Targeted progesterone supplementation improves fertility in lactating dairy cows without a corpus luteum at the initiation of the timed artificial insemination protocol

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ABSTRACT

The objectives of this study were to determine the effect of supplemental progesterone on fertility in lactating dairy cows lacking a corpus luteum (CL) at the initiation of the timed artificial insemination (AI) program. Holstein cows were subjected to the 5-d timed AI program (d –8 GnRH, d –3 and –2 PGF_{2α}, d 0 GnRH and AI). Cows had their ovaries scanned by ultrasonography on d –8 and those bearing a CL were considered to be in diestrus (DI; n = 946). Cows that lacked a CL on d –8 were assigned to remain as untreated control (CON; n = 234) or receive 2 controlled internal drug release (CIDR) inserts containing progesterone (2CIDR; n = 218) from d –8 to –3, as a single insert has been proven insufficient to modulate fertility in cows without CL. Blood was analyzed for progesterone and estradiol concentrations. Pregnancy was diagnosed on d 34 and 62 after AI. Progesterone concentrations during the timed AI program were lowest for CON, intermediate for 2CIDR, and highest for DI. Supplementation increased progesterone concentrations between d –7 and –3 compared with CON (2.65 vs. 0.51 ng/mL). Ovulation to the first GnRH was not affected by treatment. However, a greater proportion of CON and 2CIDR cows had a new CL on d –3 compared with DI cows (66.7 vs. 61.9 vs. 52.0%). In cows with a new CL, the diameter of the ovulatory follicle was larger for CON than 2CIDR, and intermediate for DI (18.7 vs. 16.5 vs. 17.7 mm). Concentrations of estradiol on d –3 did not differ among treatments; however, DI cows had greater estradiol concentrations at AI compared with CON or 2CIDR cows. Pregnancy per AI was less for CON compared with 2CIDR or DI on d 32 (30.8 vs. 46.8 vs. 49.9%) and 64 (28.6 vs. 43.7 vs. 47.3%), indicating that supplementation with progesterone reestablished fertility in cows lacking a CL on d –8. A greater proportion of nonpregnant CON cows had

a short reinsemination interval compared with 2CIDR or DI (11.1 vs. 3.5 vs. 5.7%). Treatment did not affect pregnancy loss between d 34 and 62 of gestation. A single ultrasound exam was effective in identifying a low-fertility cohort of cows based on the absence of CL at the first GnRH injection of the timed AI protocol. Progesterone supplementation with 2 CIDR inserts increased progesterone in plasma to 2.65 ng/mL and restored fertility in lactating dairy cows lacking a CL at the initiation of the timed AI program similar to that of cows in diestrus.

Key words: anovular cow, dairy cow, progesterone, reproduction

INTRODUCTION

Protocols for synchronization of the estrous cycle and timed AI have been implemented worldwide to systematically control reproduction in dairy herds. Current programs have been shown to improve pregnancy rates by assuring elevated submission to AI without reducing pregnancy per AI (P/AI) when compared with AI on estrus or natural service (Santos et al., 2009; Lima et al., 2012). Nevertheless, the potential benefits of timed AI programs are not restricted to increasing insemination rate and facilitating management. These regimens also provide a way to target cohorts of low-fertility cows and increase P/AI compared with more traditional reproductive management schemes.

Approximately 30% of postpartum, high-producing dairy cows are anovular or simply lack a corpus luteum (CL) when the synchronization protocol is initiated (Stevenson et al., 2008; Santos et al., 2009; Bisinotto et al., 2010a). For resynchronized AI, the proportion of cows not bearing a CL at the initiation of the timed AI program ranges from 22 to 46% depending on the interval from previous insemination (Fricke et al., 2003; Silva et al., 2009). Recent studies have indicated that the risk of becoming pregnant is reduced by 30% in cows lacking a CL at the initiation of the timed AI program compared with herdmates that bear a functional CL when synchronization is initiated (Bisinotto et al.,

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2010a). Interestingly, this reduction in fertility was not conditional to cyclic status. Cows that had resumed ovulation postpartum but initiated the synchronization program in the absence of a functional CL (i.e., during proestrus, estrus, or metestrus) had similar P/AI compared with anovular cows, with both having lower P/AI than cows in diestrus. Therefore, the absence of a CL at the first GnRH of the timed AI program defines a low-fertility cohort that represents nearly 30% of all cows that need to be inseminated. Notably, a single ultrasound evaluation of the ovaries can identify this low-fertility cohort to avoid more costly and labor intensive approaches to diagnose anovulatory conditions (Stevenson et al., 2008).

It is likely that the decline in fertility observed in cows undergoing the timed AI program in the absence of a functional CL at the initial GnRH injection is associated with reduced concentrations of progesterone during the development of the ovulatory follicle (Denicol et al., 2012). Low plasma concentrations of progesterone during the growth of the ovulatory follicle alter several reproductive processes associated with establishment and maintenance of pregnancy. Preovulatory suboptimal concentrations of progesterone affect uterine endometrial morphology (Shaham-Albalancy et al., 1997), which is thought to affect endometrial secretory function during early gestation and also increase the synthesis of PGF_{2 α} in response to oxytocin (Shaham-Albalancy et al., 2001; Cerri et al., 2011b). This altered responsiveness of the endometrium to oxytocin suggests that luteolytic signals might be upregulated in cows with low progesterone concentrations, thereby leading to shortened subsequent CL lifespan (Cerri et al., 2011b). Moreover, reduced concentrations of progesterone have been associated with greater LH concentrations, faster growth of the ovulatory follicle, lower intra-follicular IGF-1, and compromised embryo quality (Cerri et al., 2011a,b; Rivera et al., 2011).

