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Prolonged use of a progesterone-releasing intravaginal device (CIDR®) for induction of persistent follicles in bovine embryo recipients

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Abstract

Embryonic mortality after embryo transfer causes substantial economic losses in the cattle industry. This has been related to the inability of the corpus luteum (CL) to secrete enough progesterone (P_4) to prepare the endometrium for embryo implantation and maintain pregnancy. Thus, the objective of this experiment was to evaluate the effects of treatments that induce ovulation of a persistent follicle and formation of a larger CL that secretes more P_4 on the conception rate (number pregnant/number that received an embryo) of recipients following embryo transfer. Two hundred seventy-eight crossbred *Bos taurus* x *Bos indicus* heifers were randomly allocated to one of four groups. Group 1 (G1, n = 70) received 2 mg estradiol benzoate (EB) + 50 mg of P_4 at the time a progesterone-releasing intravaginal device (CIDR®) was inserted (Day 0), 0.53 mg of cloprostenol ($PGF_{2\alpha}$; prostaglandin $F_{2\alpha}$ analogue) at the time of CIDR® removal (Day 8), and 0.5 mg EB on Day 9. Group 2 (G2, n = 71) received a CIDR® and 2 mg of EB + 50 mg of P_4 at CIDR insertion (Day 0) and a $PGF_{2\alpha}$ treatment on Day 0 and Day 5. The CIDR® was removed on Day 14, and 0.5 mg of EB was given on Day 15. Group 3 (G3, n = 67) was similar to G2, except that an injection of $PGF_{2\alpha}$ was given on Day 5. Group 4 (n = 70) was similar to G2, however, these heifers received $PGF_{2\alpha}$ both at the time of CIDR® insertion and removal. Eight days after the second EB administration, heifers of all groups were selected to receive a frozen-thawed *in vivo* produced embryo by direct transfer. Mean (± SEM) diameter (mm) of the dominant follicle one day after CIDR® removal was larger in heifers in G2 (11.1 ± 0.3), G3 (10.6 ± 0.4), and G4 (10.6 ± 0.3) than in G1 (7.8 ± 0.4). The mean CL area (cm^2), plasma P_4 concentrations (ng/ml), and recipient selection rate (number that received an embryo/number in treatment group) was greater in G2 (2.3 ± 0.1; 3.8 ± 0.2; 77.4%) and G3 (2.4 ± 0.1; 3.8 ± 0.3; 74.6%) than in G1 (1.9 ± 0.1; 2.3 ± 0.2; 51.4%), but mean values in G4 (2.2 ± 0.1; 3.1 ± 0.3; 68.6%) were not different from those of other groups. Conception rate was lower in G2 (38.9%; 21/55) and G3 (37.1%; 19/50) than in G1 (59.1%; 21/36), but conception rate in G4 (50.0%; 21/36) was not different from that of the other groups. Pregnancy rate (number pregnant/number in treatment group) was not different among groups. These results showed that a long-term CIDR® treatment with $PGF_{2\alpha}$ administered at the beginning of the treatment effectively caused the formation and ovulation of a persistent follicle and resulted in a larger CL that provided a higher P_4 concentration. However, the induction of a persistent follicle reduced conception rates following embryo transfer.

Keywords: persistent follicle; progesterone; corpus luteum; embryo transfer

Introduction

The presence of the corpus luteum (CL) and its progesterone-secreting capacity are essential for the establishment and maintenance of early pregnancy in domestic animals (Bulman and Lamming, 1978). A greater concentration of progesterone (P_4) increases the capacity of the conceptus to produce interferon-τ (IFN), a main regulator of pregnancy recognition (Binelli *et al*., 2001). Moreover, P_4 is suggested to be a suppressor of apoptosis in bovine luteal cells (Okuda *et al*., 2004).

