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Effect of circulating progesterone on *in vitro* developmental competence of bovine oocytes

L.F.M. Pfeifer[1,6], R. Sartori[2], I. Pivato[3], R. Rumpf[4], G.P. Nogueira[5], E.G. Xavier[1], N.J.L. Dionello[1], M.N. Corrêa[1]

[1]Federal University of Pelotas, 96010-900 Pelotas, RS, Brazil.
[2]ESALQ, University of São Paulo (USP), 13418-900 Piracicaba, SP, Brazil.
[3]Company of Integrated Agricultural Development of Santa Catarina (CIDASC), 89130-000, Indaial, SC, Brazil.
[4]Embrapa Genetic Resources and Biotechnology, 70770-900 Brasília, DF, Brazil.
[5]São Paulo State University (UNESP), 16015-050, Araçatuba, SP, Brazil.

Abstract

This study evaluated the effects of systemic progesterone concentration on oocyte quality and *in vitro* embryo production. Oocytes were retrieved from 15 crossbred cows (*Bos taurus x Bos indicus*). These cows were randomly allocated into three groups to provide low; high, or very low (LP4, HP4 and VLP4, respectively) plasma progesterone concentrations and received either a previously used CIDR, two new CIDR devices, or no progesterone treatment (Day 0). The CIDR devices were replaced every 8 days along with 150 μg of D-cloprostenol injections. The ovum pick-up (OPU) procedure was performed every 4 days from Day 4 to 24. Simultaneous to OPU procedure, plasma was collected to measure progesterone and on Day 18, serial blood samples were collected to assess the pattern of LH release. Hormone concentrations were analyzed by ANOVA and the binomial variables were analyzed by Chi-square. Plasma progesterone concentration was higher in the HP4, intermediate in the LP4, and lower in the VLP4 group (3.6, 1.6, and 0.5 ng/ml; $P < 0.05$). Plasma LH was higher in the LP4, intermediary in the VLP4, and lower in the HP4 group (1.6, 1.0, and 0.8 ng/ml). A greater percentage of viable oocytes (grades I to III) was retrieved from LP4 (79.4%; 131/165) than from the HP4 (68.4%; 119/174) group ($P = 0.07$); the VLP4 group did not differ from the others (72.3%; 60/83). Furthermore, the blastocyst production and blastocyst rate was higher in LP4 (1.3 ± 0.4; 28.2%), than in HP4 (0.8 ± 0.4; 16.0%) or the VLP4 (0.4 ± 0.4; 15.0%) group ($P = 0.06$ and 0.03 for blastocyst production and rate, respectively). In conclusion, intermediate plasma P4 concentration that results in higher circulating LH in cows may improve *in vitro* embryo production.

Keywords: cattle, *in vitro* embryo production, oocyte, progesterone.

Introduction

The period of follicular growth and dominance prior to ovulation is critical for the development potential of the bovine oocyte (Blondin *et al.*, 1997; Chaubal *et al.*, 2007). The developmental competence of the oocytes is acquired gradually and increases with follicular development (Machatkova *et al.*, 2004); furthermore, *in vitro* development is related to follicle size at the time of oocyte recovery. Oocytes isolated from follicles with a diameter ≥6 mm have higher competence than oocytes from follicles <4 mm (Lequarre *et al.*, 2005). In addition, oocytes collected before follicle selection have greater *in vitro* development capacity (Hendriksen *et al.*, 2004) and development to the blastocyst stage was greater when oocytes were obtained during follicular growth, as compared with follicular dominance (Hagemann, 1999; Hagemann *et al.*, 1999; Machatkova *et al.*, 2004). Systemic progesterone concentrations also affect oocyte quality (Hagemann *et al.*, 1999; Salamone *et al.*, 1999; Hendriksen *et al.*, 2004). Progesterone appeared to enhance oocyte competence (Leibfried-Rutledge *et al.*, 1987; Blondin and Sirard, 1995), since oocytes collected in late diestrus were more competent than oocytes collected in early luteal or follicular phase (Machatkova *et al.*, 1996, 2004). Although several experiments have been done to determine how progesterone concentrations and duration of progesterone treatment affect fertility after a synchronized breeding (Roche, 1974; Austin *et al.*, 1999; Shaham-Albalancy *et al.*, 2000), there are few studies addressing the effects of progesterone on oocyte competence. Evidently, the progesterone environment and oocyte collection before the selection of the dominant follicle positively affect the quality of oocytes and the IVP results. Knowing that, a basic question still needs to be answered: to what extent do 5progesterone concentrations affect oocyte quality and embryo development. In this context, the use of ovum pick-up (OPU) guided by transvaginal ultrasound that facilitates retrieval and subsequent utilization of oocytes which have developed *in vivo* under various hormonal environments is an interesting model of study. The objective of the present study was to evaluate the effects of systemic P4 concentration on the number of follicles, the amount and quality of oocytes, and *in vitro* production of embryos.

