Concentration of progesterone during the development of the ovulatory follicle: I. Ovarian and embryonic responses

R. L. A. Cerri,* R. C. Chebel,† F. Rivera,‡ C. D. Narciso,‡ R. A. Oliveira,‡ W. W. Thatcher,‡ and J. E. P. Santos^{†1}

*Faculty of Land and Food Systems, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada

†Department of Veterinary Population Medicine, University of Minnesota, St. Paul 55108

‡Department of Animal Sciences, University of Florida, Gainesville 32611

ABSTRACT

Objectives were to evaluate the effects of differing progesterone concentrations during follicle development on follicular dynamics, fertilization, and embryo quality. Lactating Holstein cows ($n = 154$) were assigned randomly to 1 of 2 treatments. Cows underwent a pre-synchronization of the estrous cycle composed of an injection of GnRH concurrently with the placement of a progesterone insert, an injection of PGF_{2α} and insert removal 7 d later, and a second injection of GnRH 48 h later (study d -16). All cows were then submitted to a hormonal protocol identical to the presynchronization program starting on d 7 of the estrous cycle (study d -9). Cows enrolled in the high progesterone (HP) treatment received no further treatment. Cows in the low progesterone (LP) treatment received additional PGF_{2α} injections on study d -14, -13.5, and -13 and again on study d -9, -7, -6.5, and -6. Ovaries were evaluated by ultrasonography, and blood was sampled for concentrations of progesterone and estradiol throughout the study. Uteri were flushed 6 d after artificial insemination (AI) and recovered oocytes-embryos were evaluated. Concentrations of progesterone were less for LP cows from study d -7 to -2; concentrations of estradiol at PGF_{2α} and at the last GnRH of synchronization were greater for LP than HP. The proportion of cows in estrus at AI was greater for LP than for HP (38.0 vs. 5.3%). Ovulatory follicles of LP cows had larger diameters at the injections of PGF_{2α} (17.2 vs. 14.6 mm) and final GnRH (19.4 vs. 16.9%) of the synchronization, which resulted in a larger diameter of the corpus luteum 6 d after AI (24.3 vs. 22.6 mm). Double ovulation after the last GnRH of the synchronization was increased in LP (18.6%) compared with HP (4.5%). Fertilization rate was similar and averaged 82.7%. The proportion of embryos and oocytes-embryos classified as grades 1 and 2, proportion of degenerated embryos,

and unfertilized-degenerated oocytes-embryos were not different between LP and HP. Number of blastomeres did not differ between LP and HP, but the proportion of live blastomeres tended to be less for LP than HP (94.2 vs. 98.7%). Reducing progesterone concentrations during the synchronization program altered concentrations of estradiol and follicular dynamics, but resulted in similar fertilization and only minor changes in embryo quality.

Key words: dairy cow, embryo quality, progesterone, reproduction

INTRODUCTION

The decrease in fertility of the lactating dairy cow (Lucy, 2001; Santos et al., 2004) is probably multifactorial and often associated with high milk production and decreased concentrations of circulating progesterone (Wiltbank et al., 2006). Changes in follicular development partly caused by changes in steroid metabolism could affect fertilization and early embryonic development, contributing to the subfertility problem in lactating cows. Lactating dairy cows typically have lower concentrations of estradiol and progesterone in plasma compared with nonlactating dairy cows and growing heifers (Sartori et al., 2004; Wiltbank et al., 2006). Because hepatic blood flow was correlated positively with feed and energy intakes (Reynolds et al., 2003), it has been hypothesized that the greater feed intake in high-producing dairy cows could affect metabolism and concentrations of progesterone and estradiol. The decrease in circulating concentration of progesterone in the lactating dairy cow was associated with changes in the pattern of follicular wave development (Wiltbank et al., 2006) and with endometrial PGF_{2α} release (Shaham-Albalancy et al., 2001). Strategies to supplement estradiol during proestrus (Cerri et al., 2004) or to increase progesterone after ovulation (Santos et al., 2001) demonstrated some positive effects on pregnancy per AI (P/AI). Collectively, it is suggested that high-producing lactating dairy cows are not able to maintain optimal concentrations of circulating ovarian steroids for optimum fertility.

