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Follicular dynamics and pregnancy rates in *Bos taurus* x *Bos indicus* embryo transfer recipients treated to increase plasma progesterone concentrations

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Abstract

The aim of this study was to evaluate the effects of GnRH, LH, hCG or exogenous progesterone administration on plasma progesterone concentrations and pregnancy rates following embryo transfer in *Bos taurus* x *Bos indicus* cross-bred heifers. In Experiment 1, animals with body condition scores ≥3.5 (1 to 5 scale) were synchronized with two injections of a prostaglandin F2α analog 13 days apart. Heifers detected in estrus (day 0; n = 37) were randomly assigned on day 7 to receive one of five treatments: Control (2 ml saline im; n = 6), GnRH (10 μg Buserelin im; n = 8), hCG (1500 IU Chorulon im; n = 8), LH (25 mg pLH im; n = 7) or a CIDR-B device for 13 days (n = 8). Ovarian ultrasonography was performed daily from day 6 until the subsequent estrus. Heifers in the GnRH, hCG and LH groups were evaluated every 12 h between days 7 and 9 to confirm ovulation of the first-wave dominant follicle. Blood samples were collected daily for determination of P4 levels. Estrus detection was performed daily with the aid of androgenized cows. Ovulation rate for the first wave dominant follicle was 100% for heifers treated with GnRH, hCG and LH. Between days 13 and 17, the mean diameter of original CLs, diameter of accessory CLs and P4 concentrations were greater in heifers treated with hCG than in heifers in all other groups ($P < 0.05$). Duration of the luteal phase (number of days with a P4 concentration ≥1.0 ng/ml) was similar in hCG (14.3 ± 0.6), LH (13.4 ± 0.6), GnRH (13.4 ± 0.4), CIDR-B (14.5 ± 0.2) and Control (12.8 ± 0.5) groups. In Experiment 2, animals were kept on a grazing regimen at commercial farms in Brazil and were synchronized with one injection of a prostaglandin F2α analog. The same hormonal treatments as in Experiment 1 were given on day 7 after estrus at the time of transfer of frozen/thawed embryos to *Bos taurus x Bos indicus* recipients (n = 485). Pregnancy rates were higher in GnRH- (53.5%; 53/99) and hCG- (51.0%; 49/96) treated heifers ($P < 0.05$) than in control heifers (28.6%; 28/98), but were similar to heifers treated with CIDR devices (41.1%; 39/95) and LH (45.4%; 44/97). It was concluded that the improvement in conception rates in hCG treated *Bos taurus x Bos indicus* cross-bred heifers receiving frozen/thawed embryos were due to both P4-dependent and P4-independent mechanisms.

Keywords: cattle, corpus luteum, embryo transfer, pregnancy, progesterone.

Introduction

Maintenance of an elevated plasma progesterone (P4) concentration has been associated with pregnancy recognition in cattle (Mann and Lamming, 1995). Low circulating progesterone levels may decrease embryo development, thus, negatively affecting maternal recognition of pregnancy (Mann *et al*., 1999). Conversely, elevated plasma concentrations of P4 stimulate conceptus growth and its capacity of secreting interferon-τ (Mann *et al*., 1999; Binelli *et al*., 2001) and, consequently, conception rates (Macmillan *et al*., 1994; Fuentes and De La Fuentes, 1997; Baruselli *et al*., 2000; Santos *et al*., 2001).

Several strategies have been proposed to increase plasma P4 concentrations during the critical period (days 15 to 19 after insemination; Binelli *et al*., 2001; Thatcher *et al*., 2002). Such strategies include inducing the formation of an accessory corpus luteum (Schimtt *et al*., 1996a, b; Santos *et al*., 2001) and P4 supplementation during the critical period (Robinson *et al*., 1989; Macmillan and Thatcher, 1991, Tribulo *et al*., 1997). It is possible to induce the formation of an accessory CL by injecting heifers with GnRH, LH or hCG during the early luteal phase to induce ovulation of the first-wave dominant follicle (Rajamahendran and Sianangama, 1992; Schimtt *et al*., 1996a; Ambrose *et al*., 1998; Diaz *et al*., 1998; Sianangama and Rajamahendran, 1996; Santos *et al*., 2001).