Incorporation of supplemental progesterone into timed AI programs has not consistently improved fertility in lactating dairy cows lacking a CL (Bisinotto and Santos, 2011). Commercially available controlled internal drug release (CIDR) inserts release, on average, 620 mg of progesterone over a course of 7 d (Rathbone et al., 2002). This release results in circulating progesterone concentrations of only 0.8 to 1.0 ng/mL in high-producing, lactating dairy cows (Cerri et al., 2009a). This marginal increase in progesterone concentration has been shown to improve P/AI in cows in some studies (Bisinotto et al., 2010b), but might be insufficient to promote high fertility in cows without a CL at the initiation of the synchronization protocol. In fact, most of the benefit of supplemental progesterone with administration of a single insert during timed

AI programs originates from cows with a CL at the initiation of the protocol, not from anovular or cows without a CL (Bisinotto and Santos, 2011). Previous studies have suggested that increasing the amount of supplemental progesterone during the development of the ovulatory follicle, by using 2 CIDR inserts, might restore fertility in cows without CL (Lima et al., 2009; Denicol et al., 2012).

The hypotheses of the present study were that (1) cows without a visible CL at the initiation of the timed AI program have reduced P/AI compared with cows in diestrus, and (2) that progesterone supplementation via 2 CIDR inserts, to increase concentrations of progesterone in plasma in approximately 2.0 ng/mL, restores fertility in lactating dairy cows lacking a visible CL at this time. Therefore, the objectives were to investigate the effects of supplementing dairy cows without a CL at the initiation of the timed AI program with 2 CIDR inserts for sustained release of progesterone on concentrations of progesterone in plasma, ovarian responses, P/AI, and pregnancy loss.

MATERIALS AND METHODS

All procedures involving animals in this study were approved by the University of Florida Nonregulatory Animal Research System Committee.

Cows, Housing, and Diets

The study was conducted on a dairy farm in central Florida, milking 4,366 cows with yearly a rolling herd average milk yield of 10,949 kg. Weekly cohorts of cows were enrolled during 13 consecutive weeks and all inseminations were performed from January 27 to April 22, 2011. Primiparous ($n = 515$) and multiparous ($n = 883$) cows were housed separately in freestall barns equipped with sprinklers and fans. Cows received the same TMR to meet or exceed the nutrient requirements for a lactating Holstein cow producing 45 kg/d of milk with 3.5% fat and 3.2% true protein when DM intake is 25 kg/d (NRC, 2001). Diet consisted of ryegrass silage, corn silage, ground corn, citrus pulp, solvent-extracted soybean meal, expeller soybean meal, corn gluten feed, molasses, minerals, and vitamins. Cows were fed twice and milked thrice daily.

Reproductive Management

All 1,398 Holstein cows were subjected to the 5-d timed AI program (Figure 1); 628 were enrolled at the first postpartum AI and 770 cows were enrolled during a resynchronized insemination after diagnosed as non-pregnant. Cows receiving the first AI postpartum had

their estrous cycle presynchronized with 2 i.m. injections of PGF_{2α} (5 mL of Lutalyse Sterile Solution, dinoprost tromethamine, equivalent to 5 mg of dinoprost/mL; Pfizer Animal Health, Madison, NJ) administered 14 d apart, at 50 ± 3 and 64 ± 3 DIM. The timed AI protocol was initiated 12 d after the second PGF_{2α} of the presynchronization protocol, with mean and median DIM of 76 and 75, respectively. Cows receiving resynchronized inseminations were enrolled in the 5-d timed AI program following a nonpregnancy diagnosis at 34 ± 3 d after the previous insemination, with mean and median DIM of 156 and 127 DIM, respectively. The timed AI program consisted of an i.m. injection of 100 µg of GnRH (2 mL of Cystorelin, gonadorelin diacetate tetrahydrate equivalent to 43 µg of gonadorelin/mL; Merial Ltd., Duluth, GA) followed by 2 i.m. injections of PGF_{2α} 5 and 6 d later. A second i.m. injection of GnRH was administered concurrently with AI at 72 h after the first PGF_{2α}. For study purposes, the day of timed AI was labeled as study d 0 and the day of the first GnRH of the 5-d timed AI protocol as study d -8.

The tailheads of cows were painted daily with chalk starting on d -8 and removal of chalk was used as an indication of estrus. Cows detected in estrus between study d -8 and -3 were excluded from the statistical

analyses. Cows detected in estrus from d -3 to -1 were inseminated on the same day and remained in the study.

Ultrasonography of the Ovaries and Treatments

All cows in the study had their ovaries evaluated by ultrasonography using a portable ultrasound equipped with a 7.5-MHz transrectal linear transducer (Easi-Scan, BCF Technology, Rochester, MN) on study d -8, and the location of CL and follicles greater than 10 mm in diameter were recorded. Cows bearing a CL greater than 10 mm in diameter were considered to be in diestrus (**DI**; $n = 946$). Cows lacking a CL were blocked by service number (first AI vs. resynchronized AI) and parity (primiparous vs. multiparous). Within each block, cows were allocated randomly into one of 2 treatments; progesterone supplementation or control. Cows assigned to receive progesterone supplementation (**2CIDR**; $n = 218$) were treated with 2 CIDR inserts, each containing 1.38 g of progesterone (Eazi-Breed CIDR Cattle Insert, Pfizer Animal Health, Madison, NJ) from study d -8 to -3; whereas control cows (**CON**; $n = 234$) received no supplemental progesterone. The proportions of CON, 2CIDR, and DI cows

Figure 1. Diagram of activities for cows subjected to the first AI postpartum. Study d 0 is the day of insemination. Treatments: Control = cows without a corpus luteum (CL) on d -8; 2CIDR = cows without a CL on d -8 and treated with 2 controlled internal drug-release (CIDR) inserts containing progesterone from d -8 to -3; Diestrus = cows with a CL on d -8. BS = blood sampling; US = ovarian ultrasonography.

enrolled in the study in the first AI were 62.0, 64.7, and 36.1%, respectively.

A second ultrasound evaluation of the ovaries on study d -3 was performed in all CON ($n = 234$) and 2CIDR cows ($n = 218$), and in a subset of DI cows ($n = 354$). Ovulation in response to the GnRH injection on study d -8 was considered when the cow had a follicle ≥ 10 mm on d -8 and a new CL was observed on d -3. Cows with follicles < 10 mm on study d -8, but with a new CL on study d -3 were considered to have a new CL, but ovulated before study d -8.

