Timed Artificial Insemination with Estradiol Cypionate or Insemination at Estrus in High-Producing Dairy Cows

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ABSTRACT

A total of 799 Holstein cows from 3 herds were randomly assigned at 37 ± 3 d in milk (DIM) to timed artificial insemination (AI) or insemination at detected estrus. Cows were presynchronized with injections of PGF_{2 α} at 37 and 51 DIM. At 65 DIM, cows received an injection of GnRH, followed 7 d later by PGF_{2 α} . Cows in the estrus-detected group were inseminated after being observed in estrus during the 7 d after the last PGF_{2 α} . Cows in the timed AI group received an injection of 1 mg of estradiol cypionate (ECP) 24 h after the last PGF_{2 α} . If detected in estrus ≤ 24 h after ECP, cows were inseminated then or at a fixed time 48 h after ECP. Pregnancy was diagnosed by ultrasonography at 30 d and by palpation at 44 and 58 d after AI. Plasma progesterone was measured in 4 samples per cow collected on days of 1) second PGF_{2 α} , 2) GnRH, 3) third PGF_{2 α} , and 4) 48 h after third PGF_{2 α} . Cows were classified as cyclic or anovulatory based on progesterone concentrations in samples 1 and 2. Similarly, cows were classified according to progesterone concentrations in samples 2, 3, and 4 ($H = \geq 1$ ng/mL; $L < 1$ ng/mL), resulting in 8 combinations (LLL, LHL, LLH, LHH, HHH, HHL, HLH, and HLL). Conception rates and pregnancy rates were higher for cows in the timed AI group than in the estrus-detected group at 30, 44, and 58 d (e.g., at 58 d, pregnancy rates were 42.2% for multiparous cows or 34.4% for primiparous cows in the group receiving ECP and timed AI compared with only 20.8 or 18.8% for respective parity subgroups for the treatment group inseminated only at detected estrus). Pregnancy losses were 11.5% from 30 to 58 d and did not differ between treatments. Cyclic cows within both treatments had higher estrous responses, conception rates, and pregnancy rates. Cows that responded to presynchronization and to luteolysis (HHL) had the highest conception and pregnancy rates, followed by cows classified as LHL. Use of 1 mg of ECP to induce ovulation as part

of a synchrony regimen improved reproduction at first postpartum insemination in dairy cows.

(Key words: timed artificial insemination, estradiol cypionate, dairy cow, reproduction)

Abbreviation key: AOR = adjusted odds ratio, CI = confidence interval, CL = corpus luteum, ECP = estradiol cypionate.

INTRODUCTION

Synchronization protocols that allow for timed AI (Pursley et al., 1997) provide several advantages for the overall reproductive efficiency in dairy farms with inadequate detection of estrus (Tenhagen et al., 2004). Because protocols that combine synchronization of ovarian follicle emergence, corpus luteum (CL) regression, and induced ovulation result in similar or slightly lower conception rates but higher service rates compared with protocols for synchronization of estrus (Pursley et al., 1997; Cartmill et al., 2001; Chebel et al., 2004; Santos et al., 2004a; Tenhagen et al., 2004), they usually increase pregnancy rates in herds with low rates of detected estrus.

A recent study evaluated the effects of incorporating different doses of estradiol cypionate (ECP) to induce a synchronized ovulation in a timed AI protocol in dry cows and heifers (Lopes et al., 2000). Those researchers observed that ECP induced a preovulatory peak of LH and synchronized ovulation at approximately 56 h after treatment with subsequent normal concentrations of progesterone during the luteal phase (Lopes et al., 2000) and further observed that 1.0 mg of ECP in lactating dairy cows was adequate to synchronize ovulation.

In lactating cows, pregnancy rates were similar when inseminated at a fixed time following an insemination protocol known as Ovsynch, in which GnRH is used to induce ovulation, or Heatsynch, in which ECP was used to induce ovulation (Pancarci et al., 2002). Although pregnancy rates were similar between Heatsynch and Ovsynch protocols, cows not displaying signs of behavioral estrus had low conception after Heatsynch, which might be related to cyclic status. No studies have revealed reproductive responses to Heatsynch for cows

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classified according to cyclic status before the initiation of that protocol. Because high-producing, lactating dairy cows have increased steroid metabolism (Sangsri-tavong et al., 2002) and lower concentrations of estradiol before ovulation (Sartori et al., 2002), it is possible that supplemental estradiol might benefit reproductive responses as observed in beef cows (Ahmadzadeh et al., 2003).

The objectives of the current study were to determine reproductive responses during the first postpartum AI in high-producing dairy cows in 3 different dairy herds when inseminated following a timed AI protocol using ECP compared with a similar protocol for synchronization of estrus without use of ECP to induce ovulation. Moreover, fertility responses to the 2 breeding protocols were compared for cows of different parities (primiparous and multiparous) and according to cyclic status and progesterone profiles during synchronization.