Attempts have been made to improve pregnancy rates by increasing plasma P_4 concentrations (Bó *et al*., 2002). Recent studies have shown that eCG treatment given on the day of ovarian follicular wave emergence increases the number of CLs, plasma P_4 concentrations, conception rates, and pregnancy rates in bovine embryo recipients (Baruselli *et al*., 2000; Nasser *et al*., 2004). Administration of hCG on Day 7 after insemination caused ovulation of large follicles and increased P_4 plasma concentrations and pregnancy rates in dairy cows (Rajamahendran and Sianangama, 1992). Similar results were reported when hCG was administered to embryo transfer recipients at the time of transfer (Marques *et al*., 2003).

A larger pre-ovulatory follicle may generate a larger CL that will secrete more P_4 and thereby have a positive effect on pregnancy recognition (Binelli *et al*., 2001) and increase pregnancy rates following artificial insemination (Vasconcelos *et al*., 2001) or embryo transfer (Baruselli *et al*., 2000). A classical way to obtain a large pre-ovulatory follicle is to promote follicle growth under low circulating concentrations of P_4 or progestagens (Kinder *et al*., 1996). Our hypothesis

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was that treatment with low concentrations of P_4 would increase size of the pre-ovulatory follicle and of the subsequent CL. We further hypothesized that the larger CL would secrete more P_4 and have a positive effect on conception and pregnancy rates. Thus, the aim of this study was to evaluate the effects of treatments that induce formation and ovulation of a persistent follicle and subsequent formation of a larger CL (that secretes more P_4) on selection, conception, and pregnancy rates of embryo recipients.

Materials and Methods

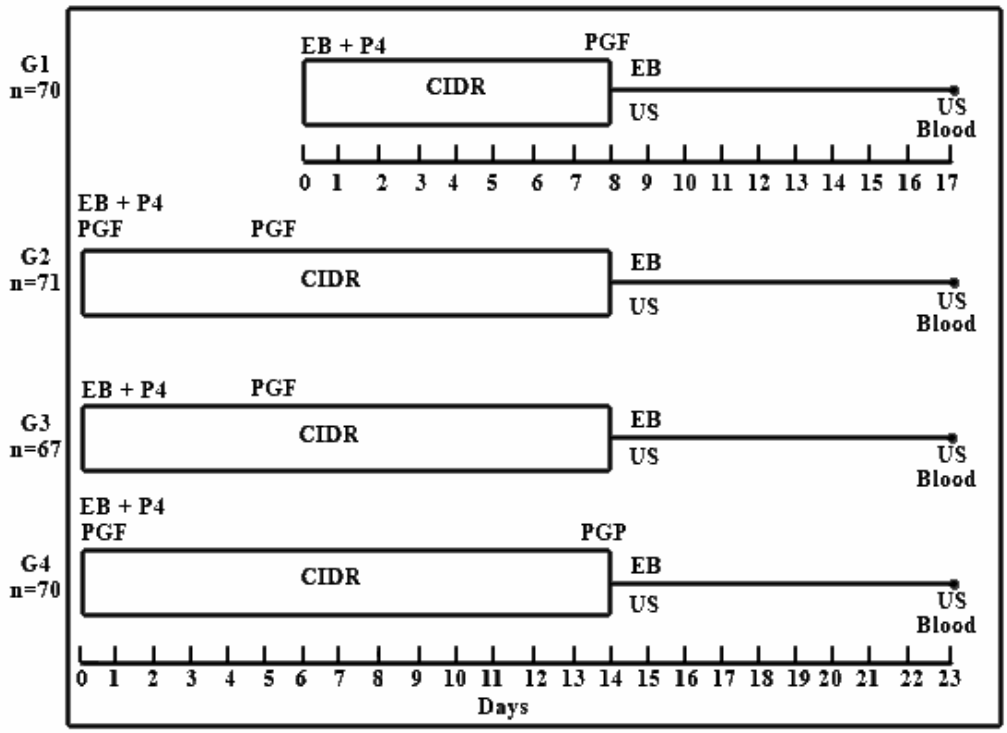
Animals

The experiment was conducted at a single farm in southeastern Brazil. Crossbred *Bos taurus* x *Bos indicus* heifers (n = 278) at unknown stages of the estrous cycle, between 22 and 30 months of age, and weighing an average of 300 kg were used. Animals were kept on native pasture with free access to mineral supplements and water.