A subset of 56 CON, 47 2CIDR, and 49 DI cows had their ovaries scanned (Aloka SSD-500 equipped with a 7.5-MHz linear transducer, Aloka Co., Tokyo, Japan) on d 0 and 2, corresponding to the day of timed AI and again 48 h later. The diameter of all follicles greater than 5 mm was measured. Ovulation to the GnRH on d 0 was defined as the disappearance of one or more follicles ≥ 10 mm in diameter within 48 h.

Blood Sampling and Analyses of Progesterone and Estradiol in Plasma

Blood was sampled from a subset of 71 cows (CON = 24; 2CIDR = 24; DI = 23) by puncture of the coccygeal vein or artery into evacuated tubes containing K_2 EDTA (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) on study d -8, -7, -5, -3, -2, 0, 2, 5, 8, 11, and 14. Samples were placed immediately in ice. Blood tubes were centrifuged at $3,000 \times g$ for 15 min at 4°C for plasma separation within 6 h of collection; plasma samples were frozen at -20°C until assayed. Concentrations of progesterone were analyzed in samples by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.15 ng/mL, calculated as 2 SD below the mean counts per minute at maximum binding. Samples with known concentrations of progesterone (1.5 and 2.5 ng/mL) were incorporated into the assay for quality control. All samples were assayed in a single assay and the intra-assay CV was 6.4%. Concentrations of estradiol were analyzed in plasma samples collected on study d -3 and 0 by RIA using a commercial kit (Estradiol Double Antibody, Siemens Healthcare Diagnostics) validated previously for use in bovine samples (Siddiqui et al., 2009). The sensitivity of the assay was 0.55 pg/mL calculated as 2 SD below the mean counts per minute at maximum binding. Samples were analyzed in duplicate and repeated when the CV between duplicates was $> 15\%$. Plasma from a cow exhibiting standing activity, charcoal-stripped plasma from a male calf, and samples with known concentrations of estradiol (2.5, 5.0, and 10.0 pg/mL) were incorporated into the assay for quality control. Samples were analyzed

in 4 assays; the intraassay CV averaged 12.5% and the interassay CV was 14.8%.

Pregnancy Diagnosis and Calculation of Reproductive Responses

Pregnancy was diagnosed by transrectal ultrasonography on d 34 after AI. The presence of an amniotic vesicle containing an embryo with a heartbeat was used as the determinant of pregnancy. Pregnant cows on d 34 were reexamined for pregnancy by transrectal palpation 4 wk later, on d 62 of gestation. Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant at d 34 or 62 after AI by the number of cows receiving AI. Pregnancy loss was calculated as the number of cows that lost a pregnancy between d 34 and 62 after AI divided by the number of cows diagnosed pregnant on d 34 after AI. Cows that were detected in estrus before study d 32 were reinseminated and considered nonpregnant. Short AI interval was defined for cows reinseminated from study d 5 to 17.

BCS and Milk Yield

Body condition of all cows was scored (Ferguson et al., 1994) on study d -8. For statistical analysis, BCS was categorized as low (≤ 2.50) or high (≥ 2.75). Yields of milk were recorded for individual cows once monthly using on-farm milk meters (Tru-Test Ltd., Manukau, New Zealand). Measurements for the first 3 mo postpartum were used to derive an individual 305-d production estimate (Wiggans and Powell, 1980). Within parity group, cows were categorized according to milk production as being above or below the average for statistical analyses.

Experimental Design and Statistical Analyses

Cows lacking a CL on study d -8 were assigned to treatments in a randomized block design. Cows were blocked by service number (first postpartum AI or resynchronized AI) and parity (primiparous or multiparous) and randomly assigned to either CON or 2CIDR. By design, contemporary herdmates that had a CL on d -8 were labeled as DI and used as a positive control to evaluate the effectiveness of progesterone supplementation in restoring fertility of cows without CL relative to a cow with CL. We have previously shown that cows without a CL at the beginning of the synchronization protocol have a 13-percentage-point decrease in P/AI compared with cows in diestrus (Bisinotto et al., 2010a). Similarly, anovular cows had a 12-percentage-point decrease in P/AI compared with estrous cyclic cows (Santos et al., 2009). It was assumed that cows

in 2CIDR would have similar P/AI to that of DI cows, and both would be 13 percentage points greater than cows on the CON group. Therefore, a sample size calculation was performed using the POWER procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC) to allow for sufficient experimental units to detect an increase in P/AI of 13 percentage points following progesterone supplementation of cows without a CL (CON vs. 2CIDR; $\alpha = 0.05$; $\beta = 0.20$). Pregnancy per AI for CON cows was assumed to be 30% (Bisinotto et al., 2010a). Under such assumptions, a total of 215 cows per treatment were calculated to be required.

Data were analyzed initially with only randomly assigned cows (CON and 2CIDR) and then with all 3 treatments (CON, 2CIDR, and DI). Because estimates of differences between CON and 2CIDR remained basically the same when both strategies were used, the approach with 3 treatments was selected to identify differences in fertility responses between CON and DI and between 2CIDR and DI.

Binary responses were analyzed by logistic regression using the GLIMMIX procedure of SAS. Parity (primiparous vs. multiparous), BCS category (≤ 2.50 or ≥ 2.75), milk yield category (above or below the mean milk production within parity), service number (first postpartum AI vs. resynchronized AI), and the respective interactions with treatment were included as covariates. For P/AI and pregnancy loss, sire, technician, and their interactions with treatment were also included in the statistical models. Multivariable logistic models were built and a backward stepwise elimination method was applied, in which covariates were continuously removed when $P > 0.10$. Treatment was forced into the final model in all analyses. To evaluate if cows in estrus at AI had differential response to treatments, an additional model was built to analyze P/AI and pregnancy loss with the effects of estrus at AI and the interaction between treatment and estrus at AI. Adjusted risk ratios (**ARR**) and respective 95% CI were estimated by analyzing binary data with a modified Poisson regression model with the GENMOD procedure of SAS using a log link function and correction for data dispersion (Spiegelman and Hertzmark, 2005; Fang, 2011).