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¹Corresponding author: jepsantos@ufl.edu

The endocrine milieu in which the preovulatory follicle grows can determine its persistency as well as the maturation of the oocyte. Low concentrations of progesterone increase LH pulse frequency and extend follicular dominance, which could be deleterious if the follicle is maintained in such an environment for a long time (Savio et al., 1993). In fact, a recent study observed that even small variations in the length of follicular dominance affect early embryo development (Cerri et al., 2009) and P/AI (Bleach et al., 2004). In addition, the increase in LH pulse frequency was thought to initiate premature oocyte maturation (Revah and Butler, 1996) and compromise early embryo development (Ahmad et al., 1995).

Oocytes from ovarian follicular waves of similar length but growing under decreased concentrations of progesterone after d 6 of emergence resumed meiotic division prematurely (Inskeep, 2004). Nevertheless, there is no clear evidence demonstrating the effect of progesterone concentration during ovarian follicle development, using an identical length of follicle growth, on fertilization and early embryonic development in high-producing dairy cows.

The objectives of the present study were to determine the effect of altering the concentrations of circulating progesterone during the development of the ovulatory follicle on ovarian structures and responses to hormonal treatments, and on fertilization and embryo quality on d 6 after AI. We hypothesized that in cows exposed to decreased concentrations of progesterone, the dominant follicle would grow at a faster rate, a larger follicle would be ovulated, and cows would have greater concentrations of estradiol during the periovulatory period compared with cows exposed to greater concentrations of progesterone. Furthermore, we expected cows exposed to lesser concentrations of progesterone to have poorer embryo quality possibly because of reduced oocyte competence caused by premature resumption of second meiotic division (Revah and Butler, 1996).

MATERIALS AND METHODS

Animals, Housing, and Diets

The University of California–Davis Institutional Animal Care and Use Committee approved all animal procedures. One hundred fifty-four (52 primiparous and 102 multiparous) lactating Holstein cows from one dairy farm located in the San Joaquin Valley of California were enrolled in the study, which was carried out in the months of February to June. The average number of lactating cows in the herd during the study was 900, and the 305-d 3.5% FCM rolling herd average was 11,590 kg/cow per year. Cows were housed in

freestall barns equipped with fans and sprinklers that were automatically activated when the temperature reached 26.7°C. All cows were fed the same diet as a TMR twice daily to meet or exceed the dietary requirements for lactating cows weighing 680 kg, consuming 24 kg of DM, and producing 45 kg of milk containing 3.5% fat and 3.1% true protein in the first 70 d of lactation (NRC, 2001).

All cows had BCS evaluated at 33 ± 3 and 65 ± 3 DIM according to Ferguson et al. (1994). Cows with BCS <2.25 at 33 ± 3 DIM were excluded from the experiment. Cows were classified according to the average BCS at 33 ± 3 and 65 ± 3 DIM. A cow was classified with a low BCS if the average BCS was ≤ 2.75 or moderate BCS if the average BCS >2.75 . Cows diagnosed with any evident health disorder (displacement of the abomasum, lameness, uterine infection, and uterine adhesions) were not enrolled in the study.

Treatments and AI

Starting at 33 ± 3 DIM, all cows had their estrous cycle presynchronized with an injection of 100 µg of GnRH (gonadorelin diacetate tetrahydrate, Merial Ltd., Iselin, NJ) and a controlled internal drug-release (CIDR; Eazi Breed, Pfizer Animal Health, New York, NY) containing 1.38 g of progesterone. Seven days later, cows received an injection of 25 mg of PGF_{2α} (dinoprost tromethamine, Pfizer Animal Health) concurrent with removal of the CIDR, and a second injection of GnRH was administered 48 h after the PGF_{2α} injection. The second GnRH injection of the presynchronization protocol was considered to be on d -16 of the study. Cows were then randomly assigned to 1 of 2 treatments (Figure 1). Cows in the high progesterone (HP) treatment ($n = 75$) were enrolled in the synchronization of ovulation protocol. Cows received an injection of 100 µg of GnRH on study d -9 concurrently with the insertion of a CIDR, an injection of 25 mg of PGF_{2α} and CIDR removal on study d -2 , and a second injection of 100 µg of GnRH on d 0; cows received fixed-time AI 12 h after the last GnRH injection. Cows assigned to the low progesterone (LP) treatment ($n = 79$) were subjected to the same ovulation synchronization protocol used for HP, but received additional injections of PGF_{2α} on study d -14 , -13.5 , and -13 and again on study d -9 , -7 , -6.5 , and -6 . These days were chosen because they would coincide with the early stages of development of a newly formed corpus luteum (CL) after the GnRH injections. Cows were observed for signs of estrus on the day of AI based on removal of tail chalk using paint sticks (All-Weather Paintstik, LA-CO Industries, Chicago, IL). All cows were inseminated by the same technician using semen from a single sire of