It was hypothesized that timed AI would increase service rate and pregnancy rates. It was also hypothesized that additional estradiol from ECP would increase expression of estrus and benefit fertility of high-producing dairy cows by increasing conception rates with no subsequent effects on late embryonic survival. It was expected that cyclic cows and those that responded to the presynchronization and to luteolysis would have higher estrous response and higher conception and pregnancy rates.

MATERIALS AND METHODS

Animals, Housing, and Feeding

A total of 799 lactating Holstein dairy cows (479 primiparous; 320 multiparous) from 3 high-producing large commercial dairy farms located in the Central Valley of California were enrolled in the study and used for statistical analyses. Lactating herd sizes of respective sites 1, 2, and 3 during the study included 850, 1440, and 1530 cows, and the 3.5% FCM rolling herd averages for 2002 were 11,320, 12,560, and 11,640 kg per cow. The numbers of lactating cows used on Sites 1, 2, and 3 were, 330, 221, and 248, respectively. At all sites, cows were housed in free-stall barns equipped with fans and sprinklers, and within each site, all cows were fed the same diet as a TMR, once daily, to meet or exceed the dietary requirements for a lactating cow weighing 680 kg and producing 45 kg of 3.5% FCM (NRC, 2001). Primiparous and multiparous cows were housed separately throughout the study, which was conducted from January to October 2002.

Body condition (Ferguson et al., 1994) of all cows was scored at 37 ± 3 DIM (study enrollment), 75 ± 3 DIM (first AI), and again at 105 ± 1 DIM (pregnancy diagnosis). Cows diagnosed with any evident health disorder

(mastitis, left displacement abomasum, lameness, uterine infection, uterine adhesions, etc.) or those with a BCS <2.25 at the beginning of the study (37 ± 3 DIM) were excluded from the experiment. A total of 115 cows contemporary to the 799 cows used were excluded from the study: 50 from site 1, 13 from site 2, and 52 from site 3. Reasons for exclusion were insemination before the synchronization protocol ($n = 7$), death ($n = 3$), owner's decision not to inseminate cows because of udder conformation ($n = 8$), owner's sale of cow before completion of synchronization protocols ($n = 70$), low BCS ($n = 8$), movement of cow to a bull pen before first insemination ($n = 13$), and contraction of uterine adhesion or lameness ($n = 6$).

Treatments and AI

Cows had estrous cycles presynchronized with 2 injections of 25 mg of $\text{PGF}_{2\alpha}$ (5 mg/mL of Lutalyse, dinoprost tromethamine; Pfizer Animal Health, New York, NY) at 37 ± 3 and 51 ± 3 d postpartum. After 14 d (65 ± 3 DIM), cows received 1 of the 2 synchronization protocols. For the Selectsynch protocol ($n = 404$), GnRH (100 μg i.m.) (50 $\mu\text{g}/\text{mL}$ of Cystorelin, gonadorelin diacetate tetrahydrate; Merial Ltd., Iselin, NJ) was administered followed 7 d later by an i.m. injection of 25 mg of $\text{PGF}_{2\alpha}$ and AI upon detection of estrus during 7 d after the last $\text{PGF}_{2\alpha}$ injection (Figure 1). For the Heatsynch protocol ($n = 395$) GnRH (100 μg i.m.) was administered followed 7 d later by an i.m. injection of 25 mg of $\text{PGF}_{2\alpha}$. Twenty-four hours after the $\text{PGF}_{2\alpha}$ injection, cows received an i.m. injection of 1 mg of ECP (2.0 mg/mL; Pfizer Animal Health) and received either AI upon detected estrus within 24 h after ECP treatment or timed AI at 48 h after the ECP treatment (Figure 1).

Presynchronization of estrous cycles with $\text{PGF}_{2\alpha}$ was implemented to improve responses to the synchronization protocols (Moreira et al., 2001). Cows were observed for signs of estrus once each morning, based on behavior and on removal of tail-head paint (Macmillan et al., 1988) applied using paintsticks (All-weather Paintstik; LA-CO Industries, Chicago, IL). For cows in the Heatsynch treatment, estrus was detected in the 2 d after ECP treatment to determine whether they were in estrus before or on the day of timed AI.

Within dairy sites 2 and 3, the same technician artificially inseminated cows once daily, in the morning, throughout the experimental period. At site 1, 2 technicians inseminated cows once daily, in the afternoon. Semen from different sires was randomly assigned to the 2 treatment groups to maintain similar distributions of sires across treatments.