Pregnancy per AI was also analyzed for the subset of 806 cows in which ovarian responses on d -8 and -3 were evaluated by ultrasonography. The statistical models were the same as indicated above, but also included the presence of a new CL on d -3 and the interaction between treatment and presence of new CL.

Continuous and discrete variables were analyzed by ANOVA using the GLIMMIX procedure of SAS fitting a normal distribution. Tests for normality of residuals and homogeneity of variances were conducted for each

variable using the guided data analysis option of SAS. Data which did not fulfill the assumptions of ANOVA were transformed accordingly. Back-transformed data using the ilink function of SAS are presented for clarity. The effects of treatment, parity, and interaction between treatment and parity were included in the model as independent variables. Concentrations of progesterone and estradiol were analyzed as repeated measures and cow nested within treatment was considered a random effect. The concentration of progesterone on the day of AI was included as a covariate in the analysis of progesterone concentrations from study d 2 to 14. The first-order autoregressive was selected as covariance structure because it presented the smallest Schwarz's Bayesian information criterion value.

Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered as tendencies.

RESULTS

The average 305-d milk yield did not differ ($P = 0.95$) among treatments, and cows averaged $10,589 \pm 58$ kg. Multiparous cows had greater ($P < 0.001$) daily milk production in the first 305 DIM than primiparous cows (36.6 ± 0.3 vs. 31.6 ± 0.4 kg/d). An interaction ($P = 0.005$) between treatment and service number was observed for BCS on study d -8. For cows at first AI postpartum, BCS was greater for DI (2.80 ± 0.02) than for CON (2.72 ± 0.03) and 2CIDR (2.74 ± 0.03); however, BCS did not differ among treatments (CON = 3.01 ± 0.03 ; 2CIDR = 3.01 ± 0.04 ; DI = 2.97 ± 0.01) in cows receiving resynchronized AI. Consequently, the proportion of cows receiving the first AI postpartum that were classified as having low BCS was greater ($P = 0.01$) for CON and 2CIDR than for DI (35.6, 32.6, and 16.1%, respectively), whereas no difference was observed for cows receiving resynchronized AI (CON = 11.4; 2CIDR = 6.5; DI = 9.6%). Of the initial 218 cows assigned to 2CIDR, 4 cows lost 1 device and 4 cows lost both devices before removal on d -3.

Ovarian Responses to the Timed AI Protocol

The ovulatory response to the first GnRH was not affected by treatment (Table 1). However, because some of the cows not bearing a CL were likely in metestrus on d -8, a greater ($P = 0.001$) proportion of CON and 2CIDR cows had a newly formed CL on d -3 compared with DI cows. Both the ovulatory response to the first GnRH on d -8 (58.2 vs. 46.7%; ARR = 1.24; 95% CI = 1.08 to 1.43) and the presence of a newly formed CL on d -3 (63.7 vs. 51.4%; ARR = 1.22; 95% CI = 1.08 to 1.39) were greater ($P < 0.001$) for cows receiv-

ing the first AI postpartum than for those receiving resynchronized AI. Only 2.3% (8 of 354) of DI cows did not present a visible CL on d -3. Therefore, in spite of a smaller occurrence of newly formed CL, the proportion of cows with a CL on d -3 was greater ($P = 0.001$) for DI compared with CON and 2CIDR cows (Table 1). Cows subjected to the first AI postpartum were more likely ($P < 0.001$) to bear a visible CL at PGF_{2α} than those receiving resynchronized AI (81.7 vs. 74.8%; ARR = 1.12; 95% CI = 1.04 to 1.20). The proportion of cows that had a synchronized ovulation within 48 h of the second injection of GnRH of the timed AI was greater ($P = 0.05$) for DI than for CON (ARR = 1.16; 95% CI = 1.03 to 1.32) and intermediate for 2CIDR cows. An interaction ($P = 0.02$) between treatment and presence of a newly formed CL on d -3 was observed for the diameter of the ovulatory follicle on d 0 (Table 1). Diameter of the ovulatory follicle did not differ among treatments in cows without a newly formed CL; however, for cows that had a newly formed CL on d -3, the diameter of the ovulatory follicle was larger ($P < 0.05$) for CON than 2CIDR (Table 1).

Concentrations of Progesterone Before and After AI

The concentration of progesterone in plasma on study d -8 was greater ($P < 0.001$) for cows in DI than for those not bearing a CL (Figure 2). As expected, CON

and 2CIDR cows had similar ($P = 0.88$) concentrations of progesterone on the d -8. The incorporation of 2 CIDR inserts to the timed AI program increased ($P < 0.05$) circulating concentrations of progesterone, which were consistently greater for 2CIDR than for CON cows throughout the timed AI program. A treatment by day interaction was observed ($P < 0.001$) for circulating progesterone from study d -7 to -3. This interaction occurred because concentrations of progesterone in CON and DI cows increased from d -7 to d -3, whereas 2CIDR cows had a slight decline in progesterone concentrations during the same period (Figure 2). Concentrations of progesterone were greater ($P = 0.02$) for DI (0.31 ng/mL) compared with 2CIDR and CON cows (0.19 ng/mL) on d 0. Treatment did not affect progesterone concentrations during the first 14 d after AI (Figure 3).