Figure 1. Diagram of the synchronization protocol and activities in the study. CIDR = controlled internal drug-release containing 1.38 g of progesterone; LP = low progesterone treatment; presynchronization and synchronization = ovulation synchronization protocols based on GnRH, PGF_{2α}, and CIDR.

proven fertility and high expected relative conception rate from field inseminations in lactating cows.

Estradiol and Progesterone Analyses

Approximately 7 mL of blood was collected by puncture of the median coccygeal vein or artery utilizing Vacutainer tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ) with sodium EDTA. Samples were immediately placed in ice, transported to the laboratory within 5 h, and centrifuged at $2,000 \times g$ for 15 min for separation of plasma. Plasma samples were frozen at -25°C and later analyzed for concentrations of progesterone and estradiol.

Blood was sampled and analyzed for concentrations of progesterone on study d -16 , -14 , -11 , -9 , -7 , -4 , -2 , 0 , 3 , 5 , and 7 , covering the entire period of ovulatory follicle development until the day of oocytes-embryos collection. Plasma samples were analyzed in duplicate for concentration of progesterone by ELISA validated by Cerri et al. (2004). Each microplate contained the standards (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 ng of progesterone/mL of plasma). The intra- and inter-assay CV were 4.7 and 13.0%, respectively. Cows with concentrations of progesterone in plasma ≥ 1.0 ng/mL at the time of the PGF_{2α} injection of the synchronization protocol and then <1.0 ng/mL 48 h later were classified as experiencing complete luteolysis. Blood samples for measurement of concentrations of estradiol were collected on study d -9 , -2 , 0 , and 7 , which coincided with the days of each hormonal treatment of the synchronization protocol and the day of uterine flush. The concentration of estradiol was measured using a

validated RIA (Kirby et al., 1997). Standards for this assay were 0.25, 0.50, 1.0, 2.5, 5.0, 7.5, 10.0, and 20.0 pg of estradiol/mL of plasma, and the intra- and inter-assay CV were 10.2 and 13.2%, respectively.

Ovarian Ultrasonography

Cows had their ovaries examined by ultrasound (Sonovet 2000, Universal Medical System, Bedford Hills, NY) equipped with a 7.5-MHz linear rectal transducer. The first 2 examinations (33 ± 3 and 40 ± 3 DIM) of the ovaries were intended to determine the cyclic status (cyclic vs. anovular) of the cow before initiation of treatments. The cow was considered cyclic if a CL was observed at one or both ultrasound examinations and considered anovular if no CL was detected. Ultrasound examinations of the ovaries were then performed at every hormonal treatment during the presynchronization and synchronization protocols and again 48 h after each GnRH injection. In addition, a final ultrasound examination of the ovaries was performed 6 d after AI at the time of uterine flush. Maps of the ovaries were drawn for each individual cow, and the size and position of follicles ≥ 5 mm in diameter and CL were recorded. Occurrence of ovulation within 48 h after each GnRH treatment was characterized by the absence of a previously recorded follicle ≥ 10 mm in diameter and later confirmed by the presence of a CL.