Most studies regarding supplementation of P4 after AI were conducted in dairy herds (Peters, 1996; Thatcher *et al*., 2002). The use of these strategies in cross-bred (*Bos taurus* x *Bos indicus*) beef heifers has not been reported.

The aim of this study was to evaluate the effects of GnRH, LH, hCG or progesterone administration at the time of direct embryo transfer on follicular development, plasma P4 concentrations and pregnancy rates in *Bos taurus x Bos indicus* cross-bred heifers. The hypotheses tested were that (1) treatments

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would effectively increase plasma progesterone concentrations and (2) pregnancy rates.

Materials and Methods

Experiment 1

Animals

Sixty two (n = 62) *Bos taurus x Bos indicus* F1 heifers, 18 to 32 months old and with a body condition score ≥3.5 (1 to 5 scale) were used. Heifers were kept on pasture and received water and mineral supplementation *ad libitum*. The experiment was conducted during the months of November and December (spring season in the Southern Hemisphere).

Estrus synchronization and treatments

Heifers were pre-synchronized with two im injections of a prostaglandin F2α analog (0.15 mg d-cloprostenol, Preloban, Hoechst) 13 days apart. After the second PGF2α injection, heat detection was performed three times a day for five days.

On day 7 of the estrous cycle (day 0 = day of estrus), heifers were assigned randomly to receive 2 ml saline im (Control group; n = 6), 10 μg Buserelin (Conceptal, Hoechst; GnRH Group; n = 8), 1500 IU hCG im (Chorulon, Intervet; hCG Group; n = 8), 25 mg pLH im (Lutropin-V, Vetrepharm; LH Group; n = 7) or an intravaginal P4-releasing device containing 1.9 g progesterone (CIDR-B, InterAg; P4 Group; n = 8) for 13 days.

Ultrasonography and monitoring of estrous behavior

Ovaries were examined daily by transrectal ultrasonography (Aloka SSD-500, linear 7.5 MHz probe). Heifers from the GnRH, hCG and LH groups were also examined every 12 h from days 7 to 9 to detect ovulation of the first wave follicle (disappearance of a dominant follicle followed by observation of an accessory CL). Diameters of follicles and CLs (original and accessory) were recorded daily. Heifers were checked for heat twice daily with the aid of an androgenized cow equipped with a chin-ball marker.

Blood sampling and hormone analysis

Jugular blood samples were collected daily from day 6 to the end of estrous cycle, centrifuged (1500 x g at 4°C for 30 min) and the plasma was stored at -20°C. Plasma P4 concentrations were analyzed using a commercial radioimmunoassay Kit (Coat-A-Count®, Diagnostic Products Corporation, Los Angeles, USA).

Experiment 2

Farms and animals

This experiment was conducted on eleven commercial farms in south-central Brazil. Embryo transfer techniques were used routinely on all farms. Cross-bred *Bos taurus* x *Bos indicus* F1 heifers (n = 485) were kept on grazing conditions and received water and mineral supplementation *ad libitum*. Embryo transfers and treatments were performed by an experienced veterinarian according to instructions from the investigators. The experiment was conducted from January to May (mid-summer to mid-fall in the Southern Hemisphere).

Estrus synchronization, treatments, embryo handling and transfer

Heifers received an injection of a prostaglandin F2α analog (0.15 mg d-cloprostenol, Preloban, Hoechst) and were heat detected twice a day. Six or 7 days after estrus, recipients were evaluated by transrectal ultrasonography. Heifers presenting a CL >18 mm in diameter were distributed to receive one of the following treatments at the time of embryo transfer: 2 ml saline im (Control group; n = 98), 10 μg Buserelin im (Conceptal, Hoechst; GnRH Group; n = 99), 1500 IU hCG im (Chorulon, Intervet; hCG Group; n = 96), 25 mg pLH im (Lutropin-V, Vetrepharm; LH Group; n = 97) or supplemental progesterone through a intravaginal progesterone-releasing device containing 1.9 g progesterone (CIDR-B, InterAg; P4 Group; n = 95) for 13 days.