[6]Corresponding author. lfmpfeifer@terra.com.br
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Materials and Methods

Cattle, treatments and ultrasonographic examinations

This study was conducted with 15 crossbred cows (*Bos taurus x Bos indicus*) 3 to 7 years old, weighing 465 to 565 Kg, body condition score between 3 and 3.5 (BCS, 0 = thin and 5 = obese; Lowman *et al.*, 1976), that were housed at the Embrapa research farm (Brasília, DF, Brazil). The animals were maintained on pasture (*Brachiaria brizanta*), receiving a mineral supplementation with *ad libitum* access to water.

Prior to the start of the experiment, ovarian function was assessed twice (10 days apart) with transrectal ultrasonography (Aloka SSD 500 with 5 MHz linear-array transducer, Aloka Co., Tokyo, Japan); all cows were considered cycling. The experimental design and treatment schedule is shown (Fig. 1). After the second ultrasound examination, all cows received a CIDR (CIDR-B®, 1.9 g, InterAg- Hamilton, New Zealand, marketed by Pfizer, New York, NY, USA) for 8 days. Two days before CIDR removal, all cows received 150 µg of D-cloprostenol im (PGF2α; Prostaglandin Tortuga®, Tortuga, Santo Amaro, SP, Brazil) to cause regression of luteal tissue, thereby avoiding the influence of endogenous progesterone.

On the day of CIDR removal (Day 0), follicular aspiration (FA) of all follicles ≥5 mm was done on all cows, as described (Martinez *et al.*, 2000), to synchronize emergence of a new follicular wave 1 day later (Martinez *et al.*, 2000). After FA, cows were randomly allocated into three treatment groups (5 cows/group) and subjected to ovum pick-up (OPU) every 4 days from Day 4 to Day 24 (Fig. 1). Therefore, these cows were randomly allocated to treatments to provide low (LP4; 1 to 2 ng/ml), high (HP4; >3 ng/ml), or very low (VLP4; <1 ng/ml) progesterone (P4) concentrations, simulating subluteal, luteal and very low P4 concentrations, respectively. Cows in these three groups received a previously used CIDR, two new CIDR devices, or no P4 treatment. The CIDR devices were replaced every 8 days along with 150 µg of D-cloprostenol im injection.