Cows in the Selectsynch treatment that were not observed in estrus in the 7 d following $\text{PGF}_{2\alpha}$ were consid-

Figure 1. Diagram of activities during the study. BS = blood sample, ECP = estradiol cypionate, P4 = progesterone analysis, and US = ultrasonographic examination of ovaries.

ered not to have responded to the synchronization protocol. Nonpregnant cows that had not been re-inseminated and those that failed to respond to the Selectsynch treatment were enrolled in the Ovsynch protocol (Pursley et al., 1997; GnRH on d 0, PGF_{2α} 7 d later, GnRH 48 h after PGF_{2α} followed by timed AI) for a timed AI during the second postpartum insemination period. Therefore, cows in the Selectsynch protocol that were not observed in estrus were all inseminated after a subsequent Ovsynch protocol, and data from those inseminations were considered to be second postpartum AI. At the second postpartum AI, it was recorded whether cows were re-inseminated upon spontaneous return to estrus or after the Ovsynch protocol, and insemination method was included in the statistical analyses of conception rate.

Blood Sampling, Progesterone Analysis, and Progesterone Classes

Four blood samples were collected from cows for determination of plasma progesterone concentrations (Figure 1). The first 2 samples were collected at 51 ± 3 and 65 ± 3 DIM, and progesterone concentrations in those 2 blood samples were used to determine whether

cows were cyclic (when concentration was ≥1.0 ng/mL in one of the 2 samples) or anovulatory (when both samples were <1.0 ng/mL) before the GnRH injection that initiated synchronization. A third blood sample was collected immediately before the final PGF_{2α} injection, and the last sample was collected 48 h after the final PGF_{2α} injection. These last 2 samples were used to determine the presence of a CL immediately before the final PGF_{2α} treatment and its regression after the final PGF_{2α} treatment.

Approximately 7 mL of blood was collected by puncture of the coccygeal vein or artery utilizing Vacutainer tubes (Becton Dickinson Vacutainer systems, Rutherford, NJ) with sodium EDTA. The samples were immediately placed in ice and later centrifuged at 2000 × g for 15 min for separation of plasma. Plasma samples were frozen at -25°C until later analysis. Progesterone was analyzed by ELISA according to Munro and Stabenfeldt (1984) with modifications. Blood was collected from a young male calf, and plasma was separated. A charcoal-stripping procedure (Sharpe and Cooper, 1984) was used to remove residual steroids from plasma that could crossreact during the assay. Charcoal-stripped plasma enriched with known concentrations of progesterone was used as a quality control in each

96-well plate to determine the efficiency of progesterone extraction. The intra- and interassay coefficients of variation were 7.0 and 13.5%, respectively.

Cows were assigned to progesterone classes based on concentrations in plasma samples collected immediately before the hormone injections in the synchronization treatments, at GnRH, at the third PGF_{2 α} , and 48 h after the third PGF_{2 α} . Samples with progesterone concentrations <1.0 ng/mL were classified as low (L) and those $\geq$ 1.0 ng/mL were classified as high (H). For samples collected 48 h after PGF_{2 α} during the synchronization protocol, a decrease in progesterone concentration of $\geq$ 60% after PGF_{2 α} was additionally considered to define low progesterone (Rivera et al., 2004). This classification system resulted in 8 possible progesterone class permutations: HHH, HHL, HLH, HLL, LHH, LHL, LLH, and LLL. Of the 799 cows in the study, progesterone classes were assigned to 641 because 158 cows had a missing blood sample either at PGF_{2 α} or 48 h later.

Ultrasonography of Ovaries, Pregnancy Diagnosis, and Reproductive Outcomes

Ovaries were examined by ultrasonography (Sonovet 2000; Universal Medical System, Bedford Hills, NY) using a 7.5-MHz linear transducer at 48 h after the PGF_{2 α} treatment before AI in both treatments. The size of the 2 largest follicles and the presence of a CL were recorded.

All cows were examined for pregnancy by transrectal ultrasonography at 30 ± 1 d after the first postpartum AI. The detection of an embryonic vesicle with a viable embryo (presence of heartbeat) was used as an indicator of pregnancy. Cows diagnosed as pregnant were palpated per rectum for detection of an embryonic vesicle to confirm pregnancy at 44 ± 1 and 58 ± 1 d of gestation. After the second AI, pregnancy was diagnosed by palpation per rectum at 38 ± 3 d after AI.

Conception rate was defined as the number of pregnant cows at any given time (30, 44, or 58 d after AI) out of the total number of cows inseminated within each treatment group. Pregnancy rate was defined as the number of pregnant cows at any given time out of the total number of cows in each treatment group. Pregnancy losses between 30 and 44 d and between 44 and 58 d after AI were evaluated. Cows diagnosed with a viable pregnancy (presence of heartbeat) at 30 d, but nonpregnant at 44 or 58 d, were considered as having experienced pregnancy loss.

In the Heatsynch treatment, 6 cows (3 primiparous; 3 multiparous) were culled from the herd, one before and 5 after the pregnancy diagnosis at 44 d after AI. In the Selectsynch treatment, only one primiparous cow

was culled from the herd before the pregnancy diagnosis at 44 d after AI.

Milk Production

Production of milk was recorded monthly for individual cows for the first 6 mo of lactation during official DHIA tests. Milk produced during the first 3 mo postpartum was used to determine the effect of milk yield on cyclicity, conception rate, pregnancy rate, and pregnancy loss. The effect of treatment on yields of milk was also evaluated for up to 4 mo after initiation of treatment.