In vivo produced embryos were obtained from superovulated *Bos indicus* cows by uterine flushing 7 days after insemination. Recovered embryos were kept in cell culture dishes containing TQC® holding solution (AB Technology, Nutricell, Brazil) during embryo evaluation. Collected embryos were classified under a stereomicroscope (50X) according to their stage of development (morula, early blatocyst or blastocyst) and grade quality (1 or 2; Stringfellow and Seidel, 1998). After classification, embryos were frozen in TQC® ethylene glycol (AB Technology, Nutricell, Brazil) using an automatic freezing machine (TK 2000®, program P1-01, BOV/E/O1; TK and Nutricell, Brazil). When the procedure was completed, the straws were transferred into liquid nitrogen (-196°C), where they were stored until needed for embryo transfer. Frozen/thawed embryos were directly transferred by non-surgical technique to the uterine horn ipsilateral to the original CL. Approximately 40 to 60 days after estrus (33-53 days after embryo transfer), heifers were submitted to pregnancy diagnosis by ultrasonography.

Statistical analyses

Statistical analyses were performed using the SAS System for Windows (SAS Institute Inc., Cary, NC, USA, 1999-2000). In Experiment 1, discrete

dependent variables [ovulation of the first wave dominant follicle, number of follicular waves, length of the luteal phase (number of days between the day of ovulation and the day when decreasing progesterone concentrations reached <1.0 ng/ml), interovulatory interval (number of days between ovulation of the first wave dominant follicle and the subsequent, natural ovulation), length of the estrous cycle, diameter of follicles, diameter of CLs and concentration of progesterone in discrete time points] were analyzed by one-way ANOVA using PROC GLM. The independent variable was treatment. Continuous dependent variables (diameter of original and accessory CLs, concentrations of progesterone) were analyzed by split-plot ANOVA using PROC GLM and PROC MIXED. Independent variables were treatment, animal nested within treatment, day and interactions. Mean comparisons were performed by the Tukey test ($P < 0.05$). In Experiment 2, the proportion of heifers becoming pregnant after embryo transfer was analyzed by logistic regression using PROC GLIMMIX. Independent variables were farm, treatment and interaction.

Results

Experiment 1

Two heifers from the hCG group were excluded from the analysis because they presented an interovulatory interval >30 days. Variables regarding duration of the estrous cycle and follicular function are presented in Table 1. Treatments did not affect interovulatory interval or length of the estrous cycle. Rate of ovulation of the first-wave dominant follicle was 100% for heifers in the GnRH, hCG and LH groups. Number of follicular waves during the synchronized estrous cycle was similar among groups. Maximum diameter of the second wave dominant follicle of heifers that presented a third follicular wave was similar among groups.

Table 1. Follicular dynamics (mean ± SEM) during the estrous cycle in *Bos taurus* x *Bos indicus* heifers according to treatment on day 7 of the estrous cycle.

Treatment		Control	GnRH	hCG	LH	P4 Device	P-value
Number of animals		6	7	6	6	8	
Number of waves (%)	Two waves	33.3 (2/6)	___	33.3 (2/6)	___	37.5 (3/8)	
	Three waves	66.7 (4/6)	100 (7/7)	66.7 (4/6)	100 (6/6)	62.5 (5/8)	
Duration of luteal phase (days)		12.8 ± 0.5	13.4 ± 0.4	14.3 ± 0.6	13.4 ± 0.6	14.5 ± 0.2	0.10
Duration of interovulatory interval (days)		___	15.2 ± 0.7	16.8 ± 0.7	15.7 ± 1.0	___	0.34
Duration of estrous cycle (days)		21.2 ± 0.6	21.7 ± 0.6	23.2 ± 0.6	22.3 ± 1.2	23.1 ± 0.5	0.19
Diameter of dominant follicle on day 7 (mm)		11.7 ± 0.2^{ab}	10.9 ± 0.5^{b}	11.2 ± 0.5^{ab}	12.5 ± 0.6^{a}	11.3 ± 0.3^{ab}	<0.05
Largest diameter of ovulatory first-wave follicle (mm)		___	11.3 ± 0.3^{a}	11.5 ± 0.4^{ab}	12.6 ± 0.5^{b}	___	<0.05
Diameter of the largest follicle of the second wave (mm)		10.0 ± 0.8	10.0 ± 0.3	10.9 ± 0.7	10.0 ± 0.5	9.6 ± 0.4	0.50