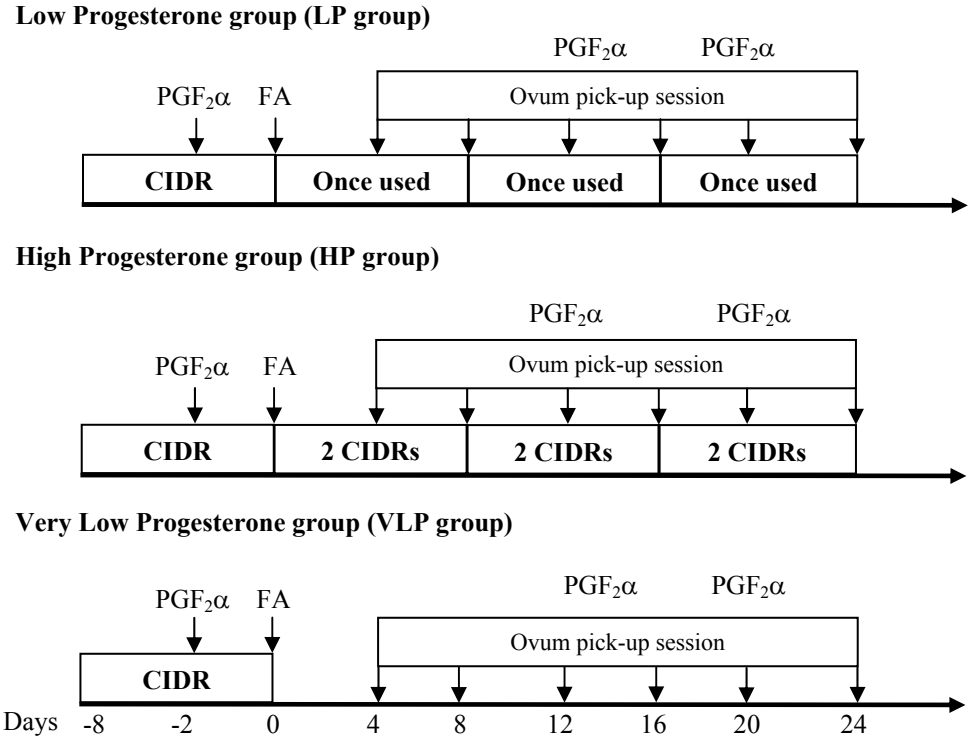

Figure 1. Time-line for treatment of the Low (LP4), High (HP4), and Very low (VLP4) circulating progesterone (P4) groups. All cows received a new CIDR at the beginning of the experiment. Two days before CIDR removal (Day -2), all the cows received 150 µg of D-cloprostenol (PGF; Prostaglandin Tortuga®, Tortuga, Santo Amaro, SP, Brazil), im. On Day 0 the CIDR (CIDR- B®, 1.9 g, InterAg- Hamilton, New Zealand) was removed, follicular aspiration (FA) of all follicles ≥5 mm was done and the cows received the following treatments: LP4 group - animals receiving a previously used CIDR, HP group - animals receiving two new CIDR devices, and VLP4 group - animals that did not receive P4 treatment. The CIDR devices were replaced every 8 days along with 150 µg of D-cloprostenol injections. The ovum pick-up sessions were performed every four days from Day 0 to 24.

Blood sample collection

Blood samples were collected by caudal venipuncture into heparinized 10 ml tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) that were immediately centrifuged (1500 x g for 15 min) and plasma harvested and stored at -20ºC. Blood samples for P4 concentrations were collected at every OPU session (Fig. 1). However, to assess LH release, blood samples were collected every 15 min for 6 h on Day 18 from three cows of each group. Just before this serial sampling period, each animal was fitted with an indwelling catheter (Angiocath, 16 gauge, 8.26 cm; Becton Dickinson Vascular Access, Sandy, UT, USA), held in place with Kamar adhesive glue (Kamar Products, Inc., Steamboat Springs, CO, USA), Vetwrap Bandaging Tape (3M, Animal Care Products, St. Paul, MN, USA) and 2-inch elastic medical tape. The end of the catheter was sealed with an iv cap and the catheter was filled with heparinized saline (0.9% sodium chloride with 0.1% heparin). Before sample collection, the solution was removed and discarded, and following collection, catheters were flushed with fresh solution to prevent blood clot formation. Sampling was not done on one cow from VLP4 group (due to loss of catheter function).