Oocyte-Embryo Collection and Evaluation

Cows were flushed on d 6 after AI by a transcervical procedure using a silicone Foley catheter (18 French,

30 mL, 56 cm; Minitube of America Inc., Verona, WI). The balloon of the Foley catheter was placed approximately 3 cm past the external intercornual ligament of the uterine horn ipsilateral to the CL. Approximately 300 mL of a flushing solution (ViGro Complete Flush Solution, Bioniche Life Sciences Inc., Belleville, ON, Canada) was used in the uterine horn in 20 cycles of infusion/recovery of 15 mL each. For cows with CL on both ovaries, both horns were flushed as described previously. Oocytes-embryos collected were evaluated for fertilization and grade quality (1 = excellent and good, 2 = fair, 3 = poor, and 4 = degenerated) as described by the International Embryo Transfer Society (IETS, 1998). Embryos were stained with 5 µg/mL propidium iodide (Sigma, St. Louis, MO) to determine the number of nonviable blastomeres and then with 5 µg/mL Hoechst 33342 (Molecular Probes Inc., Eugene, OR) to determine the number of accessory spermatozoa using epifluorescence microscopy (Olympus IX51, Olympus America Inc., Melville, NY; 365 nm excitation, >400 nm emission). The zona pellucida was then dissolved with a solution of 0.02 *N* HCl in 0.1% Tween-20 (Sigma). The embryo was again stained with 5 µg/mL Hoechst 33342 and the blastomeres spread in a glass slide and counted using epifluorescence microscopy.

Experimental Design and Statistical Analysis

The experimental design was a randomized complete block design. Lactating Holstein cows were blocked at 33 ± 3 DIM according to parity (primiparous vs. multiparous) and BCS (low vs. moderate) and, within each block, randomly assigned to 1 of the 2 treatments.

Dichotomous outcomes were evaluated by logistic regression using the LOGISTIC procedure of SAS software ver. 9.2 (SAS Institute Inc., Cary, NC). Count data such as the number of accessory spermatozoa and total blastomeres were analyzed by the GENMOD procedure of SAS using a Poisson distribution. The models used included the effects of treatment, parity, cyclic status, and average BCS.

Concentrations of progesterone and estradiol were analyzed by ANOVA for repeated measures using the MIXED procedure of SAS. The covariance structure with the smallest Akaike's information criterion was used for measurements utilized in the MIXED model. The models included the effects of treatment, time of measurement, parity, BCS, and interactions, with cow nested within treatment as the random error. Analysis of the dominant follicle diameter was performed by ANOVA with the GLM procedure of SAS using a model that included the effects of treatment, parity, cyclic status, and BCS. Treatment differences with $P \leq$

0.05 were considered significant and from $0.05 < P \leq 0.10$ were designated as tendency.

RESULTS

The mean and median lactation number did not differ among treatments and were 2.5 ± 0.1 and 2.0, respectively. Similarly, the mean and median BCS on the day of study enrollment were not different among treatments and were, respectively, 2.92 ± 0.38 and 3.00. The proportion of cows classified as cyclic before the initiation of the synchronization protocols was not different among treatments and averaged 79.9%.

Ovarian Structures and Responses to the Synchronization of Ovulation Protocol

Diameter of the dominant follicle at the first GnRH injection of the synchronization protocol did not differ between treatments and averaged 16.8 ± 0.4 mm (Table 1). Proportion of ovulation and double ovulation to the first GnRH injection of synchronization protocol was not different between treatments and averaged 91.4 and 8.0%, respectively. Cows in LP had fewer ($P = 0.001$) CL at PGF_{2α} injection of the synchronization protocol compared with the HP cows. Diameter of the dominant follicle at PGF_{2α} injection was larger ($P = 0.001$) for LP compared with HP, and remained larger ($P = 0.001$) at the final GnRH injection of the synchronization program. Ovulation to the second GnRH injection of synchronization protocol did not differ between LP and HP; however, the proportion of double ovulation increased ($P = 0.01$) in LP compared with HP. The proportion of cows in estrus at AI was greater ($P = 0.001$) for LP than HP. The diameter of the largest CL 6 d after AI was greater ($P = 0.03$) for LP than HP.

Concentrations of Progesterone and Estradiol

Concentrations of progesterone were less ($P = 0.001$) for LP compared with HP from study d -7 to -2, which coincided with the interval from the first GnRH to the PGF_{2α} injections of the synchronization protocol (Figure 2). Both study day ($P = 0.001$) and an interaction between treatment and study day ($P = 0.001$) were detected.