Experimental Design and Statistical Analyses

The experimental design was a randomized complete block design (Kuehl, 1994). Weekly, within each dairy, cows were blocked according to lactation number, BCS at 37 ± 3 d postpartum, and milk yield in the first month postpartum and, within each block, were randomly assigned to one of the 2 treatments.

A sample size calculation was performed (Win Epi-scope Version 2.0, 2000) to estimate the number of experimental units required to provide sufficient power to detect differences in conception rates at first AI, as well as in pregnancy loss after first AI. The number of experimental units per treatment was determined to provide enough replicates for significance ($\alpha = 0.05$; $\beta = 0.20$) when a 7% unit difference in conception rate between the 2 treatments was observed, given that conception ranged from 30 to 45%. Assuming that 35 to 45% of the cows would be pregnant after the first AI, the sample size was calculated to provide sufficient experimental units to determine statistical differences ($\alpha = 0.05$; $\beta = 0.20$) in pregnancy loss between 30 and 58 d of gestation when an 8% unit difference in pregnancy loss between the 2 treatments was observed, given that pregnancy losses ranged from 10 to 25%.

Binomially distributed data, such as cyclicity, estrous response, conception rate, pregnancy rate, and pregnancy loss, were analyzed by logistic regression using the LOGISTIC procedure of SAS (2001). A backward stepwise regression model was used (Allison, 1999), and the full model included the effects of treatment, dairy, parity, BCS at the time of AI, BCS change between study enrollment and AI, cyclicity status (anovulatory vs. cycling), milk production during the first 3 mo postpartum, and the interactions between treatment and the respective explanatory variables. Variables were continuously removed from the model by the Wald statistic criterion if the significance was >0.20 . Additional statistical analyses were performed with 641 cows to evaluate the effect of progesterone class on fertility re-

Table 1. Effect of synchronization protocol before first postpartum AI on reproductive responses of lactating dairy cows.

Item	Treatment ¹				<i>P</i> ²		
	Selectsynch		Heatsynch		TRT	Parity	TRT × Parity
	Primiparous	Multiparous	Primiparous	Multiparous			
Cyclic, %	70.7 (171/242)	86.3 (139/161)	65.4 (155/237)	80.3 (126/157)	0.04	0.02	0.65
Detected in estrus, %	59.5 (144/242)	70.4 (114/162)	80.4 (189/235)	81.8 (121/148)	0.0001	0.82	0.14
Conception rate, %							
30 d	35.9 (51/142)	35.1 (39/111)	39.4 (93/236)	48.4 (76/157)	0.03	0.01	0.47
44 d	31.9 (45/141)	31.5 (35/111)	36.2 (85/235)	43.5 (67/154)	0.03	0.09	0.69
58 d	31.9 (45/141)	29.7 (33/111)	34.3 (80/233)	42.2 (65/154)	0.05	0.05	0.30
Pregnancy rate, %							
30 d	21.3 (51/240)	24.5 (39/159)	39.4 (93/236)	48.4 (76/157)	0.0001	0.006	0.74
44 d	18.8 (45/239)	22.0 (35/159)	36.2 (85/235)	43.5 (67/154)	0.0001	0.05	0.90
58 d	18.8 (45/239)	20.8 (33/159)	34.3 (80/233)	42.2 (65/154)	0.0001	0.02	0.66
Pregnancy loss, %							
30 to 44 d	10.0 (5/50)	10.3 (4/39)	7.6 (7/92)	8.2 (6/73)	0.90	0.45	0.90
44 to 58 d	0.0 (0/45)	5.7 (2/35)	3.6 (3/83)	3.0 (2/67)	0.80	0.40	—
30 to 58 d	10.0 (5/50)	15.4 (6/39)	11.1 (10/90)	11.0 (8/73)	0.85	0.67	0.89

¹All cows were presynchronized with 2 injections of PGF_{2α}. Selectsynch = GnRH at 65 ± 3 DIM, PGF_{2α} 7 d later; and breeding on observed estrus; Heatsynch = GnRH at 65 ± 3 DIM, PGF_{2α} 7 d later, estradiol cypionate (ECP) 24 h after PGF_{2α}, and AI at estrus until 24 h or timed AI at 48 h after ECP.

²TRT = Treatment (Selectsynch vs. Heatsynch).

sponses according to the statistical model described previously.

Milk production and BCS data were analyzed by ANOVA for repeated measures (Littell et al., 2002) using the MIXED procedure of the SAS (2001) program. The model included the effects of treatment, month postpartum, interaction between treatment and month postpartum, dairy, parity, interaction between treatment and dairy, and interaction between treatment and parity, with cow nested within treatment as the random error. Production in the first 2 mo postpartum, immediately before treatments, was used for covariate adjustment during analysis of milk production, which included data from mo 3 to 6 postpartum. The covariance structures (unstructured, compound symmetry, toeplitz, and autoregressive) for the MIXED models were evaluated (Littell et al., 2002), and the autoregressive structure was chosen as the one that best fit the data based on the Schwarz Bayesian criterion. Size of the largest follicle 48 h after PGF_{2α} was analyzed by ANOVA (Littell et al., 2002) using the GLM procedure of SAS (2001).