Hormone analyses

Progesterone concentrations were determined with solid phase radioimmunoassay (Coat-A-Count®, Diagnostic Products Corporation, Los Angeles, CA, USA), as described by Peter and Bosu (1987), with a minimum detection limit of 0.1 ng/ml. The assay for plasma LH concentrations was adapted from Bolt and Rollins (1983) and Bolt *et al.* (1990) and expressed in terms of NIDDK-bLH-4. The minimum detection limit was 0.06 ng/ml, with a standard curve ranging from 0.06 to 8 ng/ml. For each hormone, all samples were analyzed in a single assay. The intra-assay coefficients of variation were 8.2 and 8.7%, for P4 and LH respectively.

Ovum pick-up procedure

Oocytes were retrieved by transvaginal FA, as described (Petyim *et al.*, 2003). Briefly, the cows were restrained in the chute and given 5 ml of epidural anesthesia (Lidocaine 2%, Lidovet, RJ, Brazil). The OPU procedure was performed using an ultrasound guided (Aloka SSD 500 with 5 MHz convex sectorial transducer, Aloka Co., Japan) system containing disposable needles (needle for follicular aspiration Handle Cook®, Spencer, IN, USA), 18 gauge in diameter, connected to a vacuum system (follicular pump for follicular aspiration, Handle Cook®) corresponding to approximately 75 to 85 mm Hg (15 ml/min). All follicles >3 mm in diameter were aspirated. The follicles were visualized on the monitor, counted, and recorded.

Aspirated follicular fluid was collected into a 50 ml Falcon tube (Corning®). Dulbecco PBS supplemented with 10% of fetal calf serum (FCS), gentamycin and heparin 100 IU/ml was used for washing. Immediately following aspiration, the filter (Milipore® embryos filter) was washed and its contents poured into a square grid dish to facilitate locating oocytes under a stereomicroscope.

Cumulus oocyte complexes (COCs) were examined under a stereomicroscope and classified into four categories based on the homogeneity, morphology of the cytoplasm and the compactness of the cumulus investment, as follows: Grade I, >3 layers of compact cumulus cells with a homogeneous, evenly granulated cytoplasm; Grade II, three layers of cumulus cells, cytoplasm generally homogeneous; Grade III, one or two layers of cumulus cells, cytoplasm of irregular appearance with dark areas; and Grade IV, completely denuded oocytes or oocytes with expanded cumulus. All oocytes, except those classified as Grade IV, were subjected to *in vitro* embryo production (IVP) as Grade IV oocytes have very limited development potential (Crosby *et al.*, 1981; Staigmiller and Moor, 1984; Fukui, 1990).

Each cow underwent seven OPU sessions (Fig. 1), however only data from the last five sessions were used in the study.

In vitro embryo production

Protocols for *in vitro* maturation, fertilization and culture (IVM, IVF and IVC) were done according to Dode *et al.*, 2002. Collected COCs were washed and transferred to maturation medium, which consisted of TCM 199 Earl's salt (Gibco BRL®), 24 IU/ml of luteinizing hormone (LH, Sigma®), 10 µg/ml follicle stimulant hormone (FSH, Sigma®), 1 µg/ml L-glutamine (Sigma®), 100 IU/ml penicillin (Sigma®), and 50 µg/ml streptomycin (Sigma®). The COCs were matured in a 4-well plate containing 2 ml of maturation medium, coated with 2 ml of paraffin oil. Then they were incubated for 22 h at 39ºC and 5% CO_2. At the end of the maturation period, the oocytes were washed and transferred to droplets of 200 µl fertilization medium. The medium used was TALP (Parrish *et al.*, 1995) supplemented with 21.1 µM penicillin (Sigma®), 10.4 µM hypotaurine (Sigma®), 1 µM epinephrine (Sigma®), and 10 µg/ml heparin (Sigma®). For IVF, doses of semen from the same bull, with proven *in vitro* fertility were used throughout the experimental period. The sperm selection followed the method of Percoll gradient, using 2 ml of Percoll 45% and 2 ml of Percoll 90% (Parrish *et al.*, 1995). The semen was thawed in warm water (36ºC), placed on Percoll gradient, and centrifuged at 700 x g for 20 min at 30ºC. The