Concentrations of estradiol differed ($P = 0.001$) with treatment and study day, and an interaction ($P = 0.001$) between treatment and study day was also observed (Figure 3). Concentrations increased during proestrus and they were greater ($P = 0.001$) for LP than for HP. This difference between treatments was observed on study d -2 and 0, which coincided with the days of

Table 1. Effect of treatment on ovarian dynamics during the synchronization protocol

Item ¹	Treatment ²		AOR ³	95% CI	P-value
	HP	LP			
Cows, n	75	79	—	—	—
First GnRH					
DF, mm	16.9 ± 0.4	16.7 ± 0.4	—	—	0.55
Ovulation, %	93.1	89.7	1.5	0.4–4.9	0.54
Double ovulation, %	7.3	8.6	1.0	0.3–3.5	0.98
PGF _{2α}					
DF, mm	14.6 ± 0.7	17.2 ± 0.6	—	—	0.001
CL, n	1.9 ± 0.5	1.0 ± 0.4	—	—	0.001
Second GnRH					
DF, mm	16.9 ± 0.5	19.4 ± 0.4	—	—	0.001
Ovulation, %	90.4	96.1	0.4	0.1–1.7	0.16
Double ovulation, %	4.5	18.6	0.2	0.1–0.8	0.01
Detected estrus at AI, %	5.3	38.0	0.1	0.1–0.3	0.001
CL 6 d after AI, mm	22.8 ± 0.5	24.3 ± 0.4	—	—	0.03

¹DF = diameter of the dominant follicle; CL = corpus luteum.

²HP = high progesterone; LP = low progesterone.

³AOR = adjusted odds ratio. The LP treatment was utilized as the referent group.

induced luteal regression with PGF_{2α} and the day of induced ovulation with the final GnRH of the synchronization protocol, respectively. Cows in LP treatment that had double ovulation after AI had greater ($P < 0.01$) concentration of estradiol at AI (5.6 ± 0.2 vs. 4.9 ± 0.1 pg/mL) and increased ($P = 0.03$) the proportion of cows detected in estrus (32.1 vs. 10.6%) compared with single ovulating cows.

Oocytes-Embryos Evaluation

The proportion of oocytes-embryos recovered relative to the number of CL was not different between treatments and averaged 53.2% (Table 2). The average fertilization rate was 82.7%, which was also not different between treatments. The critical number of accessory spermatozoa that resulted in the highest sensitivity and

Figure 2. Concentrations of progesterone in plasma of cows in high (HP) and low (LP) progesterone treatments. Effects of treatment ($P = 0.001$), study day ($P = 0.001$), and interaction between treatment and study day ($P = 0.001$). Within a study day, the asterisk (*) denotes a difference between treatments ($P < 0.01$).

Figure 3. Concentrations of estradiol in plasma of cows in high (HP) and low (LP) progesterone treatments during the synchronization protocol and on the day of uterine flush. Effect of treatment ($P = 0.001$), study day ($P = 0.001$), and interaction between treatment and study day ($P = 0.001$). Within a study day, the asterisk (*) denotes a difference between treatments ($P < 0.01$).

specificity for fertilization was 4. Using this cut-off, the sensitivity and specificity were 68.7 (95% CI = 56.2 to 79.4) and 100% (95% CI = 76.7 to 100.0), respectively, and the area under the curve was 0.92 (95% CI = 0.84 to 0.97; $P < 0.001$). In summary, 68% of the fertilized oocytes had >4 accessory spermatozoa, whereas 100% of the nonfertilized oocytes had ≤ 4 accessory spermatozoa per nonfertilized oocyte.