Estrous cycle synchronization protocols

Animals were allocated randomly to one of four groups (Fig. 1). A conventional protocol for timed embryo transfer using a CIDR device for 8 days was adopted for heifers in Group 1 (G1, n = 70), which served as a negative control. On Day 0, heifers received 2 mg of estradiol benzoate (EB) and 50 mg of P_4 i.m. (Index Farmaceutica, São Paulo, Brazil), and at the same time, they received a progesterone-releasing intravaginal device containing 1.9 g of P_4 (CIDR®; Pfizer Animal Health, São Paulo, Brazil). Eight days later, at the time of CIDR® removal, heifers in G1 received 0.53 mg of cloprostenol i.m. ($PGF_{2\alpha}$; prostaglandin $F_{2\alpha}$ analogue; Ciosin®, Schering-Plough Coopers, São Paulo, Brazil) and on Day 9, received 0.5 mg EB im (Estrogin®, Farmavet, São Paulo, Brazil).

Figure 1. Schedule of the treatments. Heifers received a CIDR device for 8 and 14 d combined with $PGF_{2\alpha}$ injected at different times, for induction of persistent follicles



EB + P4: injection of 2mg estradiol benzoate + 50mg P4
PGF: injection of PGF analogue
EB: injection of 0.5mg estradiol benzoate
US: ultrasonographic examination
Blood: collection of blood samples

Heifers in Group 2 (G2, n = 71) received a CIDR® device in addition to 2 mg of EB and 50 mg of P_4 on Day 0 and an injection of $PGF_{2\alpha}$ i.m. on Day 0 and Day 5. In G2, the CIDR® remained in place for 14 days, and 0.5 mg EB was administered i.m. on Day 15. The injection of $PGF_{2\alpha}$ on Day 0 and 5 would assure that animals in this group would have follicle development occuring under low P_4 concentrations throughout the

treatment. Luteolysis would be induced in animals with a mature CL at the first $PGF_{2\alpha}$ injection (Day 0), while animals that were in metestrus and did not respond to the first $PGF_{2\alpha}$ injection could respond to the second injection on Day 5.

Heifers in Group 3 (G3, n = 67) were treated similarly to those in G2, except that only one i.m. injection of $PGF_{2\alpha}$ was administered on Day 5. Such combination of treatments should have induced luteolysis in most animals. Therefore, some animals would have high P_4 concentrations at the beginning of treatment (Day 0 to Day 5). The objective of this group was to evaluate the effect of high P_4 concentrations at the beginning of treatment on formation of persistent follicles. If efficiency of formation of persistent follicles was maintained, this simpler protocol with one less $PGF_{2\alpha}$ injection could be recommended for practical use. The treatment performed on Group 4 (G4, n = 70) was similar to that performed on G2; however, heifers in G4 received $PGF_{2\alpha}$ both at the time of CIDR® insertion (Day 0) and removal (Day 14). The rationale for this was that animals that did not respond to the first $PGF_{2\alpha}$ injection could have follicles developing under high P_4 concentrations, which would not favor growth of a persistent follicle. The objective of including this group was to test the efficacy of a protocol with reduced animal handling on persistent follicle formation. Administration of treatments in G1 through G4 was timed such that CIDR removal occurred on the same day in all groups. Eight days after the last EB injection, scheduled embryo transfer was performed in selected heifers.

Ultrasonographic examinations and embryo transfer

One day after CIDR removal and at the scheduled time of embryo transfer, ovarian status was assessed in all heifers by transrectal ultrasonography (Pierson and Ginther, 1988) using an ultrasound scanner equipped with a 5.0 MHz linear-array transducer (Model SSD 500; Aloka, Tokyo, Japan). Heifers were examined one day after CIDR® removal to evaluate the diameter of the dominant follicle (DF) and to determine CL area on the day of embryo transfer.