supernatant was then removed, leaving only the pellet. The pellet was washed again, resuspended with 2 ml of TALP-sp and centrifuged at 700 x g for 5 min at 30ºC, and then resuspended in fertilization medium. After evaluating concentration, the semen was added to the fertilization drop in a final concentration of 1 x 10^6 sperm/ml. After 22 h of co-incubation, oocytes were vortexed to remove cumulus cells and excess sperm and washed once in a synthetic fluid cultivation medium (oviduct supplemented with essential and non-essential amino acids - SOFaa) and placed into the final culture drop (SOFaa). The SOFaa medium, 0.34 mM of sodium tri citrate (Sigma®), 2.77 mM myo-inositol (Sigma®) and 5% FCS was used for *in vitro* culture (Holm *et al.*, 1999). Cleavage rates were evaluated 48 h post insemination and embryo development rates were recorded on Day 7.

Statistical analyses

The statistical model included animal, body condition score (BCS), collection session, treatment, and their interactions. No effects of collection, animal, BCS and their interactions were detected and they were excluded from the final statistical model. Dependent variables, i.e. total number of follicles, total number of oocytes, concentrations of P4 and LH, were analyzed by Analysis of Variance and the Least Square Means procedure of SAS® was used for comparing the treatment means. Binomial dependent variables, such as oocyte recovery rate, percentage of oocyte quality I to III, cleavage rate, and blastocyst rate were analyzed by Chi-square. Mean plasma LH concentrations and frequency of LH pulses (pulses/8 h) were calculated for each sequential blood sampling period. An LH pulse was defined as an increase in LH concentrations that exceeded the previous nadir by two intra-assay standard deviations (Schillo *et al.*, 1988).

Animal welfare

The Committee for Ethics in Animal Experimentation from the Federal University of Pelotas has approved all procedures performed in this experiment.

Results

Follicular, oocytes and embryo production results

Number of follicles aspirated, oocytes recovered, oocytes subjected to IVP, cleavage structures, and blastocysts developed are shown for each group (Table 1). Per cow per OPU session performance (mean ± SEM) on the basis of follicular response, oocytes retrieved, oocyte cleavage and blastocyst production is also presented in Table 1. Groups that received exogenous progesterone (LP4 and HP4 groups) differed in the number of follicles/cow/OPU from the VLP4 group ($P < 0.001$), with no significant difference between LP4 and HP4. Similarly, the average of collected oocytes/cow/OPU was higher in the LP4 and HP4 groups than VLP4 group ($P = 0.02$; Table 1). The oocyte recovery rate was higher in the HP4 group, intermediate in the LP4 group and lower in the VLP4 group ($P \leq 0.06$; Table 1). The LP4 group had greater percentage of viable oocytes than HP4 ($P = 0.07$); VLP4 group was intermediate and did not differ from the others (Table 1). Rates of cleavage and blastocyst formation were calculated based on the number of viable oocytes subjected to IVF. Although there was no difference in numbers of cleaved oocytes/cow/OPU among treatment groups, oocyte cleavage rate tended ($P = 0.07$) to be lower in the VLP4 group. In contrast, the LP group tended ($P = 0.06$) to be higher for blastocyst production than HP and VLP groups. Furthermore, the blastocyst production/cow/OPU and blastocyst rate was higher in the LP4, than in the HP4 or VLP4 group ($P = 0.06$ and 0.03 for blastocyst production and rate, respectively; Table 1).

Table 1. Results (mean ± SEM, or %) of total number of aspirated follicles, recovered and cleaved oocytes, and blastocyst production after five ovum pick-ups (OPU) per donor cow, in cows (n = 5 per group) with low (1.0 to 2.0 ng/ml; LP4), high (>3.0 ng/ml; HP4), or very low (<1.0 ng/ml; VLP4) circulating progesterone (P4) concentrations.