Expression of Estrus and Concentration of Estradiol at AI

Although the overall proportion of cows detected in estrus at AI was similar among treatments (Table 2), an interaction ($P < 0.001$) between treatment and the presence of a newly formed CL on d -3 influenced detection of estrus. For cows without a new CL, detection of estrus was less for CON than 2CIDR and DI (21.8 vs. 41.0 vs. 47.0%). On the other hand, for cows with

Table 1. Effect of the presence of a corpus luteum (CL) and progesterone supplementation for cows without a CL at the initiation of the timed AI program on ovarian and endocrine responses in lactating dairy cows subjected to the 5-d timed AI program

Item	Treatment ¹			P-value
	CON	2CIDR	DI	
Ovulation to GnRH d -8, ² % (no./no.)				
All cows	55.1 (129/234)	56.0 (122/218)	51.4 (182/354)	0.51
Cows with follicles ≥ 10 mm	57.9 (129/223)	57.8 (122/210)	52.2 (182/349)	0.31
New CL on d -3, ³ % (no./no.)	66.7 (156/234) ^a	61.9 (135/218) ^a	52.0 (184/354) ^b	0.01
Cows with CL on d -3, % (no./no.)	66.7 (156/234) ^b	61.9 (135/218) ^b	97.7 (346/354) ^a	0.01
Number of CL per cow on d -3	0.72 $\pm$ 0.04 ^b	0.64 $\pm$ 0.04 ^b	1.54 $\pm$ 0.03 ^a	0.01
Ovulation to GnRH d 0, ⁴ % (no./no.)	87.5 (49/56) ^b	89.4 (42/47) ^B	98.0 (48/49) ^{a,A}	0.05
Ovulatory follicle on d 0, ⁵ mm				
Cows with new CL on d -3	18.7 $\pm$ 0.5 ^a	16.5 $\pm$ 0.6 ^b	17.7 $\pm$ 0.6 ^{a,b}	0.02
Cows without new CL on d -3	17.0 $\pm$ 0.9	19.2 $\pm$ 0.8	19.0 $\pm$ 0.9	0.17
Estradiol, pg/mL				
d -3	3.19 $\pm$ 0.44	2.76 $\pm$ 0.40	2.26 $\pm$ 0.40	0.30
d 0	4.81 $\pm$ 0.44 ^b	4.04 $\pm$ 0.40 ^b	6.17 $\pm$ 0.40 ^a	0.001

^{a,b}Values with different superscripts differed ($P < 0.05$).

^{A,B}Values with different superscripts tended to differ ($P < 0.10$).

¹All cows were subjected to the 5-d timed AI protocol (d -8 GnRH, d -3 and -2 PGF_{2α}, d 0 GnRH and timed AI). CON = cows without a CL on d -8; 2CIDR = cows without a CL on d -8 and treated with two controlled internal drug-release inserts containing progesterone from d -8 to -3; DI = cows with a CL on d -8 (diestrus).

²Follicle ≥ 10 mm present on d -8 and appearance of a new CL in the same ovary on d -3.

³New CL detected on d -3 that was not present on d -8.

⁴Follicle ≥ 10 mm present on d 0 that disappeared on d 2.

⁵Only cows that ovulated in response to the injection of GnRH on d 0 were considered.

Figure 2. Concentrations of progesterone in plasma according to study day. Cows received GnRH on d -8, PGF_{2α} on d -3 and -2, and GnRH and AI on d 0. Treatments: CON (▼; cows without a corpus luteum on d -8; n = 24); 2CIDR (□; cows without a corpus luteum on d -8 treated with 2 controlled internal drug-release inserts containing progesterone from d -8 to -3; n = 24); DI (●; cows with a corpus luteum on d -8, diestrus; n = 23). Concentrations of progesterone from d -7 to -3 averaged 0.51 ± 0.27 , 2.65 ± 0.26 , and 3.40 ± 0.25 ng/mL for CON, 2CIDR, and DI, respectively. Effects of treatment ($P < 0.001$), day ($P = 0.01$), and interaction between treatment and day ($P < 0.001$). Within day, concentrations of progesterone differed (a, b, c; $P \leq 0.05$).

Figure 3. Concentrations of progesterone in plasma during the first 14 d after AI. Study d 0 refers to day of AI. Treatments: CON (▼; cows without a corpus luteum on d -8; n = 24); 2CIDR (□; cows without a corpus luteum on d -8 treated with 2 controlled internal drug-release inserts containing progesterone from d -8 to -3; n = 24); DI (●; cows with a corpus luteum on d -8, diestrus; n = 23). Effects of treatment ($P = 0.99$), day ($P < 0.001$), and interaction between treatment and day ($P = 0.95$).

However, on d 0, when AI was performed, CON and 2CIDR cows had lower ($P < 0.001$) concentrations of estradiol than DI cows.

a newly formed CL, detection of estrus was greater for CON, followed by 2CIDR, and then DI (40.4 vs. 33.3 vs. 25.0%).

Circulating concentrations of estradiol did not differ among treatments on d -3, when the first PGF_{2α} of the 5-d timed AI protocol was administered (Table 1).

Pregnancy per AI, Short AI Interval, and Pregnancy Loss

Treatment affected ($P < 0.01$) P/AI on d 34 and 62 after insemination (Table 2). Pregnancy per AI for 2CIDR and DI did not differ; however, they were both

Table 2. Effect of the presence of a corpus luteum (CL) and progesterone supplementation for cows without a CL at the initiation of the timed AI program on fertility responses in lactating dairy cows subjected to the 5-d timed AI program

Item	Treatment ¹			P-value
	CON	2CIDR	DI	
Estrus at AI, ² % (no./no.)	34.2 (80/234)	36.2 (79/218)	35.0 (331/946)	0.80
Short AI interval, ³ % (no./no.)	11.1 (18/162) ^a	3.5 (4/116) ^b	5.7 (27/474) ^b	0.02
Pregnant, % (no./no.)				
d 34	30.8 (72/234) ^b	46.8 (102/218) ^a	49.9 (472/946) ^a	<0.01
d 62	28.6 (67/234) ^b	43.7 (94/215) ^a	47.3 (445/941) ^a	<0.01
Pregnancy loss, ⁴ % (no./no.)	6.9 (5/72)	5.1 (5/99)	4.7 (22/467)	0.72

^{a,b}Values with different superscripts differed ($P < 0.05$).

¹All cows were subjected to the 5-d timed AI protocol (d -8 GnRH, d -3 and -2 PGF_{2α}, d 0 GnRH and timed AI). CON = cows without a CL on d -8; 2CIDR = cows without a CL on d -8 and treated with 2 controlled internal drug-release inserts containing progesterone from d -8 to -3; DI = cows with a CL on d -8 (diestrus).