Variables related to luteal function are presented in Table 2. Treatments did not affect duration of the luteal phase. Diameter of the corpora lutea on day 7 of the estrous cycle was larger for heifers in the Control group and smaller for heifers in the LH group ($P < 0.05$). However, concentrations of plasma progesterone on day 7 were similar among groups. Diameter of corpora lutea ($P < 0.01$) and accessory corpora lutea between days 13 and 17 were larger in the hCG group when compared to the other groups (Fig. 1 and 2).

Average progesterone concentrations were higher in heifers treated with hCG. However, progesterone concentrations in the GnRH, LH and P4 groups did not differ from the Control group ($P > 0.05$; Table 2; Fig. 3).

Experiment 2

There was no significant effect of farm ($P = 0.15$) or interaction between farm and treatment ($P = 0.95$). However there was a significant effect of treatment on conception rates of embryo recipients ($P < 0.005$). Conception rates of GnRH- and hCG-treated heifers were significantly higher ($P < 0.001$) than those of control heifers. No differences were found when pregnancy rates for the LH and P4 treatments when compared with the other groups ($P > 0.05$; Table 3).

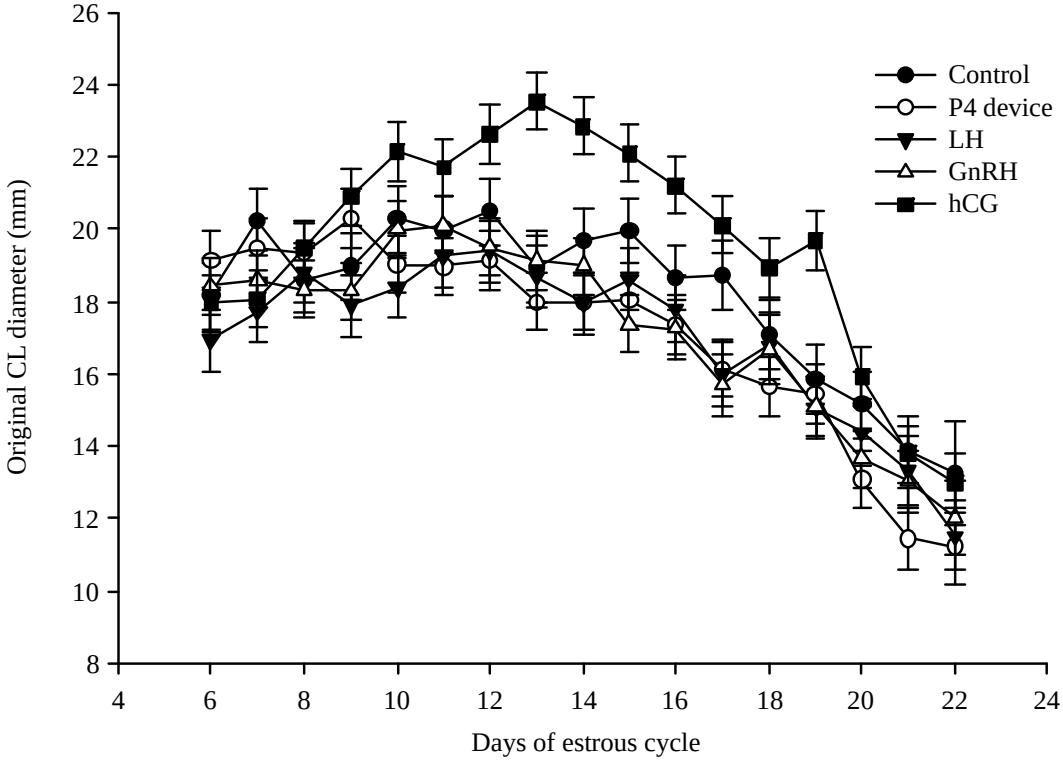

Figure 1. Corpora lutea diameter (mean ± SEM) in *Bos taurus* x *Bos indicus* heifers according to the treatment on day 7 of the estrous cycle.