	LP4	HP4	VLP4	P-value
Aspirated follicles; n	10.8 ± 0.5^a	10.1 ± 0.6^a	7.1 ± 0.6^b	< 0.001
Recovered oocytes; n	6.6 ± 0.8^a	7.0 ± 0.8^a	3.4 ± 0.7^b	0.02
Recovery rate; % (n/n)	60.9^a (165/271)	68.8^b (174/253)	46.6^c (83/178)	≤ 0.06
Oocytes Grade I to III; % (n/n)	79.4^a (131/165)	68.4^b (119/174)	72.3^{ab} (60/83)	0.07
Cleaved oocytes[1]; n	3.2 ± 0.5	3.0 ± 0.5	1.2 ± 0.5	0.40
Cleavage rate; % (n/n)	67.5^a (79/117)	63.0^a (75/119)	50.0^b (30/60)	0.07
Blastocysts (n)	1.3 ± 0.4^a	0.8 ± 0.4^b	0.4 ± 0.4^b	0.06
Blastocyst rate; % (n/n)	28.2^a (33/117)	16.0^b (19/119)	15.0^b (9/60)	0.03

[1]Cleavage was calculated on the number of the oocytes subjected to the IVF procedure (Grades I, II and III only).

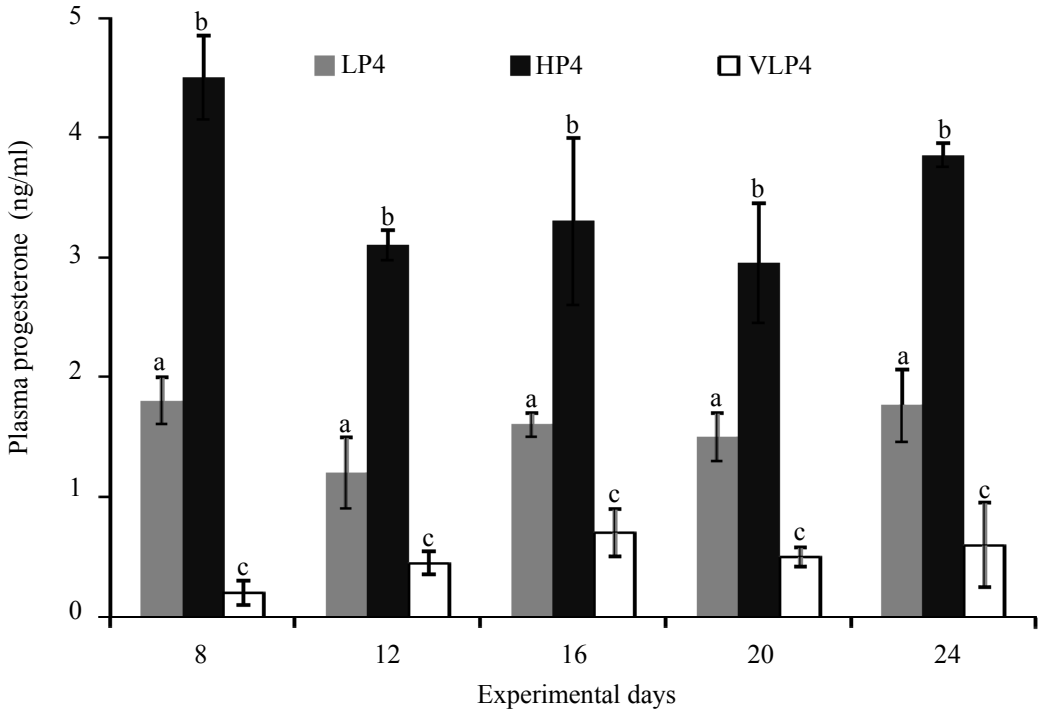

Figure 2. Plasma progesterone concentrations (ng/mL; mean ± SEM) according to each ovum pick-up (OPU) session in cows treated with a used intravaginal P4 releasing device (LP4; n = 5), two new devices (HP4; n = 5), or not submitted to P4 treatment (VLP4; n = 5). All cows were also treated with $PGF_{2\alpha}$ every 8 days.
[a,b,c]Treatments with different letters within the same session are significantly different ($P < 0.05$).