Only heifers with a CL greater than 1.0 cm^2 in area (Baruselli *et al*., 2003) received a frozen-thawed embryo that was transferred (by the same veterinarian) non-surgically into the uterine horn ipsilateral to the CL 8 days after EB administration. Selection rate was calculated as the number of animals that received embryo/number of animals in the treatment group.

Embryos were produced by superovulation, collected, and frozen in 1.5 mol/L glycerol. Embryos were thawed and rehydrated using the three-step rehydration method (Elsden, 1984). After rehydration, embryos were evaluated, and only those that maintained quality grades 1 or 2 (based on IETS standards) were transferred into recipient heifers. Embryos were randomized across all treatments for grade and stage of development (morula and early blastocyst).

Heifers also were examined by transrectal ultrasonography 30 days after embryo transfer to obtain pregnancy status. This evaluation was necessary to determine the conception and pregnancy rates that were defined as the number of animals pregnant/number of animals that received an embryo and the number of animals pregnant/number of animals in the treatment group, respectively.

Blood sampling and P_4 assay

Blood samples for P_4 analysis were collected from all heifers by jugular venipuncture on the day of embryo transfer. Samples were collected into heparinized tubes (Vacutainer, Becton Dickinson & Company, USA) and placed on ice immediately. Within 6 hours after collection, plasma was separated by centrifugation (Centrífuga Excelsa Baby I, Fanem®, São Paulo - Brazil) and stored at -20°C until hormone analysis. Progesterone concentration in plasma was analyzed using a commercial radioimmunoassay kit (Coat-A-Count®, Diagnostic Products Corporation, Los Angeles, CA, USA). The sensitivity (or limit of detection) of the P_4 assay was 0.07 ng/ml, and the inter- and intra-assay coefficients of variation were 6.66 and 8.98%, respectively.

Statistical analysis

Data were analyzed using the SAS System for windows (SAS Institute Inc., Cary, NC, USA, 2000). Dependent variables (diameter of dominant follicle, area of CL, and plasma progesterone concentrations) were analyzed by least squares ANOVA using the PROC GLM procedure. Data were tested previously for homogeneity of variance (F-max test) and normality of residuals (Shapiro-Wilk test) and whenever necessary, were log10- or square-root-transformed to meet the assumptions of ANOVA. The independent variable was treatment group. Means were compared pair-wise using the Tukey test. For sake of clarity, means (± SEM) are presented untransformed. Pearson's correlation coefficients were calculated using PROC CORR to evaluate association between dependent variables. Categorical dependent variables (selection, conception, and pregnancy rates) were analyzed by Chi-square using PROC FREQ. Rates for each treatment were compared pair-wise.

Results

Mean diameter of the dominant follicle one day after CIDR® withdrawal was larger in heifers that were treated with the CIDR® for 14 days than those that were treated for 8 days ($P < 0.0001$; Table 1). The CL area and plasma P_4 concentrations were greater in heifers in G2 and G3 than in those in G1 ($P < 0.0002$). Values were intermediate in G4 and not different from those of heifers in the other three treatment groups.

Recipient selection rate (heifers with CL larger than 1.0cm^2) was greater in G2 and G3 than in G1 ($P < 0.05$; Table 2). Conception rates were less ($P < 0.05$) in G2 and G3 compared to G1. Group 4 did not differ when compared with the other groups for both selection and conception rates. There were no differences found in pregnancy rates among groups.

Significant correlations were found between the mean diameter of dominant follicle one day after withdrawal of the CIDR® and area of the CL on the day of embryo transfer ($r = 0.35$; $P = 0.0004$). Furthermore, CL area and plasma P_4 concentration ($r = 0.43$; $p < 0.0001$) and diameter of dominant follicle and plasma P_4 concentration ($r = 0.39$; $P < 0.0001$) were also correlated.