Treatment differences with $P \leq 0.05$ were considered significant, and $0.05 < P \leq 0.10$ were designated as a tendency to differ.

RESULTS

The mean BCS for cows in the Heatsynch and Selectsynch treatments throughout the study were similar and averaged 3.01 (±0.02) and 3.04 (±0.02), respectively ($P = 0.20$). The proportion of cows with high (≥1.0

ng/mL) progesterone at the GnRH injection of the synchronization protocol tended to be lower for cows in the Heatsynch protocol than in the Selectsynch protocol (65.0% vs. 68.0%; $P = 0.09$). However, the proportion of cows with high progesterone at the last PGF_{2α} injection (86.2% vs. 85.0%; $P = 0.52$) and at 48 h after the last PGF (7.4% vs. 7.4%; $P = 0.81$), along with the size of the largest follicle 48 h after the last PGF_{2α} (17.9 vs. 18.2 mm; $P = 0.52$), were all similar for cows on the Heatsynch and Selectsynch protocols.

The proportion of cyclic cows was greater for Selectsynch than for Heatsynch ($P = 0.04$) and for multiparous than for primiparous cows (Table 1; $P = 0.02$). In addition to parity, BCS at AI and dairy site affected proportions of cyclic cows. As BCS at AI increased, the proportion of cyclic cows also increased ($P < 0.001$). Cows were 2.15 times more likely to be cyclic as BCS at AI increased one unit (adjusted odds ratio [AOR] = 2.15; 95% confidence interval [CI] = 1.35, 3.42). The estrous response after the PGF_{2α} was greater ($P < 0.0001$) for cows on the Heatsynch protocol than on the Selectsynch protocol, but no effect of parity was observed. An interaction between treatment and cyclic status was observed for percentages of cows detected in estrus ($P = 0.004$; Table 2), because Heatsynch markedly increased detection of estrus in anovular cows.

Conception rates at 30, 44, and 58 d after AI were higher for cows subjected to the Heatsynch protocol than for cows inseminated following induced estrus with the Selectsynch protocol ($P < 0.05$; Table 1). Similar to conception rates, pregnancy rates were higher for cows on the Heatsynch protocol than for cows on the

Table 2. Effect of cyclic status on reproductive responses in lactating dairy cows at first AI.

Item	Cyclic status ¹				$P_{<^3}$		
	Cyclic		Anovulatory		TRT	Cyc	TRT × Cyc
	Selectsynch ²	Heatsynch ²	Selectsynch	Heatsynch			
Detected in estrus, %	71.6 (222/310)	83.3 (225/270)	37.6 (35/93)	75.9 (85/112)	0.0001	0.0001	0.004
Conception rate, %							
30 d	35.8 (78/218)	47.5 (133/280)	35.3 (12/34)	32.1 (36/112)	0.03	0.02	0.29
44 d	32.3 (70/217)	43.1 (119/276)	29.4 (10/34)	29.5 (33/112)	0.03	0.05	0.45
58 d	31.3 (68/217)	41.6 (114/274)	29.4 (10/34)	27.7 (31/112)	0.05	0.02	0.49
Pregnancy rate, %							
30 d	25.5 (78/306)	47.5 (133/280)	13.0 (12/92)	32.1 (36/112)	0.0001	0.0004	0.61
44 d	23.0 (70/305)	43.1 (119/276)	10.9 (10/92)	29.5 (33/112)	0.0001	0.003	0.47
58 d	22.3 (68/305)	41.6 (114/274)	10.9 (10/92)	27.7 (31/112)	0.0001	0.001	0.55
Pregnancy loss, %							
30 to 44 d	9.1 (7/77)	7.8 (10/129)	16.7 (2/12)	8.3 (3/36)	0.90	0.88	0.92
44 to 58 d	2.9 (2/70)	2.6 (3/117)	0 (0/10)	6.1 (2/33)	0.80	0.50	—
30 to 58 d	11.7 (9/77)	10.2 (13/127)	16.7 (2/12)	13.9 (5/36)	0.85	0.56	0.90

¹Cyclic = progesterone ≥ 1.0 ng/mL in one of the 2 samples before the synchronization protocol; anovulatory = progesterone < 1.0 ng/mL in both samples.

²All cows were presynchronized with 2 injections of PGF_{2 α} . Selectsynch = GnRH at 65 ± 3 DIM, PGF_{2 α} 7 d later; and breeding on observed estrus; Heatsynch = GnRH at 65 ± 3 DIM, PGF_{2 α} 7 d later, estradiol cypionate (ECP) 24 h after PGF_{2 α} , and AI at estrus until 24 h or timed AI at 48 h after ECP.