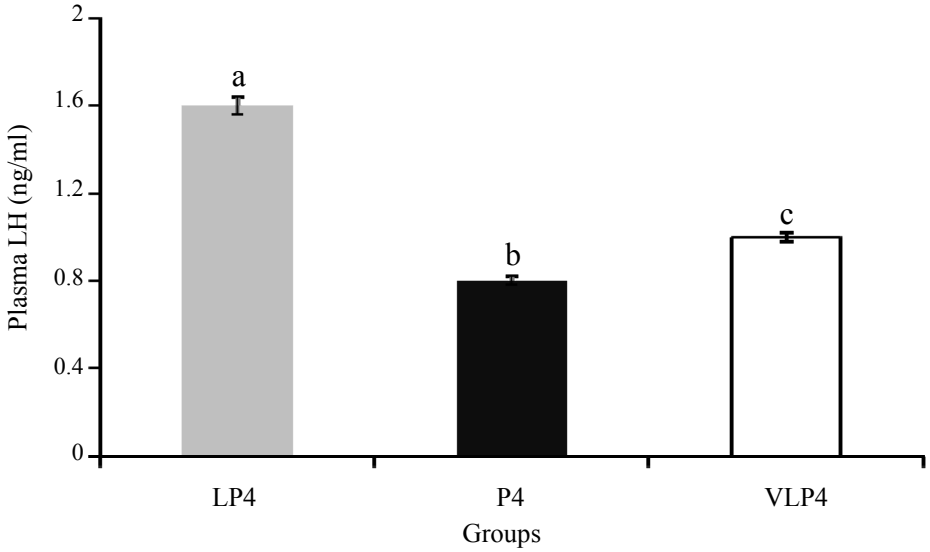

Figure 3. Plasma LH concentrations (ng/ml; mean ± SEM) of samples collected every 15 min for 6 h between two ovum pick-up sessions (Day 18) in cows treated with an intravaginal P4 releasing device (LP4; n = 70 samples from 3 cows), two new devices (HP4; n = 67 samples from 3 cows), or not submitted to P4 treatment (VLP4; n = 42 samples from 2 cows).
a,b,cTreatments with different letters are different ($P < 0.05$).

Hormone concentrations

There was a difference ($P < 0.05$) among groups in the plasma progesterone concentrations during the entire experimental period (Fig. 2). In that regard, the average of progesterone concentrations during the experiment was 1.58, 3.57, and 0.48 ng/ml, for the LP4, HP4, and VLP4 groups, respectively. Similarly, there was a difference ($P < 0.05$) among groups in plasma LH concentration (the LP4 group had higher concentrations and the HP4 had lower concentrations; Fig. 3). In contrast, no difference in the number of LH peaks was detected among the groups ($P > 0.05$). The LP4 group had 2.3 ± 0.6, whereas the HP4 group had 2.0 ± 1.0 and the VLP4 group had 2.0 ± 0.0 LH peaks during the sampling period.

Discussion

The aim of this study was not to improve the IVP results or OPU procedure technique, instead, this experiment was done in order to answer a basic question: to what extent progesterone concentration affects the competence of oocytes collected 3 days after the wave emergence. This experimental design using OPU to recover oocytes that have developed under different progesterone environments provides a good model for the study of its effects on oocyte competence. By using this model, we were able to demonstrate the influence of progesterone concentration on oocyte competence. Performing OPU every 4 days was an effective interval to optimize oocyte retrieval and avoid the detrimental effect of follicular dominance on oocyte quality (Hagemann *et al.*, 1999; Salamone *et al.*, 1999; Hendriksen *et al.*, 2004). Since a new follicular wave emerged 24 h after follicle ablation (Bergfelt *et al.*, 1994; Martinez *et al.*, 2000). In the present study, OPU was performed approximately 3 days after wave emergence, just around the time of the selection of the dominant follicle (Sartorelli *et al.*, 2005). In contrast, performing OPU after selection would have reduced the number of visible follicles, oocytes collected, and blastocysts produced per cow per session (Machatkova *et al.*, 2000).

