2 factorial arrangement of treatments. The effect of treatments on probability of pregnancy varied according to follicle diameter ($P = 0.04$). The regression equations were as follows: **CP** = $\exp(-0.07x^2 + 1.99x - 13.49)/1 + \exp(-0.07x^2 + 1.99x - 13.49)$; **CP + iP4** = $\exp(-0.06x^2 + 1.63x - 9.97)/1 + \exp(-0.06x^2 + 1.63x - 9.97)$; **NoCP** = $\exp(0.27x - 3.94)/1 + \exp(0.27x - 3.94)$; **NoCP + iP4** = $\exp(0.44x - 5.79)/1 + \exp(0.44x - 5.79)$.

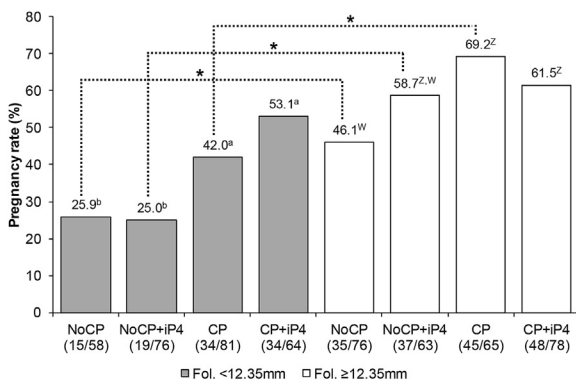


Fig. 2. Effect of classes of follicle diameter at TAI (D0) on pregnancy rates of suckled multiparous beef cows ($n = 561$) synchronized with E2/P4 based protocol and assigned to receive nothing or 1 mg of CP on D-2 and placebo or 150 mg of iP4 on D4 on a 2 by 2 factorial arrangement of treatments. The effect of treatments on pregnancy rates varied according to follicle diameter ($P = 0.06$). *Significant difference ($P < 0.01$) for comparisons between class of follicle within each group. ^{a,b}Values without a common superscript differed ($P \leq 0.05$) for comparisons performed within class of follicles <12.35 mm. ^{Z,W}Values without a common superscript differed ($P \leq 0.05$) for comparisons performed within class of follicles ≥ 12.35 mm.

classes of follicles (i.e. <12.35 and ≥ 12.35 mm), demonstrated that except for the CP + iP4 group, every other group presented a smaller pregnancy rate in the class of follicles <12.35 mm. This indicated that the positive effect of iP4 supplementation for CP treated cows occurred for cows with smaller follicles (<12.35 mm).

Contrary to the follicle analysis, the interaction between the covariate CL size and the variables CP and iP4 on conception rate was not significant ($P = 0.79$). Regardless of treatment, the conception rate was affected by CL size ($P = 0.007$; Fig. 3). Curiously, the equation that better explained the relationship between the covariate CL size and pregnancy rate was of a cubic order. When CLs were categorized according to the median (1.17 cm²) in the classes <1.17 and ≥ 1.17 cm², again, independent of treatment an effect of CL size was found ($P = 0.002$). The pregnancy rate of cows with

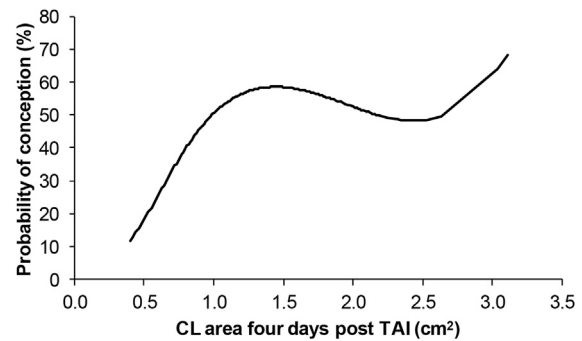


Fig. 3. Relationship between CL area four days post TAI (D0) and the probability of pregnancy of suckled multiparous beef cows ($n = 553$) synchronized with E2/P4 based protocol and assigned to receive nothing or 1 mg of CP on D-2 and placebo or 150 mg of iP4 on D4 on a 2 by 2 factorial arrangement of treatments. Independent of treatments, the CL area affected ($P = 0.007$) the probability of pregnancy in a cubic order [$\exp(0.85x^3 - 4.95x^2 + 8.98x - 4.86)/1 + \exp(0.85x^3 - 4.95x^2 + 8.98x - 4.86)$].