Embryo quality did not differ between LP and HP (Table 2). Mean and median numbers of blastomeres from collected embryos did not differ between LP and HP, with mean and median numbers of cells of 49.5 ± 3.4 and 54, respectively. The percentage of live blastomeres tended ($P = 0.09$) to be less for LP compared with HP cows. No differences were observed in the median and mean number of accessory spermatozoa between LP and HP and the proportion of oocytes-embryos containing ≥ 1 accessory spermatozoon. Embryos had greater ($P < 0.01$) median numbers of accessory spermatozoa than oocytes (11.0 vs. 1.0). Furthermore, only 42.9% of the oocytes had ≥ 1 accessory spermatozoon, whereas 100% of the embryos had ≥ 1 accessory spermatozoon ($P < 0.01$). Embryos of grade 1 had greater ($P = 0.01$) numbers of accessory spermatozoa (21.3) compared with grade 2 (10.0), grade 3 (11.5), degenerated (13.1), and unfertilized oocytes (1.1).

DISCUSSION

Different concentrations of progesterone during ovarian follicular development had major effects on follicular growth, incidence of double ovulation, concentration of estradiol during proestrus, and detection of estrus at AI. Fertilization and embryo development, however, were mostly unaffected by treatment. The present study aimed to develop ovulatory follicles under different concentrations of circulating progesterone while maintaining a similar follicle dominance length. We expected ovulatory follicles from cows in the LP group to reach larger diameters (as in fact occurred) and, therefore, to reach estrus and ovulation earlier than cows in the HP treatment, which could shorten the dominance length and therefore affect early embryo quality and development (Cerri et al., 2009). Timed ovulation and AI were used to keep a consistent interval between luteolysis and AI and ovulation.

Sequential PGF_{2α} injections given early during CL development were intended to partially compromise the synthesis of progesterone by the CL, but not necessarily to promote complete luteolysis (Shaham-Albalancy et al., 2001; Beltman et al., 2009). The protocols used for HP and LP treatments successfully promoted high and low circulating concentrations of progesterone, respec-

Table 2. Effect of treatment on recovery, fertilization, and embryo quality in lactating dairy cows

Item	Treatment ¹		AOR ²	95% CI	P-value
	HP	LP			
Oocytes-embryos, n	36	45	—	—	—
Recovery, %	50.7	55.3	0.8	0.4–1.6	0.59
Oocytes-embryos					
Fertilization, %	80.6	84.4	0.8	0.2–2.6	0.72
Grades 1 and 2, %	58.3	53.3	1.2	0.5–3.0	0.64
Degenerated, %	16.7	17.8	0.9	0.2–3.2	0.86
Unfertilized-degenerated, %	36.1	33.3	1.1	0.4–2.9	0.89
Embryos					
Embryo, n	29	38	—	—	—
Grades 1 and 2, %	72.4	63.2	1.4	0.5–4.3	0.41
Degenerated, %	20.7	21.0	1.0	0.3–3.5	0.95
Blastomeres					
Mean $\pm$ SEM	49.9 $\pm$ 3.9	46.2 $\pm$ 3.4	—	—	0.44
Median, n	53	54	—	—	0.96
Live, % $\pm$ SEM	98.7 $\pm$ 2.1	94.2 $\pm$ 1.9	—	—	0.09
Accessory spermatozoa					
Mean $\pm$ SEM	11.2 $\pm$ 3.6	16.2 $\pm$ 3.1	—	—	0.23
Median, n	6.0	9.0	—	—	0.94
Oocyte-embryos with ≥ 1 , %	94.4	86.7	2.6	0.5–9.8	0.26

¹HP = high progesterone; LP = low progesterone.

²AOR = adjusted odds ratio. The LP treatment was utilized as the referent group.

tively, during the development of the ovulatory follicle. Concentrations of progesterone did not differ between treatments until after the day of the first GnRH of the synchronization protocol; however, the mean concentration of progesterone in HP cows was more than 3 times greater compared with LP treatment (5.2 ± 0.2 vs. 1.7 ± 0.1 ng/mL). The difference in concentration of progesterone at the time of PGF_{2 α} during the synchronization protocol was the result of the previously formed CL regressing and the development of the newly formed CL being compromised in LP cows after receiving sequential doses of PGF_{2 α} . Moreover, the sequential PGF_{2 α} injections used in the LP treatment also compromised the steroidogenic capacity of the newly developed CL formed after the first GnRH of the synchronization protocol. This effect has been demonstrated previously (Shaham-Albalancy et al., 2001; Beltman et al., 2009), confirming the planned response for the experimental protocol used.