³TRT = Treatment (Selectsynch vs Heatsynch); Cyc = cyclic status (cyclic vs. anovulatory).

Selectsynch protocol at 30, 44, and 58 d after AI. The increase in pregnancy rates was associated with the higher service rate (100% vs. 63.9%; $P < 0.001$) and conception rates for cows on the Heatsynch protocol than for those on the Selectsynch protocol. Parity affected conception and pregnancy rates, but no interaction between treatment and parity was observed for those outcomes. Fertility responses were higher for cows on Heatsynch than for cows on Selectsynch at all 3 sites, and no interactions between treatment and site were observed for conception and pregnancy rates. Pregnancy losses during the first 58 d of gestation were similar for cows inseminated at detected estrus or fixed time with the Heatsynch protocol or upon detected estrus after the Selectsynch protocol. Similarly, pregnancy loss was not affected by parity or cyclic status. Cyclic cows had higher conception ($P < 0.05$) and pregnancy rates ($P < 0.003$) than anovular cows at 30, 44, and 58 d after AI (Table 2).

Milk production was negatively associated with reproductive performance of dairy cows. When cows were stratified according to milk production above or below the mean milk yield for primiparous and multiparous cows, cows with production below the mean had higher conception rates at 30 d (44.2% vs. 36.0%; $P = 0.01$), 44 d (39.9% vs. 32.5%; $P = 0.08$), and 58 d (39.6% vs. 30.3%; $P < 0.01$) than those with production above the mean. Similar to conception rates, milk production also affected pregnancy rates, and cows with production below the mean milk yield had higher pregnancy rates than those with production above the mean milk yield at 30

d (36.4% vs. 29.1%; $P = 0.03$), 44 d (32.8% vs. 26.6%; $P = 0.10$), and 58 d after AI (32.5% vs. 24.5%; $P < 0.01$).

Treatment and parity also affected the proportion of cows re-inseminated after the first postpartum AI (Table 3). Treatment had no effect on the proportion of cows re-inseminated by 17 d ($P = 0.11$), but more Heatsynch cows were re-inseminated by 21 d ($P < 0.03$) and by 30 d ($P < 0.007$) at the time of pregnancy diagnosis. More nonpregnant multiparous cows vs. primiparous cows were reinseminated ($P < 0.009$), and the interval between the first and second postpartum AI was shorter for multiparous cows than for primiparous cows ($P < 0.04$). Although treatment affected conception rates at first AI, no treatment effect was observed for conception rates to second AI ($P = 0.21$) when cows were inseminated either at spontaneous estrus or at timed AI following resynchronization with the Ovsynch protocol.

When cows on the Heatsynch protocol were grouped according to estrual responses after ECP treatment (Table 4), those displaying estrus either at 24 h after ECP or at timed AI had higher conception rates at 30, 44, and 58 d of gestation than those that were not observed in estrus ($P < 0.05$). Despite the positive effects on conception, Heatsynch cows exhibiting signs of estrus experienced similar pregnancy losses compared with cows not in estrus when timed inseminated.

Follicle size affected the proportion of animals that expressed estrus (Figure 2). Increasing follicle size increased estrus response ($P < 0.0001$), and this effect was not dependent upon treatment because no interaction was observed ($P = 0.51$). However, follicle sizes 48

Table 3. Effect of insemination protocol at first postpartum AI on returns to estrus and conception rate at second AI in nonpregnant cows at first AI.

Item	Treatment ¹				<i>P</i> < ²		
	Selectsynch		Heatsynch		TRT	Parity	TRT × Parity
	Primiparous	Multiparous	Primiparous	Multiparous			
Reinseminated, %							
By 17 d	1.1 (1/91)	11.3 (8/71)	4.9 (7/142)	17.3 (14/81)	0.11	0.001	0.23
By 21 d	12.1 (11/91)	29.6 (21/71)	20.4 (29/142)	28.4 (23/81)	0.03	0.009	0.04
By 30 d	56.0 (51/91)	73.2 (52/71)	50.0 (71/142)	66.7 (54/81)	0.007	0.002	0.56
Rebreeding interval, d							
By 30 d (±SEM)	22.4 ^b (±0.8)	20.6 ^{ab} (±0.9)	21.3 ^{ab} (±0.7)	19.6 ^a (±0.9)	0.13	0.04	0.91
All cows (±SEM)	30.2 ^b (±1.1)	25.6 ^a (±1.4)	31.0 ^b (±0.9)	26.3 ^a (±1.3)	0.47	0.001	0.97
Conception rate, %	32.8 (62/189)	21.4 (25/117)	24.7 (35/142)	24.7 (19/77)	0.21	0.01	0.60

¹All cows were presynchronized with 2 injections of PGF_{2α}. Selectsynch = GnRH at 65 ± 3 DIM, PGF_{2α} 7 d later; and breeding on observed estrus; Heatsynch = GnRH at 65 ± 3 DIM, PGF_{2α} 7 d later, estradiol cypionate (ECP) 24 h after PGF_{2α}, and AI at estrus until 24 h or timed AI at 48 h after ECP.