CL ≥ 1.17 cm² was greater than those with CL < 1.17 cm² (57.3% [160/279] vs. 41.7% [115/276]), which reflects the effects observed in Fig. 3 more clearly.

In addition, at the start of the estrus synchronization protocol 45.4% [292/643] of multiparous and 8.8% [17/193] of primiparous cows presented CL, however, the presence or absence of a CL did not affect ($P > 0.10$) P/AI.

Interactions between CP addition prior to TAI and iP4 supplementation at initial diestrus on luteal function and lifespan of suckled beef cows.

In the absence of CP supplementation, iP4 injection on day 4 caused the greatest proportion of early luteolysis (26 and 20% for structural and functional luteolysis, respectively, for the NoCP + iP4 group; Table 3). The addition of CP prior to TAI prevented the incidence of early luteolysis triggered by iP4 supplementation on day 4 ($P < 0.05$). Indeed, CP + iP4 treatment resulted in a proportion of cows showing early luteolysis similar to that of groups that did not receive iP4 supplementation.

For cows with a functional CL on day 15, i.e. no structural or functional luteolysis detected, no effect of iP4 supplementation was observed on CL area or blood flow (Table 3). However, a CP by iP4 interaction approaching significance ($P = 0.08$) indicated that the NoCP group presented a lower ($P < 0.05$) serum P4 concentration than the CP group (Table 3). In addition, cows showing estrus had greater serum P4 concentrations (6.77 ± 0.43 vs. NoEstrus: 5.38 ± 0.24 ng/mL; $P = 0.006$).

4. Discussion

The main motivation of the present study was to verify whether the combined supplementation of CP and iP4 would increase P/AI in suckled beef cows. Expectation was that supplementation of CP at the beginning of proestrus (day of P4-releasing device removal) in cows supplemented with long-acting P4 at early diestrus would prevent the incidence of iP4-induced early luteolysis. In general, we found that CP treatment: 1) improved the incidence of estrus; 2) increased size of CL at early diestrus; 3) increased P4 concentrations at late diestrus; 4) prevented iP4-induced early onset of luteolysis and 5) improved fertility of both multiparous and primiparous beef cattle submitted to TAI, independent of iP4. The fact that early luteolysis was suppressed but P/AI was not further increased after combined CP and iP4 supplementation was intriguing. Further examination of the data after partitioning cows according to size of the preovulatory follicle revealed that the fertility response to CP and iP4 is complex. Complexity is probably

Table 3

At day 15, effects of CP addition 2 days prior TAI and/or iP4 supplementation 4 days post-TAI on incidence of luteolysis, CL area, CL blood flow and serum P4 concentrations of suckled beef cows.

Variables on D15	CP		NoCP		P value		
	NoiP4	iP4	NoiP4	iP4	CP	iP4	CP*iP4
Structural lut., % (n/n) ¹	6.4 ^b (3/47)	8.3 ^b (4/48)	8.0 ^b (4/50)	26.0 ^a (13/50)	.	.	.
Functional lut., % (n/n) ²	2.1 ^b (1/47)	4.2 ^b (2/48)	8.0 ^{a,b,y} (4/50)	20.0 ^{a,x} (10/50)	.	.	.
CL area (cm ²)	2.80 ± 0.08	2.67 ± 0.08	2.65 ± 0.08	2.67 ± 0.09	0.34	0.59	0.29
CL blood flow, % ³	44.8 ± 0.02	43.8 ± 0.02	44.1 ± 0.02	44.1 ± 0.02	.	.	.
P4 conc. (ng/mL) ⁴	6.14 ± 0.35 ^a	5.84 ± 0.35 ^{a,b,x}	4.95 ± 0.35 ^{b,y}	5.84 ± 0.39 ^{a,b,x}	0.11	0.37	0.08

Abbreviations: Structural lut. = structural luteolysis; Functional luteolysis = functional luteolysis; P4 conc. = concentrations of P4

Suckled beef cows were assigned to receive an intravaginal P4 implant device plus 2 mg of EB at initiation of synchronization protocol for TAI (D–10). Devices were removed 8 days later (D–2) and animals received 300 IU of eCG and 0.53 mg of PGF and were selected to receive nothing or 1 mg of CP. On D4, animals were further sub-divided to receive placebo or 150 mg of iP4, on a 2 by 2 factorial arrangement of treatments. On D0, all cows were TAI and received 100 µg of a GnRH analogue. On D15, CLs were examined by ultrasonography to determine CL area, proportion of the CL area that contained colour-doppler signals of blood flow and a blood sample was collected for P4 assay. Cows that had undergone structural or functional luteolysis were removed from the analysis of CL area, CL blood flow and P4 concentrations.