Despite the differences in progesterone concentrations during development of the dominant follicle, which resulted in more double ovulation and larger ovulatory follicle, the concentration of progesterone on d 6 after AI was similar between LP and HP. This was surprising considering that LP cows ovulated larger follicles and had larger CL on d 6 after AI. Previous reports (Vasconcelos et al., 2001; Mussard et al., 2007; Cerri et al., 2009) observed that ovulation of larger follicles was positively correlated with concentration of progesterone during early and middle diestrus of the subsequent estrous cycle. Follicles reached a greater di-

ameter in previous studies because of the longer period of follicular growth as opposed to just the difference in concentration of progesterone induced in the current study. Using a similar experimental protocol, Cerri et al. (2011) observed that progesterone concentrations in the first 16 d of the estrous cycle subsequent to induced ovulation did not differ between cows that developed the ovulatory follicle under low or high concentrations of progesterone in spite of a larger CL diameter. It is possible that maturity or age of the ovulatory follicle, and not only diameter, might influence the subsequent steroidogenic capacity of the resulting CL.

Cows in the LP treatment ovulated larger follicles and had a greater incidence of double ovulation, and in the LP group more cows were in estrus near AI. The lower concentration of progesterone in the LP treatment probably decreased the negative feedback on the release of GnRH and LH, thereby enhancing the stimulatory effect of LH on follicle development, as well as increasing the selection of co-dominant follicles that acquire ovulatory capacity (Wiltbank et al., 2006). In a companion study (Cerri et al., 2011) using the same experimental protocol, the basal concentration of LH in plasma tended to be greater for LP than for HP cows, which probably explains the faster follicle growth. The exact mechanism by which low progesterone during follicle selection results in more co-dominance is unknown. Progesterone might influence the pattern of FSH release, although this has not been confirmed (Adams et al., 1992). Conversely, Lopez et al. (2005) studied the hormonal concentrations of cows experiencing single or

multiple ovulations. They found increased concentrations of FSH and LH before the deviation in cows with multiple ovulations. Cows with multiple ovulations had less concentration of inhibin, which might explain the increased concentrations of FSH observed for multiple ovulating cows (Lopez et al. 2005). In addition, low concentrations of progesterone alter the pulse frequency of LH release, which might mediate selection of co-dominant follicles. These changes in gonadotropins, either individually or simultaneously, might explain the greater incidence of co-dominance in cows developing follicles under low concentrations of progesterone. In heifers, which typically have greater concentrations of progesterone compared with lactating cows (Sartori et al., 2004), administration of exogenous progesterone did not influence FSH concentrations (Adams et al., 1992). Moreover, although progesterone administration altered LH concentrations in heifers (Ginther et al., 2001), it did not affect the development of the largest subordinate follicle. In those studies (Adams et al., 1992; Ginther et al., 2001), heifers were used as an experimental model, and heifers not treated with progesterone were likely have higher progesterone concentrations than lactating dairy cows. It seems that the low concentration of progesterone during development of the dominant follicle influences the release patterns of FSH and LH; however, additional mechanistic studies are needed to determine the exact reason for the increased co-dominance in cows with low progesterone.

The greater proportion of cows detected in estrus near AI in the LP treatment was probably a direct effect of larger ovulatory follicles with greater steroidogenic activity, as observed by the greater concentration of estradiol during proestrus. Previous studies also demonstrated a positive relationship between the diameter of the ovulatory follicle and expression of estrous behavior (Cerri et al., 2004; Galvão et al., 2004) and the concentration of estradiol near AI (Rutigliano et al., 2008). Furthermore, double-ovulating cows had greater concentrations of estradiol at AI and a greater proportion of cows detected in estrus compared with single-ovulating cows, which probably favored the detection of estrus in LP cows as well.