²TRT = Treatment (Selectsynch vs. Heatsynch).

^{a,b,c}Values with different superscripts in the same row differ (*P* < 0.05).

h after PGF_{2α} were 17.9 and 18.5 mm for Heatsynch and Selectsynch cows, respectively, and did not differ (*P* = 0.52) between treatments.

Of 641 cows classified according to progesterone concentrations above or below 1.0 ng/mL at GnRH, PGF_{2α}, and 48 h later, 20 were HHH, 368 were HHL, 2 were HLH, 56 were HLL, 24 were LHH, 136 were LHL, 2 were LLH, and 33 were LLL. Progesterone class affected conception and pregnancy rates (Figures 3 and 4). As expected, cows that responded to the presynchronization and experienced luteolysis within 48 h of the last PGF_{2α} treatment (HHL) had the highest conception and pregnancy rates, followed by LHL. Although a small number (*n* = 48; 7.5%), those cows in which CL regression did not occur within 48 h of the last PGF_{2α} treatment (HHH, HLH, LHH, and LLH), either because of asynchrony of the estrous cycle or lack of response to PGF_{2α}, had the lowest conception and pregnancy rates. At 30 d after AI, conception and pregnancy rates for those 48 cows were 12.1 and 8.5%, respectively.

Body condition score at AI affected conception and pregnancy rates of dairy cows. As BCS at AI increased, conception rates also increased, so that at 58 d after AI every unit increase in BCS was associated with 1.65 times higher conception rates (AOR = 1.65; 95% CI = 1.06, 2.57; *P* = 0.03). Similarly, the OR for pregnancy rates at 58 d after AI increased with every unit of BCS at AI (AOR = 1.66; 95% CI = 1.07, 2.55; *P* = 0.02).

Milk production in the months following treatments was not affected by Heatsynch or Selectsynch (41.5 vs. 41.6 kg/d; *P* = 0.64), but an interaction was observed between treatment and month postpartum (*P* < 0.002) in that production was lower for cows on the Heatsynch protocol (43.1 ± 0.4 kg/d) than for cows on the Selectsynch protocol (44.8 ± 0.4 kg/d) in mo 3 when ECP was administered to Heatsynch cows (*P* < 0.01).

DISCUSSION

In the current study, administration of 1 mg of ECP to induce estrus and synchronize ovulation to allow for

Table 4. Effect of estrus on reproductive responses to the Heatsynch protocol in lactating dairy cows.

Item	Heatsynch ¹			<i>P</i> <
	Estrus and AI before timed AI	Estrus at timed AI	No estrus at timed AI	
Conception rate, %				
30 d	40.6 ^a (28/69)	51.2 ^a (124/242)	23.6 ^b (17/72)	0.002
44 d	36.8 ^a (25/68)	46.7 ^a (112/240)	21.1 ^b (15/71)	0.003
58 d	35.3 ^a (24/68)	44.5 ^a (106/238)	21.1 ^b (15/71)	0.05
Pregnancy loss, %				
30 to 44 d	7.4 (2/27)	8.2 (10/122)	6.3 (1/16)	0.86
44 to 58 d	4.0 (1/25)	3.6 (4/110)	0 (0/15)	—
30 to 58 d	11.1 (3/27)	11.7 (14/120)	6.3 (1/16)	0.98

¹Heatsynch = GnRH at 65 ± 3 DIM, PGF_{2α} 7 d later; estradiol cypionate (ECP) 24 h after PGF_{2α}; and AI at estrus until 24 h or timed AI at 48 h after ECP.

^{a,b}Values with different superscripts in the same row differ (*P* < 0.05).

Figure 2. Percentages of cows detected in estrus in relation to size (mm) of largest follicle 48 h after $\text{PGF}_{2\alpha}$ in lactating dairy cows submitted to Selectsynch or Heatsynch protocols. Increasing follicle size increased percentage detected in estrus ($P < 0.0001$) independent of treatment ($P = 0.51$).

Figure 4. Pregnancy rates (PR) at 30 and 58 d after insemination (includes all cows in the study) according to progesterone classes (H = ≥ 1.0 ng/mL; L = < 1.0 ng/mL) at the time of GnRH, $\text{PGF}_{2\alpha}$, and 48 h after $\text{PGF}_{2\alpha}$ in lactating dairy cows submitted to Selectsynch or Heatsynch protocols. Because of few observations each in HHH, HLH, LHH, and LLH progesterone classes, those groups were combined (no Luteolysis; n = 48 cows) for comparisons; HHL = 368 cows; HLL = 56 cows, LHL = 136 cows; and LLL = 33 cows. Effect of progesterone class on PR at 30 ($P < 0.0001$) and 58 d ($P < 0.005$). ^{a,b,c} Bars with distinct superscripts differ ($P < 0.05$).