¹Structural luteolysis: CL area <2.0 cm² and blood flow rate ≤25%.

²Functional luteolysis: concentration of P4 < 1.0 ng/mL. The analyses were performed using Chi-Square test;

³Analyzed by Kruskal Wallis test of SAS. The sum of scores were 3928.50, 3744.0, 3800.50 and 3233.0 for the CP, CP + iP4, NoCP and NoCP + iP4 respectively (P = 0.93);

^{a,b} Values without a common superscript differed between treatments (P ≤ 0.05).

^{x,y} Values without a common superscript tended to differ (0.05 > P ≤ 0.10).

due to the isolated and combined effects of CP and iP4 on the preovulatory follicle, the subsequent CL, the reproductive tract and the embryo/conceptus functions.

The CP supplementation prevented the incidence of iP4-stimulated early luteolysis. Shortened luteal lifespan in P4-supplemented cows [24,28,40] is one explanation for the historically inconsistent fertility results obtained in response to supplementation of P4 at early diestrus. Here, we successfully demonstrated that an injection of CP 48 h before TAI suppressed the increased incidence of early luteolysis associated with P4 supplementation. Early luteal regression has two main explanations: advanced release of PGF2α pulses from the endometrium, leading to regression, and luteal insufficiency or sub-functionality. Regarding the endometrium, although we did not evaluate the molecular mechanisms underlying this response, we propose that the CP effect might be a consequence of the supplemental pre-ovulatory E2 stimulating an increase in the expression of endometrial ESRα and PGR [41,42] at estrus. We rationalized that perhaps a greater starting abundance of PGR would take longer to become down-regulated during early diestrus, and this might counteract the acceleration of PGR loss from the uterine epithelia caused by supplemental P4 [37]. In such scenario, timing of onset of the luteolytic cascade could be reset to that expected on a normal estrous cycle, which would provide enough time for anti-luteolytic signalling from the conceptus [38,39]. This preposition needs specific experimental testing.

Regarding a CP effect on luteal function, we observed that P4 concentration on day 15 of the CP group was 19% greater than NoCP group, and this was consistent with a 13% greater CL volume on day 4. Interestingly, increases in CL growth and function occurred despite the fact that pre-ovulatory follicle sizes were similar among groups. These responses could be related to the longer duration of the LH surge stimulated by CP in comparison to the surge stimulated by the GnRH injection alone. The LH remains elevated up to 16.5 h after CP-stimulation [46] instead of 6h when a GnRH analogue is injected [50]. Indeed, variations in the magnitude of the LH surge around the time of ovulation were reported to be important for early luteal function [51,52]. In this regard, a possible cause of early luteolysis onset caused by P4 supplementation is because of failures on CL development due to inadequate luteotropic support [28,53]. Administration of human [53] or equine [28] chorionic gonadotrophin concurrent with P4 supplementation

prevented early onset of luteolysis in cows. Thus, we infer that CLs from CP-group may be more resilient to the LH pulse frequency inhibition caused by supplemental P4. The possible association between the LH-release dynamics during proestrus and the incidence of short luteal lifespan warrants further investigation.