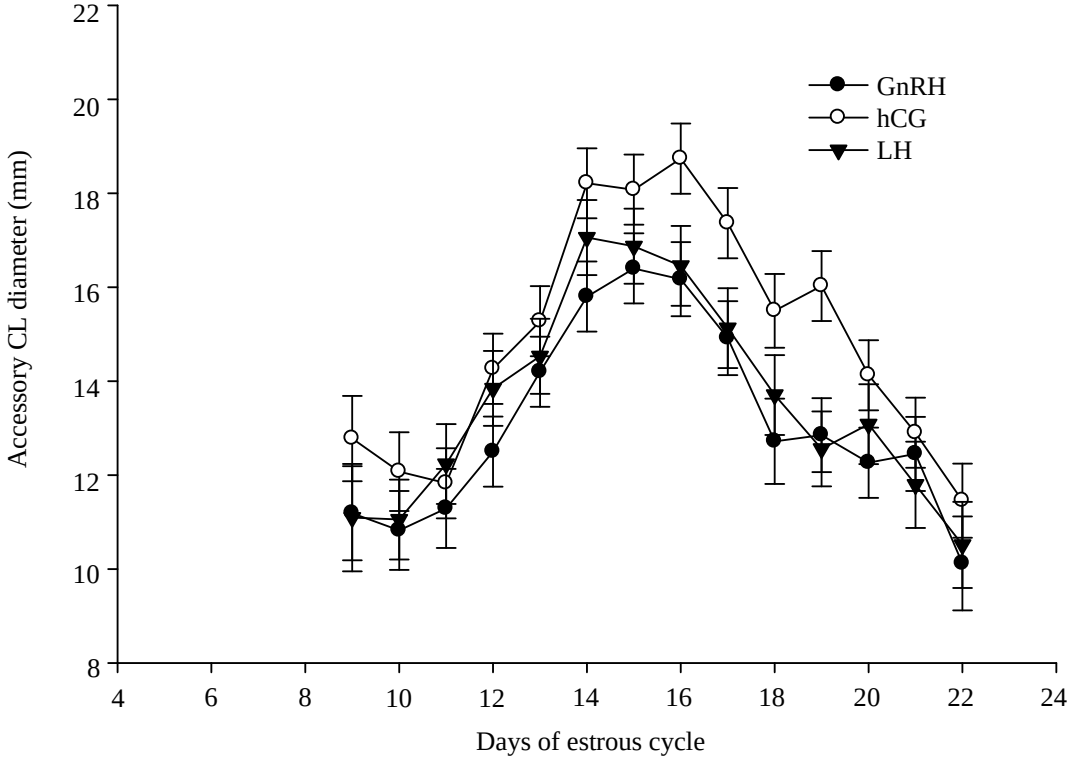

Figure 2. Accessory corpora lutea diameter (mean ± SEM) after induction of ovulation of first-wave dominant follicle in *Bos taurus* x *Bos indicus* heifers according to the treatment on day 7 of the estrous cycle.

Table 2. Diameter of corpora lutea, accessory corpora lutea and plasma progesterone concentration at day 7 and between days 13 and 17 of estrous cycle (mean ± SEM) in *Bos taurus* x *Bos indicus* heifers according to the treatment on day 7 of the estrous cycle.