The growth of the ovulatory follicle under different concentrations of progesterone did not affect fertilization or the number of accessory spermatozoa in spite of the greater concentrations of estradiol during proestrus and the greater proportion of cows in estrus near AI for LP than HP cows. Although the importance of estradiol on sperm transport has been known for some time (Hawk, 1983), the current findings suggest that concentrations of estradiol during proestrus did not affect fertilization. In fact, regardless of treatment, concentrations of estradiol on the days of PGF_{2α} and AI were

similar among cows having embryos graded as 1, 2, 3, or degenerated oocytes (data not shown). Even when lactating cows were supplemented with estradiol during proestrus, fertilization remained unaltered (Cerri et al., 2009). Collectively, these data suggest that concentrations of estradiol during the periovulatory period do not seem to be a major factor influencing fertilization success when lactating dairy cows maintain concentrations during proestrus and AI ranging between 4 and 6 pg/mL. The discriminating border of accessory spermatozoa that achieved the highest sensitivity (detection of fertilized oocytes) and specificity (detection of unfertilized oocytes) for fertilization was 4. Results from the current study are in close agreement with our previous work (Cerri et al., 2009), which indicated that fertilization can be achieved with a relatively small number of accessory spermatozoa. Previous reports have observed a positive relationship between number of accessory spermatozoa and fertilization and embryo quality in dairy cows (DeJarnette et al., 1992; Nadir et al., 1993). In fact, grade 1 embryos had a greater number of accessory spermatozoa compared with all other embryo grades and unfertilized oocytes.

We initially hypothesized that follicles growing under a low progesterone environment would be exposed to suboptimal conditions for oocyte maturation that could impair fertilization and embryo development. In a companion study using the same experimental procedures (Cerri et al., 2011), cows with high progesterone had the dominant follicle with greater concentration of total IGF-1 and a numerically greater concentration of free IGF-1 but lower concentrations of estradiol compared with cows with low circulating progesterone concentrations. Shaham-Albalancy et al. (2000), using a similar experimental design, showed greater concentrations of estradiol in the follicular fluid early in follicle development, which could be deleterious to oocyte maturation and further embryo development. Despite the changes in follicular fluid composition, altering progesterone during the development of the ovulatory follicle did not influence early embryo quality. Only a tendency for a greater percentage of live blastomeres in the HP treatment was observed. Conversely, lactating cows super-stimulated with FSH when progesterone concentrations were high yielded a greater proportion of embryos graded as 1 and 2 than cows super-stimulated under low progesterone concentrations (Rivera et al., 2011). Similarly, when the ovulatory follicle developed under low concentrations of progesterone, P/AI was less and pregnancy loss greater than that of cows developing the ovulatory follicle under high concentrations of progesterone (Cunha et al., 2008; Bisinotto et al., 2010). The positive effects of greater concentrations of progesterone during super-stimulation might indicate a pro-

protective mechanism of progesterone on embryo quality when cows receive exogenous FSH and the concurrent high concentrations of estradiol. Excessive exposure to estradiol has been reported as deleterious to the oocyte and early embryo (Inskeep, 2004). The lack of effect of progesterone in the current study suggests that the suppression in fertility observed in cows developing the ovulatory follicle under low progesterone (Cunha et al., 2008; Bisinotto et al., 2010) might be the consequence of changes that occur in the embryo after d 6 or on the endometrium. In fact, low progesterone results in changes in the responsiveness of the endometrium to oxytocin and more short estrous cycles (Shaham-Albalancy et al., 2001; Cerri et al., 2011).

CONCLUSIONS

This study used oocytes-embryos from single-ovulating cows to evaluate the effects of reduced concentrations of progesterone during growth of ovulatory follicles with similar length of dominance on fertilization and early embryo development. Decreased concentrations of progesterone had major effects on follicular dynamics, concentrations of estradiol during proestrus, and expression of estrus at AI. On the other hand, maintaining concentrations of progesterone in plasma between 1 and 2 ng/mL during the development of the ovulatory follicle did not affect embryo quality except for a small decrease in the proportion of viable blastomeres. Therefore, concentrations of progesterone during follicle development do not seem to be a major factor affecting fertilization and early embryo development up to d 6 after AI as initially hypothesized. Responses to low concentrations of progesterone that disrupt pregnancy in dairy cows are likely related to embryonic effects observed after d 6 or to changes in the periovulatory concentrations of hormones that affect subsequent uterine function and embryonic development past d 6 after AI.

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