In the present paper, lack of a main effect of iP4 supplementation and lack of an effect of the interaction CP by iP4 on P/AI and conception rate led to the conclusion that iP4 did not affect fertility. However, a closer look at the data, after stratification by size of the pre-ovulatory follicle, allowed the identification of sub-populations of cows responsive to supplemental P4. Specifically, when follicles were ≥12.35 mm, pregnancy rates increased from 46.1 to 58.7% after administration of iP4 to cows not treated with CP. Furthermore, when follicles were <12.35 mm, pregnancy rates increased from 42.0 to 53.1% after administration of iP4 to cows treated with CP (Fig. 2). Thus, in a scenario of presumed low E2 exposure during proestrus, for example in the presence of smaller pre-ovulatory follicles, (Fig. 1), the CP treatment favoured the iP4 supplementation fertility response (i.e. CP + iP4 group); in contrast, CP reduced iP4 benefits when concentrations of E2 were already elevated during proestrus, such as in the presence of a larger follicle. Furthermore, when the pre-ovulatory E2 milieu was generated only by size of follicle (i.e., NoCP groups), the response to iP4 stimulus was verified only for cows with larger follicles. In support of this observation, Madsen et al. [43] demonstrated that lack of E2 exposure during proestrus period impaired the capability of ovariectomized beef cows treated with P4 to sustain a pregnancy. The authors verified that previous E2 exposure during the proestrus period was required to sustain embryonic growth and or/placental attachment. Moreover, Davoodi et al. [15] observed that at similar P4 concentrations during diestrus, beef cows that showed estrus at TAI presented longer conceptuses compared to those that did not come in estrus. Collectively, consistent with previous reports, we show here that P4 supplementation may exert embryotropic [22], luteolytic [24,40] or no effect on the probability of conception of an individual cow. Furthermore, such effects are modulated by exposure to follicular, endogenous and exogenous estradiol. The interacting effects of E2 and P4 coordinate shifts in sub-populations of cows between pregnant and non-pregnant, to yield the overall pregnancy rate. This phenomenon is well illustrated on Fig. 1.

Interpretation of this complex scenario may help us to

understand why, contrary to our initial hypothesis, the suppression of short luteal lifespan by CP (Table 3) did not improve the proportion of pregnant cows after iP4 supplementation (Table 1 and Fig. 2). For example, the 18% increase in the incidence of early luteolysis in the NoCP + iP4 vs. the NoCP group (Table 3) did not impair P/AI (Table 1). Indeed, numerically, the P/AI of NoCP + iP4 group (37.1%) was even greater than NoCP (32.9%). However, magnitude of iP4 effects were clearly affected by size of the pre-ovulatory follicle. For example, the iP4 benefits for NoCP treated cows with follicles ≥ 12.35 mm (12.6% increase in pregnancy rate; Fig. 2) probably counterbalanced the overall iP4-luteolytic effect. The numerical improvement on P/AI indicates that iP4 benefits in cows with follicles ≥ 12.35 mm compensated partially the fertility limitations, associated with sub-maximal exposure to pre-ovulatory E2 and incidence of early luteolysis. In fact, the pregnancy rates of NoCP + iP4 group (58.7%) was intermediary between groups NoCP (46.1%) and CP (69.2%), Fig. 2. For the CP treated cows, the absence of iP4-induced early luteolysis (Table 3) associated to iP4 fertility benefits (11.1% increase in pregnancy rate; Fig. 2) for cows with follicles < 12.35 mm did not result in gains in the P/AI (Table 1). This was probably due to the reduced benefits of iP4 when follicles were larger (Fig. 1). In this regard, for animals treated with CP, the numerical decrease (7.7%) in the pregnancy rate of cows with follicles ≥ 12.35 mm treated with iP4 (Fig. 2) suggest that the positive effect of iP4 was abrogated in these sub-populations of cows, but this was not because of iP4-induced early luteolysis. Thus, our findings indicated that success of iP4 supplementation in TAI programs depends on the endocrine profiles resulting from the TAI programs. This implies that an iP4 treatment designed specifically to certain populations of cows within TAI programs should be considered in future investigations.

The present results involving CP supplementation and size of follicles at TAI confirm, respectively, the strong positive association between (i) the circulating estradiol concentrations during proestrus and fertility [5,8,10,44] and (ii) the follicle diameter at TAI and fertility [1–3,6].

In conclusion, the present data highlighted the complex interplay among endogenous and exogenous estradiol and supplemental P4 on beef cow fertility. The association of supplemental E2 and P4 can increase pregnancy rates in sub-populations of cows with smaller follicles at TAI and this may be partially due to a decrease in the incidence of early onset of luteolysis. The precise mechanisms by which CP induces these effects need to be determined. From a practical standpoint, exogenous E2 addition at the beginning of proestrus reduces the occurrence of early luteolysis in P4-supplemented cows at early diestrus and, strategies associating E2 and P4 supplementation have a potential positive impact on the reproductive performance of suckled beef cows.

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