Treatment	Control	GnRH	hCG	LH	P4 Device	P-value
Number of animals	6	7	6	6	8	
CL diameter at day 7 (mm)	20.2 ± 0.6^{a}	18.6 ± 0.7^{ab}	18.1 ± 0.7^{ab}	17.7 ± 0.8^{b}	19.5 ± 0.8^{ab}	P < 0.05
Plasma progesterone concentration at day 7 (ng/ml)	2.8 ± 0.7	2.5 ± 0.4	2.7 ± 0.3	3.3 ± 0.6	3.0 ± 1.0	NS
CL diameter at day 13 to 17 (mm)	19.1 ± 0.3^{a}	17.7 ± 0.3^{b}	22.2 ± 0.5^{c}	17.7 ± 0.3^{b}	17.5 ± 0.3^{b}	P < 0.05
Mean diameter of accessory CL between days 13 and 17 (mm)	___	15.9 ± 0.3^{a}	17.3 ± 0.5^{b}	16.1 ± 0.3^{a}	___	P < 0.05
Plasma progesterone concentration between days 13 and 17 (ng/ml)	4.8 ± 0.3^{b}	4.8 ± 0.3^{b}	8.4 ± 0.6^{a}	6.1 ± 0.5^{b}	4.8 ± 0.3^{b}	P < 0.01

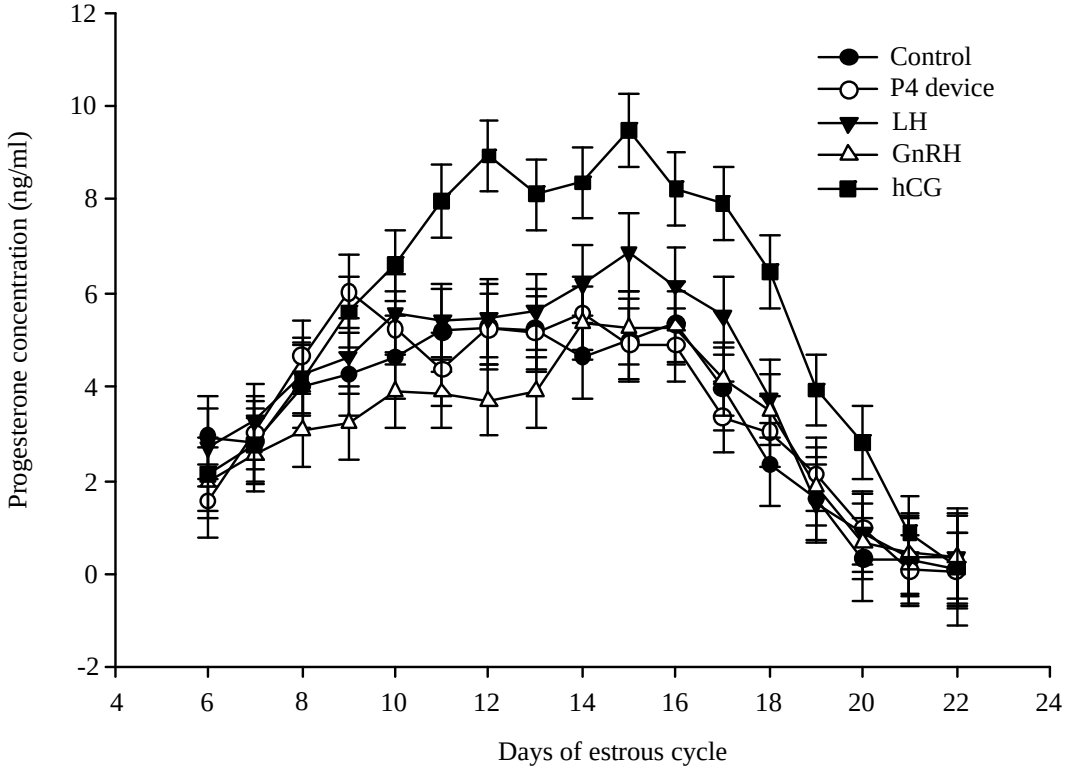

Figure 3. Plasma progesterone concentration (mean ± SEM) in *Bos taurus taurus* x *Bos taurus indicus* heifers according to the treatment on day 7 of the estrous cycle.

Table 3. Conception rates in *Bos taurus* x *Bos indicus* heifers relative to treatment at the moment of embryo transfer.