Table 1. Mean diameter (± SEM) of the dominant follicle 24 hours after CIDR removal, CL area and plasma P_4 concentrations on the day of embryo transfer in heifers treated with a CIDR® for 8 or 14 d associated with $PGF_{2\alpha}$ injections in different moments.

Groups	n	DF diameter 24h after CIDR removal (mm)	CL area on the day of embryo transfer (cm^2)	Plasma P_4 on the day of embryo transfer (ng/ml)
G1 (CIDR® for 8 d + $PGF_{2\alpha}$ on day 8)	70	7.8 ± 0.4^b	1.9 ± 0.1^b	2.3 ± 0.2^b
G2 (CIDR® for 14 d + $PGF_{2\alpha}$ on Day 0 and Day 5)	71	11.1 ± 0.3^a	2.3 ± 0.1^a	3.8 ± 0.2^a
G3 (CIDR® for 14 d + $PGF_{2\alpha}$ on Day 5)	67	10.6 ± 0.4^a	2.4 ± 0.1^a	3.8 ± 0.3^a
G4 (CIDR® for 14 d + $PGF_{2\alpha}$ on Day 0 and Day 14)	70	10.6 ± 0.3^a	$2.2 \pm 0.1^{a,b}$	$3.1 \pm 0.3^{a, b}$

Means in the same column with different superscripts differ ($P < 0.05$).

Table 2. Selection, conception and pregnancy rates in bovine embryo recipients treated with CIDR® for 8 and 14 d associated with $PGF_{2\alpha}$ injections in different moments.

Groups	n	Selection rate[1]	Conception rate[2]	Pregnancy rate[3]
G1 (CIDR® for 8 d + $PGF_{2\alpha}$ on Day 8)	70	51.4% (36/70)^b	59.1% (21/36)^a	30.0% (21/70)
G2 (CIDR® for 14 d + $PGF_{2\alpha}$ on Day 0 and Day 5)	71	77.4% (55/71)^a	38.9% (21/55)^b	29.6% (21/71)
G3 (CIDR® for 14 d + $PGF_{2\alpha}$ on Day 5)	67	74.6% (50/67)^a	37.1% (19/50)^b	28.4% (19/67)
G4 (CIDR® for 14 d + $PGF_{2\alpha}$ on Day 0 and Day 14)	70	68.6% (48/70)$^{a, b}$	50.0% (24/48)a, b	34.3% (24/70)

Proportions in the same column with different superscripts differ ($P < 0.05$).
[1](number that received an embryo)/(number in treatment group).
[2](number pregnant)/(number that received an embryo).
[3](number pregnant)/(number in treatment group).

Discussion

The use of a long-term CIDR® treatment associated with $PGF_{2\alpha}$ at the beginning of treatment resulted in a larger dominant follicle after CIDR® removal. This was probably due to the stimulation of follicle growth in a low P_4 environment that was induced by the treatment with progestin in the absence of a CL (Savio *et al*., 1993; Sanchez *et al*., 1995).

When the administration of P_4 results in subluteal concentrations of circulating P_4 (1 to 2 ng/ml of plasma), LH pulses occur with greater frequency than during the mid-luteal phase (Kinder et al., 1996). This pattern in the release of LH results in the prolonged growth and maintenance of a dominant follicle and elevated plasma concentrations of 17ß-estradiol (Sirois and Fortune, 1990). The development of a persistent ovarian follicle has a detrimental effect on conception rate after artificial insemination because of an altered oviductal environment (Binelli *et al*., 1999), premature maturation of the oocyte (Mihm *et al*., 1994; Revah and Butler, 1996), or both. However, this would not necessarily be detrimental for embryo transfer in which the oocyte from a persistent follicle would not be fertilized.