²Evaluated based on removal of tail chalk at timed AI.

³Nonpregnant cows on d 34 after AI that were reinseminated from 5 to 17 d after previous AI.

⁴Calculated as [(no. of cows pregnant on d 34 that were not pregnant on d 62/no. of pregnant cows on d 34) × 100]. Five diestrus and three 2CIDR cows left the study before reconfirmation of pregnancy on d 62 after AI.

Table 3. Effect of the presence of a corpus luteum (CL) and progesterone supplementation for cows without a CL at the initiation of the timed AI program on fertility responses according to service number¹

Item	First postpartum AI			Resynchronized AI			<i>P</i> -value ²		
	CON	2CIDR	DI	CON	2CIDR	DI	TRT	AI	TRT × AI
Pregnant, % (no./no.)									
d 34	34.3 (50/146)	49.7 (70/141)	54.3 (185/341)	25.0 (22/88)	41.6 (32/77)	47.4 (287/605)	<0.01	<0.01	0.27
d 62	32.2 (47/146)	46.4 (64/138)	51.5 (175/340)	22.7 (20/88)	39.0 (30/77)	44.9 (270/601)	<0.01	<0.01	0.41
Pregnancy loss, % (no./no.)	6.0 (3/50)	4.5 (3/67)	4.9 (9/184)	9.1 (2/22)	6.3 (2/32)	4.6 (13/283)	0.72	0.60	0.83

¹All cows were subjected to the 5-d timed AI protocol (d -8 GnRH, d -3 and -2 PGF_{2α}, d 0 GnRH and timed AI). CON = cows without a CL on d -8; 2CIDR = cows without a CL on d -8 and treated with two controlled internal drug-release inserts containing progesterone from d -8 to -3; DI = cows with a CL on d -8 (diestrus).

²TRT = effect of treatment; AI = effect of service number (first AI vs. resynchronized AI); TRT × AI = interaction between TRT and AI.

greater ($P < 0.01$) than the CON. In fact, supplementing progesterone in cows without a CL on d -8 increased the risk of pregnancy on d 62 by 58% (ARR = 1.58; 95% CI = 1.30 to 1.93). No interactions between treatment and parity, milk yield, or BCS were observed for P/AI. Cows receiving the first AI postpartum, which was preceded by presynchronization with PGF_{2α}, had greater P/AI on d 32 and 64 than cows receiving resynchronized inseminations (Table 3). The benefits of supplementing progesterone to cows without a CL on d -8 were similar whether they received the first or resynchronized AI, and no differences were observed between 2CIDR and DI cows according to service number. When detection of estrus at AI was included in the statistical models, an interaction ($P < 0.01$) between treatment and estrus at AI was observed to affect P/AI on d 34 and 62 (Table 4). Cows in estrus had greater P/AI in all treatments; however, the benefits to pregnancy from supplemental progesterone were observed only among cows that were not detected in estrus at AI.

For the subset of 806 cows with ovarian responses on d -8 and -3, presence of a new CL influenced ($P < 0.01$) P/AI on d 34 and 62 after insemination. Furthermore, an interaction ($P < 0.01$) between treatment and presence of a new CL on d -3 was observed because the increment in P/AI for cows supplemented with progesterone was greater among those that did not have a new CL than in those with a new CL on d -3. On d 62, in cows with a new CL detected on d -3, the P/AI did not differ between 2CIDR and DI (47.4 vs. 51.1%), but were both greater ($P < 0.01$) than CON (37.2%). Similarly, for cows without a new CL on d -3, the P/AI did not differ between 2CIDR and DI (37.8 vs. 45.3%), but were both greater ($P < 0.01$) than CON (11.5%).

The proportion of cows that were reinseminated from d 5 to 17 after AI was greater ($P = 0.02$) for CON than DI and 2CIDR cows (Table 2). For the subset of 806 cows with ovarian responses on d -8 and -3, a tendency for an interaction ($P = 0.08$) between treatment and presence of new CL on d -3 was observed for the proportion of cows with short AI interval. For cows

Table 4. Effect of the presence of a corpus luteum (CL) and progesterone supplementation for cows without a CL at the initiation of the timed AI program on fertility responses according to detection of estrus at AI¹

Item	Cows in estrus at AI ²			Cows not in estrus at AI			<i>P</i> -value ³		
	CON	2CIDR	DI	CON	2CIDR	DI	TRT	E	TRT × E
Pregnant, % (no./no.)									
d 34	50.0 (40/80)	48.1 (38/79)	52.0 (172/331)	20.8 (32/154)	46.0 (64/139)	48.8 (300/615)	<0.01	<0.01	<0.01
d 62	48.8 (39/80)	46.8 (37/79)	51.4 (169/329)	18.2 (28/154)	41.9 (57/136)	45.1 (276/612)	<0.01	<0.01	<0.01
Pregnancy loss, % (no./no.)	2.5 (1/40)	2.6 (1/38)	0.6 (1/170)	12.5 (4/32)	6.6 (4/61)	7.1 (21/297)	0.72	<0.01	0.58

¹All cows were subjected to the 5-d timed AI protocol (d -8 GnRH, d -3 and -2 PGF_{2α}, d 0 GnRH and timed AI). CON = cows without a CL on d -8; 2CIDR = cows without a CL on d -8 and treated with two controlled internal drug-release inserts containing progesterone from d -8 to -3; DI = cows with a CL on d -8 (diestrus).

²Detection of estrus was based on removal of tail chalk at timed AI.

³TRT = effect of treatment; E = effect of detection of estrus at AI; TRT × E = interaction between TRT and E.

with a new CL, treatment had no effect ($P = 0.97$) on short AI interval (CON = 4.6 vs. 2CIDR = 4.8 vs. DI = 5.3%). Conversely, for cows without a new CL on d -3, those in 2CIDR or DI groups had a lower ($P < 0.01$) incidence (2.0 and 3.3%) of short AI interval than CON (19.4%).