Treatment	Control	GnRH	hCG	LH	P4 Device
Conception rates, % (pregnant/transferred)	28.6% $(28/98)^{a}$	53.5% $(53/99)^{b}$	51.0% $(49/96)^{b}$	45.4% $(44/97)^{ab}$	41.1% $(39/95)^{ab}$

Discussion

In the present experiment, it was possible to increase pregnancy per embryo transfer through the strategic administration of luteotropic hormones at the time of transfer. Administration of treatments was operationally simple, and, except for the P4 group, did not alter the embryo transfer routine. Increases in pregnancy per embryo transfer in response to the treatments may have been due to (1) supplemental

progesterone provided by the accessory CLs originated by ovulation of the first-wave dominant follicles (hCG, GnRH and LH groups) and by the progesterone releasing device (P4 group) and (2) changes in follicular dynamics.

In Experiment 1, treatment with GnRH, LH and hCG on day 7 induced ovulation of the first-wave dominant follicle and formation of an accessory CL in 100% of heifers. Similar results were reported for dairy cows and heifers on day 5 of the estrous cycle (Schmitt *et al*., 1996b; Diaz *et al*., 1998; Santos *et al*., 2001) and for dairy cows on day 7 of the estrous cycle (Rajamahendran and Sianangama, 1992; Sianangama and Rajamahendran, 1996; Vasconcelos *et al*., 1999). Increases in pregnancy per AI have been reported in response to the supplemental progesterone provided by an accessory CL (Rensen and Roussel, 1982; Niemann *et al*., 1985; Macmillan *et al.*, 1986; Stubbings and Walton, 1986; Rajamahendran and Sianangama, 1992; Sianangama and Rajamahendran, 1992; Sheldon and Dobson, 1993; Drew and Peters, 1994; Peters, 1996; Baruselli *et al*., 2000; Binelli *et al*., 2001; Santos *et al*., 2001; Thatcher *et al*., 2001, 2002). These collective pregnancy responses are in accordance with the data reported for Experiment 2 (hCG and GnRH groups). Higher progesterone concentrations during early pregnancy may stimulate endometrial secretions (Geisert *et al*., 1992) that favor embryo development, stimulate higher interferon-τ secretion (Mann and Lamming, 2001) and consequently increase embryo capacity to block luteolytic mechanisms (Lukaszewska and Hansel, 1980; Lamming *et al*., 1989; Mann and Lamming, 1995; Mann *et al.*, 1999). Freezing of embryos reduces conception rates 8.5 to 14% in comparison to fresh embryos, probably due to damage to embryonic cells caused by the freezing/thawing process (Hasler, 2001; Spell *et al.*, 2001). Such damage could reduce embryonic growth and promote lower interferon-τ secretion. It is possible that higher progesterone concentrations could improve anti-luteolytic mechanisms in frozen/thawed embryos. Treatment with hCG induced larger original and accessory CLs and increased progesterone concentration between days 13 and 17 of the estrous cycle. This effect may be due to the longer half-life of hCG in blood and a slower turnover of the hCG-LH receptor complex on the surface of granulosa cells, which could increase the number of large luteal cells, responsible for approximately 80% of progesterone synthesis (Schmitt, *et al*., 1996a, b).

In Experiment 1, higher plasma progesterone concentrations were observed for hCG-treated compared to GnRH-treated heifers. It was expected that pregnancy per embryo transfer also would be greater for heifers in the hCG group. However, pregnancy per embryo transfer was similar between hCG and GnRH groups in Experiment 2. It is possible that treatment with GnRH increased plasma progesterone concentrations in field conditions as discussed for Schmitt *et al.* (1996a, b). This could also explain the numerical increases in pregnancy per embryo transfer for the LH group compared to the Control group (16.8%).

Pregnancy per embryo transfer was numerically higher for heifers in the P4 group compared to the Control group. This was similar to data reported by Macmillan *et al.* (1994), in which embryo recipients treated with an intravaginal progesterone device (CIDR) had a 12.8% higher pregnancy rate per embryo transfer. However, Burke *et al.* (1999) did not observe a difference in plasma progesterone concentrations between cows that received and did not receive the CIDR device. The authors suggested that an increment in clearance and/or a reduction in luteal production could explain this observation.