In previous studies, it was demonstrated that size of the dominant follicle was associated with subsequent CL size (Vasconcelos *et al*., 2001). Furthermore, it was proposed that a large persistent

follicle could generate a large CL that would secrete more P_4 and have a positive effect on pregnancy rates in embryo transfer programs (Baruselli *et al*., 2000; Bó *et al*., 2002). However, the present study revealed the opposite result. Conception rates were decreased in heifer embryo transfer recipients with larger corpora lutea produced by the ovulation of persistent follicles, the result of long-term P_4 treatment. These results are further supported by those of previous studies in which persistent follicles were obtained by treating *Bos indicus* heifers with norgestomet ear implants for 9 days associated with $PGF_{2\alpha}$ treatment at implant insertion (Moura *et al*., 2001). These persistent follicles generated larger corpora lutea with greater capacity to secrete P_4, but the conception rate following embryo transfer was 38.4% (13/33) while the conception rate in the control group was 47.6% (10/21). Previous studies showed that the induction of persistent follicles did not influence pregnancy rates (Wehrman *et al*., 1997); however, the selection of recipients was evaluated based on detection of estrus and not by the presence of CL (as performed in the present study).

Mean diameter of the dominant follicle and CL area were positively correlated as well as CL area and plasma P_4 concentration as initially hypothesized (Baruselli *et al*., 2000; Vasconcelos *et al*., 2001). The P_4-secreting capacity of the CL is essential for maintenance of pregnancy (Hansel and Convey, 1983; Geisert *et al*., 1992) as P_4 is an important regulator of luteolytic mechanisms (Silvia *et al*., 1991; Silvia, 1999). One possible mechanism through which P_4 stimulates maintenance of pregnancy may be through stimulation of interferon-τ, the main regulator of pregnancy recognition, by the growing conceptus (Mann *et al*., 1999; Binelli *et al*., 2001) or blocking $PGF_{2\alpha}$ production (Mann and Lamming, 1995). It was, therefore, expected that animals with a larger CL and a greater capacity to secrete P_4 would have a correlated increase in conception rates. However, groups that had greater mean area of the CL and greater plasma P_4 concentration (G2 and G3) had a lower conception rate when compared to G1.

Low conception rates found in groups G2 and G3 could be attributed to several factors, such as low concentrations of progesterone and high concentrations of estradiol-17β during the cycle previous to embryo transfer. Persistence of ovarian follicles and associated elevated concentrations of estradiol-17β for prolonged periods preceding ovulation may alter uterine function in the subsequent cycle. An inadequate or adverse intrauterine environment may increase embryonic losses and decrease pregnancy rates (Wehrman *et al*., 1997). For example, oxytocin is an acute stimulus for production of $PGF_{2\alpha}$ from the bovine uterus (Bogaki *et al*., 2002), and low P_4 concentrations during the luteal phase of one estrous cycle, similar to those that promote the development of a persistent ovarian follicle (Sirois and Fortune, 1990), markedly increase PGFM concentrations in response to exogenous oxytocin in the subsequent cycle (Shaham-Albalancy *et al*., 2001). A greater uterine $PGF_{2\alpha}$ secretion at the time of pregnancy recognition could lead subsequently to early luteal regression and termination of pregnancy. Mechanisms by which a lower P_4 concentration in one estrous cycle affects uterine function in the late luteal phase of the subsequent estrous cycle have not been elucidated but may be related to alterations in endometrial oxytocin receptor concentration or function (Wathes and Lamming, 1995). It is also possible that high estradiol concentrations, found during persistent follicle development, combined with lower P_4 concentrations may have enhanced the stimulatory effect on the endometrial oxytocin receptor and, subsequently, increased $PGF_{2\alpha}$ secretion (Beard and Lamming, 1994). Moreover, Shaham-Albalancy et al. (1997) reported that vaginal P_4 supplementation has a delayed effect on endometrial morphology, and that low plasma P_4 levels obtained by partial luteolysis did not have any effect on the endometrium, unlike the low plasma P_4 induced by using a vaginal device.

In conclusion, a long-term treatment with a P_4-releasing intravaginal device (CIDR®) in the absence of a CL induced the formation of a persistent follicle, resulting in a larger CL that provided greater P_4 concentrations than a CL that developed following the conventional 8-day CIDR treatment. However, the induction of a persistent follicle reduced conception rates following embryo transfer.

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