Pregnancy loss between d 34 and 62 of gestation did not differ with treatments (Table 2) in both the first and the resynchronized AI (Table 3). Cows that showed estrus at AI had smaller ($P < 0.01$) pregnancy loss than those not detected in estrus (7.4 vs. 1.2%; Table 4).

DISCUSSION

As initially hypothesized, cows without a CL at the initiation of the timed AI program had a marked reduction in fertility compared with those synchronized during diestrus. Supplementation with 2 CIDR inserts containing progesterone elevated concentrations in plasma to 2.65 ng/mL during the growth of the ovulatory follicle and reestablished P/AI similar to that of DI cows. Interestingly, the benefits to improving fertility with 2CIDR were observed in cows at first postpartum AI and during a resynchronized insemination. Although cows in the current study were not evaluated by ultrasonography twice to detect estrous cyclicity, approximately 70% of the cows without a CL on the first GnRH of the timed AI program, after receiving 2 treatments with PGF_{2α}, are expected to be anovular (Bisinotto et al., 2010a). Therefore, the improvements in P/AI observed with supplemental progesterone were likely observed in anovular as well as estrous cyclic cows on stages of the estrous cycle in which no active CL is present.

In many farms, population-driven approaches are typically used to control reproduction. However, strategic therapies applied to identifiable cohorts of low-fertility cows are expected to further improve reproductive performance. For instance, nearly 30% of all cows subjected to Presynch/Ovsynch programs for first AI postpartum (Stevenson et al., 2008; Bisinotto et al., 2010a) and to resynchronization protocols (Fricke et al., 2003; Silva et al., 2009) do not bear a functional CL at the initial GnRH of timed AI programs. Therefore, a single ultrasound evaluation of the ovaries is sufficient to cluster anovular cows and estrous cyclic cows not in diestrus, both of which have low fertility (Bisinotto et al., 2010a). In fact, our findings agree with those of previous reports indicating that cows initiating the timed AI protocol in the absence of a functional CL have reduced P/AI compared with herdmates in diestrus (Bisinotto et al., 2010a; Denicol et al., 2012). In the current study, the relative risk of being pregnant on d 62 after AI for cows in DI was 66% greater than

that of CON cows (ARR = 1.66; 95% CI = 1.36–2.01). Interestingly, when cows without a CL at d -8 received 2 CIDR inserts to supplement progesterone, the concentrations of progesterone in plasma increased with an average concentration of 2.65 ng/mL during the entire treatment period, which reestablished P/AI similar to that of DI cows.

Studies have clearly documented the importance of adequate circulating progesterone during the development of the ovulatory follicle. Fonseca et al. (1983) were among the first to observe that concentrations of progesterone during the 12 d preceding the first postpartum AI were positively associated with pregnancy at first insemination. Xu et al. (1997) demonstrated that cows inseminated after receiving PGF_{2α} on d 5 to 9 of the estrous cycle had reduced fertility compared with cows inseminated after PGF_{2α} administered from d 10 to 19 of the estrous cycle. Interestingly, when cows were supplemented with progesterone before the treatment with PGF_{2α}, the stage of the estrous cycle had no influence on P/AI. These data indicate that progesterone concentrations during the development of the ovulatory follicle are critical to achieve high fertility in dairy cows (Bisinotto et al., 2010a). When the estrous cycle of cyclic cows was manipulated such that the Ovsynch was initiated during proestrus or estrus, P/AI dramatically decreased compared with cows in diestrus (Cunha et al., 2008; Bisinotto et al., 2010a). Interestingly, when cows initiating the Ovsynch protocol in proestrus received 2 CIDR inserts containing progesterone, P/AI was restored to values similar to those of cows initiating the Ovsynch in diestrus (Denicol et al., 2012). An alternative approach would be to presynchronize the estrous cycle such that the proportion of cows in diestrus at the beginning of the timed AI protocol is maximized.

Progesterone concentrations in lactating dairy cows fluctuate between 4.0 and 5.8 ng/mL during mid diestrus (Sartori et al., 2004), which are about 5-fold greater than the increase resultant from the use of a single progesterone insert administered to high-producing cows (Cerri et al., 2009a). Elevated portal blood flow associated with increased steroid catabolism by the liver reduces the concentrations of progesterone in lactating cows, and has been suggested to be responsible for the lower concentrations of progesterone in high-producing lactating dairy cows receive a single CIDR (Cerri et al., 2009a). When a single CIDR insert was administered to dairy cows, the amount of progesterone released was approximately 88.6 mg/d, which was similar between inserts containing 1.38 or 1.90 g of progesterone (Rathbone et al., 2002). Although the

resulting concentrations of progesterone will vary with animal type, in high-producing cows the release of 88.6 mg/d usually increases progesterone in plasma by approximately 0.8 to 1.0 ng/mL (Cerri et al., 2009a; Lima et al., 2009). Previous studies in which a single CIDR was incorporated into timed AI programs demonstrated inconsistent responses in anovular cows (Bisinotto and Santos, 2011). The incremental progesterone from a single insert might be insufficient to optimize follicle or oocyte maturation during the final stages of development before AI. In fact, a recent review of the literature that included 10 studies indicated that P/AI in high-producing lactating dairy cows treated with a single CIDR insert increased 5 percentage points compared with untreated controls (Bisinotto and Santos, 2011). Nevertheless, the benefit to P/AI was observed mostly in estrous cyclic cows, with only a minimal response noted in anovular cows. Limited data from Lima et al. (2009) suggested that increasing the amount of supplemental progesterone, by using 2 CIDR, restored P/AI in cows without a CL at the beginning of the time of AI protocol similar to that of estrous cyclic cows. A similar response was not observed when a single CIDR insert was used. It is interesting to note that, for cows in estrus on the day of insemination, the P/AI did not differ among treatments and they were all high. In general, cows that come in estrus on the day of timed AI have increased fertility (Santos et al., 2010), in part because of improved luteal regression and synchronization of ovulation at AI. Only 35.1% of all cows were detected in estrus on the day of AI and no differences were observed among treatments. Interestingly, in CON cows, detection of estrus was greater when they ovulated to the initial GnRH on d -8, a response that was not observed in 2CIDR and DI. It is possible that ovulation to the initial GnRH, which typically improves embryo quality (Cerri et al., 2009b), and changes in the follicular environment in the subgroup of CON cows that displayed estrus were sufficient to result in high P/AI. Conversely, for cows not in estrus, supplemental progesterone during growth of the ovulatory follicle was extremely beneficial to fertility.