The role of progesterone in fertility and pregnancy maintenance in embryo recipients is controversial (Hasler *et al*., 1980; Payas *et al.*, 1989; Spell *et al*., 2001). Compared to controls, neither Ellington *et al.* (1990) nor Tríbulo *et al.* (1997) observed changes in pregnancy per embryo transfer after treatment of recipients with a CIDR device (69.4 *vs.* 62.5%) or with GnRH (66 *vs*. 73%). It is possible that strategies to increase plasma progesterone during the luteal phase may not affect pregnancy per embryo transfer when basal fertility is greater than 60%.

Pregnancy per embryo transfer in the Control group (28.6%) was lower than observed in other studies (Hasler *et al*., 1980, 1987; Hasler, 2001; Spell *et al*., 2001), which reported rates greater than 50% using *Bos taurus* recipients with good nutritional management. A study conducted in Brazil reported pregnancy per embryo transfer to be 29.4% after fixed timed embryo transfer in cross-bred recipients using frozen/thawed embryos (Baruselli *et al*., 2001).

It is possible that treatments used in the present study provided additional benefit to the *Bos taurus* x *Bos indicus* cattle. It has been described in several studies that *Bos indicus* cattle seem to have smaller maximum diameters of the dominant follicle and the CL than *Bos taurus* cattle (Rhodes *et al*., 1995; Figueiredo *et al*., 1997; Bó *et al*., 2003). Additionally, progesterone content of the CL was lower in *Bos indicus* than in *Bos taurus* cows (Segerson *et al*., 1984; Figueiredo *et al*., 1997). Perhaps because of the natural lower progesterone-producing potential in those animals, positive effects of progesterone supplementation could be noted more clearly in cross-bred *Bos taurus x Bos indicus* than *Bos taurus* cattle.

Maximum diameter of the second-wave dominant follicle was similar among groups. These results vary from those observed by other investigators (Bergfelt *et al*., 1991; Adams *et al*., 1992; Fortune, 1993). They reported a reduction of the maximum diameter of the dominant follicle associated with high concentrations of plasma progesterone (Roberson *et al*.,

1989). In the present experiment, post-treatment concentrations of progesterone were similar (except for the hCG group); thus, similar maximum size of second wave follicles was probably due to similar concentrations of progesterone during the growth of these follicles.

Mechanisms alternative to increased progesterone concentrations may explain the differences in pregnancy rates between control animals and animals that were stimulated to ovulate the first wave dominant follicle. It is possible that advanced emergence of the second wave of follicle growth, due to ovulation of the first wave follicle, may have programmed follicle growth so that there was no dominant, estrogen-active follicle at the beginning of the critical period for maternal recognition of pregnancy (days 15 to 19 after estrus). This physiological context is less stimulatory to the onset of luteolysis. A delayed luteolytic stimulus allows for more time for the conceptus to grow and for conceptus-induced anti-luteolytic mechanisms to take place. This sequence of events may increase pregnancy per embryo transfer.

There was no effect of day of the estrous cycle on conception rates when transfers were performed, stage of embryo development, quality of embryo or technician which performed embryo transfer, as reported by others (Spell *et al*., 2001). There was also no effect of farm on conception rates, which is in variance to data from Hasler (2001). It must be noted that in the present experiment, due to variability of experimental numbers in each farm, statistical analyses had limited power to detect potential farm effects, which were indeed detected numerically.

It was concluded that treatment with GnRH, LH or hCG on day 7 of the estrous cycle efficiently induced ovulation of the first-wave dominant follicle and formation of accessory corpora lutea in *Bos taurus x Bos indicus* embryo recipient heifers. The hCG treatment induced a significant increase in plasma progesterone concentrations. These treatments improved conception rates in *Bos taurus x Bos indicus* cross-bred heifers receiving frozen/thawed embryos. Further experiments are needed to evaluate whether these treatments would increase pregnancy rates in recipients with conception rates equal to or greater than 50%.

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