No ovulations were detected and no corpora lutea were formed throughout the entire experimental period. Thus, the CIDR devices were essentially the only source of progesterone. It was noteworthy that higher progesterone concentrations somehow favored follicular recruitment, since the HP4 and LP4 groups had more follicles aspirated than the VLP4 group, and consequently more oocytes were recovered. The mechanism of stimulation of small antral follicles remains unclear (Cushman *et al.*, 2001; Roth *et al.*, 2001) and we did not find any literature describing the effects of P4 on follicle recruitment. Although cows had been randomly allocated, perhaps there were inherent differences among animals that inadvertently affected the outcome. Unfortunately, a crossover experimental design to avoid the intrinsic individual animal effect was not done, as we were concerned about the animal welfare aspects of numerous OPU sessions.

The difference in oocyte recovery rates between the progesterone-supplemented groups and VLP4 was attributed to the number of follicles aspirated; since the HP4 group had more follicles, it was easier to obtain oocytes. Although Petyim *et al.* (2003) similarly reported an effect of the number of follicles on oocyte recovery rate, in contrast, Viana *et al.* (2003) did not detect a consistent association between the number of follicles and COCs collected.

Based on the higher quality of oocytes recovered from the LP4 group than those recovered from the HP4 group, we inferred that there was an association between systemic progesterone concentrations and oocyte quality. Similarly, Vassena *et al.* (2003) reported better oocyte quality following recovery on Day 5 of the estrous cycle (when P4 concentrations were still low). In contrast, De Wit *et al.* (2000) did not detect a difference in the quality of oocytes collected in the early luteal phase (from 0 to 7 days of the estrous cycle) or those collected in the late luteal phase (from 8 to 17 days of the cycle). In addition, Chaubal *et al.* (2007) did not detect differences in oocyte quality in cows with or without exogenous progesterone during a superstimulatory treatment prior to OPU. Nonetheless, in that study, FSH treatment probably inhibited LH secretion (Goodman and Karsch, 1980). In that regard, the detrimental effect of the suppression of LH pulsatility on follicular development and oocyte maturation has been reported (Roberge *et al.*, 1995; Chaubal *et al.*, 2007).

Better oocyte quality in the LP4 group compared to the other two groups might be associated with the plasma concentrations and pulse frequencies of LH, which were highest in the LP4 group; perhaps LH promoted oocytes maturation, thereby enhancing oocyte quality (Roberge *et al.*, 1995; Chaubal *et al.*, 2007). In that regard, small pulses of LH for a longer period improved oocyte quality (Greve *et al.*, 1995), as previously suggested (Jones, 2004; Mehlmann, 2005). Presumably, the once used CIDR mimicked P4 concentrations present during the early luteal phase; oocytes collected during this phase were of better quality than those collected at other phases.

Although, it is clear that there is an inverse association between systemic progesterone concentrations and LH pulse frequency (Rahe *et al.*, 1980), the mean LH concentrations detected in this study, as well as the literature data regarding the relationship between LH and progesterone, are controversial. According to some authors, LH pattern release is not directly dependent on the concentration of progesterone. Low levels of progesterone can enhance LH release without changing the numbers of LH peaks. Sanchez *et al.* (1995) reported that the administration of

low concentrations of progesterone to mature heifers without a CL resulted in LH pulse frequencies typical of the follicular phase (1 pulse/hour).

The LP4 group had the best oocyte quality and blastocyst production; this association between oocyte quality and embryo production was consistent with previous studies (Rizos *et al.*, 2002a, b, 2003). In a twice-weekly OPU session, oocytes may not be getting enough time in the follicular microenvironment to achieve developmental competence (Vassena *et al.*, 2003). However, under a higher LH concentration (LP4 group), oocyte quality was not compromised.

In conclusion, the oocytes recovered from cows with a low progesterone environment were of better quality and yielded more embryos than oocytes from donors with either high or very low progesterone environments.

Acknowledgments

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