Reducing concentrations of progesterone during the development of the ovulatory follicle increased basal LH concentrations, which in turn hastened follicle growth (Cerri et al., 2011b). Cows with low progesterone concentrations had larger ovulatory follicles associated with reduced total IGF-1 and greater estradiol concentrations in the follicular fluid (Cerri et al., 2011b). Such increase in LH availability has also been associated with advances in oocyte maturation, marked by early resumption of meiosis and germinal vesicle breakdown (Revah and Butler, 1996). In lactating dairy cows, starting the superstimulatory treatment with FSH in the absence of

a functional CL reduced embryo quality on d 7 after AI (Rivera et al., 2011). In the same study, incorporating 2 CIDR during the superstimulatory treatment increased progesterone concentrations to approximately 3.0 ng/mL and reestablished embryo quality similar to that of cows initiating the FSH treatment in diestrus (Rivera et al., 2011). Collectively, these data indicate that the low concentrations of progesterone, when the ovulatory follicle undergoes its final stages of development, influences the biochemical composition of the follicular fluid, alters oocyte quality, and reduces subsequent embryo quality. Moreover, preovulatory suboptimal concentrations of progesterone have been implicated with premature upregulation of components of the luteolytic cascade and increased synthesis of $\text{PGF}_{2\alpha}$ in response to oxytocin, which might shorten CL lifespan during the following estrous cycle (Shaham-Albalancy et al., 2001; Cerri et al., 2011b). This response might partially explain the greater proportion of CON cows with an inter-AI interval of 5 to 17 d compared with 2CIDR and DI cows.

As anticipated, the diameter of the ovulatory follicle in cows with a new CL on d -3, which are those that initiated a new follicular wave at the beginning of the timed AI program, was larger for CON than 2CIDR cows. Because 2CIDR cows had increased concentrations of progesterone in plasma compared with CON, it is suggested that the rate of follicle growth was attenuated by progesterone. Cows that commence the timed AI in the presence of luteal concentrations of progesterone have smaller ovulatory follicles than those that develop the ovulatory follicle during metestrus (Rutigliano et al., 2008; Bisinotto et al., 2010a). Nevertheless, it is unclear why the diameter of the ovulatory follicle in DI cows was intermediate and did not differ from CON and 2CIDR given that these cows had the greatest concentrations of progesterone in plasma. The lack of differences in ovulatory follicle diameter for cows without a newly formed CL was expected because of the possible lack of synchronous follicle growth when cows do not ovulate to the initial GnRH of the timed AI program.

Lack of synchronous response to the timed AI program in cows without a CL is unlikely to be responsible for major declines in fertility (Bisinotto et al., 2010b), although ovulation to the final GnRH was less than that of diestrus cows. In particular, the ovulatory response to the initial GnRH is typically high, usually greater than 85% in anovular cows (Gümen et al., 2003) and cows treated during proestrus (Bisinotto et al., 2010a). In the current study, presence of a new CL in cows not in diestrus averaged 64.4%, which is less than observed for anovular cows (Gümen et al., 2003), but similar to the findings of Denicol et al. (2012). It is

unclear what happens to follicle growth in cows without a CL that do not respond to the initial GnRH of the timed AI protocol. Cows that do not ovulate to the initial GnRH of the timed AI program have impaired overall synchrony of the estrous cycle (Vasconcelos et al., 1999; Rutigliano et al., 2008) and extended the period of follicle dominance (Cerri et al., 2009b), which are detrimental to fertility (Vasconcelos et al., 1999; Rutigliano et al., 2008; Santos et al., 2010). In fact, P/AI in CON cows decreased dramatically when a new CL was not observed on d -3 (from 37 to 11%). These results illustrate the importance of follicle turnover at the initiation of synchronization programs to optimize fertility. Furthermore, DI and 2CIDR cows that had a newly formed CL ovulated a smaller dominant follicle at AI than cows in the same treatments but without a newly formed CL. These data suggest that either the period of follicle dominance or the rate of growth of the ovulatory follicle was reduced, which typically benefit P/AI.

Results presented in this study support the development of CIDR inserts targeted to anovular or nondiestrus, high-producing cows. The CIDR insert releases approximately 88.6 mg/d of progesterone and this amount is sufficient to increase concentrations in plasma in approximately 1 ng/mL. Therefore, doubling the release to at least 170 mg/d should complement endogenous secretion of progesterone from a newly formed CL and increase concentrations in plasma to >2.50 ng/mL, which is expected to improve P/AI in anovular to resemble that of cows in diestrus.

CONCLUSIONS

Results from the current study support targeted supplementation with progesterone to improve P/AI in lactating dairy cows. Cows without a CL at the beginning of the timed AI protocol had increased risk of short reinsemination interval and a marked decrease in P/AI compared with cows initiating the timed AI in diestrus. Noteworthy was that supplementing progesterone with 2 CIDR to cows without a CL on d -8 increased progesterone concentrations to 2.65 ng/mL and restored P/AI similar to that of cows in diestrus, which is typically not observed when a single insert is used in high-producing lactating dairy cows. Treatment with 2 CIDR altered the diameter of the ovulatory follicle in cows that had a synchronized follicle wave after the initial GnRH of the timed AI. Collectively, these data indicate that incorporation of 2 CIDR during the timed AI protocol in cows without a CL increase progesterone concentrations similar to that of cows in early to mid diestrus, influences the rate of ovulatory follicle

growth, and reestablishes P/AI similar to that of cows in diestrus.

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