Figure 3. Conception rates (CR) at 30 and 58 d after insemination (CR30 and CR58, respectively; includes only cows that were inseminated) according to progesterone classes (H = ≥ 1.0 ng/mL; L = < 1.0 ng/mL) at the time of GnRH, $\text{PGF}_{2\alpha}$, and 48 h after $\text{PGF}_{2\alpha}$ in lactating dairy cows submitted to Selectsynch or Heatsynch protocols. Because of few observations each in HHH, HLH, LHH, and LLH progesterone classes, those groups were combined (no Luteolysis; n = 33 cows) for comparisons; HHL = 314 cows; HLL = 48 cows, LHL = 109 cows; and LLL = 20 cows. Effect of progesterone class on CR at 30 ($P < 0.002$) and 58 d ($P < 0.02$). ^{a,b,c} Bars with distinct superscripts differ ($P < 0.05$).

timed AI improved reproductive performance at first postpartum AI in high-producing lactating dairy cows in 3 dairy herds. Improvements in reproductive performance were associated with increased conception rates for cows on the Heatsynch treatment and with similar pregnancy losses to cows inseminated at estrus following the Selectsynch protocol. Interestingly, cows on the Heatsynch protocol had increased conception rates despite the lower proportion of cyclic cows.

Previously, Pancarci et al. (2002) compared reproductive performance of dairy cows when subjected to timed AI following the Ovsynch or Heatsynch protocols and observed similar pregnancy rates for cows in both ovulation synchronization treatments, although conception differed according to whether cows were detected in estrus. Generally, timed AI protocols result in increased pregnancy rates because all eligible cows are inseminated, but conception rates are either similar to or lower than those of cows inseminated based upon detection of estrus (Pursley et al., 1997; Cartmill et al., 2001; Chebel et al., 2004; Santos et al., 2004a; Tenhagen et al., 2004). Pregnancy rates in cows inseminated following the Heatsynch protocol increased not only because of higher service rate, but also from increased conception rates observed for cows receiving ECP at all 3 sites. Because lactating dairy cows have lower circulating

concentrations of estradiol during proestrus than heifers (Sartori et al., 2002) and because estradiol during proestrus influences sperm transport and might inhibit PGF_{2 α} secretion in the subsequent estrous cycle (Mann and Lamming, 2000), it is possible that increased conception rates observed for cows on the Heatsynch protocol are the result of prolonged exposure to higher estradiol during proestrus.

Recent studies have demonstrated the importance of the length of proestrus on the reproductive performance of cows. Peters and Pursley (2003) administered the final GnRH injection of the Ovsynch protocol either concomitantly with PGF_{2 α} or 2 d later, and timed AI was performed 16 h after the final GnRH injection in both groups. Synchronization rate and regression of the CL did not differ between treatments, but cows receiving the second GnRH simultaneously with PGF_{2 α} treatment ovulated a smaller follicle and had lower pregnancy rates than those receiving GnRH 2 d after PGF_{2 α} treatment. When the final GnRH of the Ovsynch protocol was given at 0, 12, 24, or 36 h after the PGF_{2 α} treatment, synchronization rate was similar for all 4 treatments, but more cows receiving GnRH at 0 h experienced short luteal phase than those receiving GnRH at 36 h after PGF_{2 α} injection, and pregnancy rate increased linearly as the interval from PGF_{2 α} to GnRH increased. Although these data do not distinguish the effects of length of proestrus with exposure to estradiol from the effect of size of the ovulatory follicle, preliminary results from Mussard et al. (2003) indicate that reducing the length of proestrus reduces pregnancy rates because of shorter luteal cycles, independently of size of the ovulatory follicle. Induction of ovulation of follicles of similar size, but exposed to different lengths of proestrus, influenced conception rates (Mussard et al., 2003). When embryos were transferred into recipient heifers 7 d after a spontaneous ovulation or induced with GnRH when the follicle reached 10 mm, pregnancy rate was higher for recipient heifers that ovulated spontaneously (Mussard et al., 2003). These results suggest that reductions in fertility when cows were exposed to short proestrus were mediated by uterine effects, and not because of smaller, incompetent follicles. However, when small follicles (≤ 11 mm) were induced to ovulate with GnRH at the moment of timed AI, late embryonic losses were increased in beef cows (Perry et al., 2003).

Mann and Lamming (2000) demonstrated that cows exposed to low estradiol concentrations were more likely to experience subsequent premature luteolysis. In that study, oxytocin-binding activity was increased in endometrial explants, and greater release of PGF_{2 α} metabolite was detected after a challenge with oxytocin *in vivo*. Furthermore, estradiol production by the pre-ovulatory follicle has been correlated with the number

of progesterone receptors present in the uterine endometrium (Zollers et al., 1993), and treatment with estradiol was required before progesterone treatment was capable of inhibiting the release of uterine prostaglandin induced by oxytocin in ovariectomized cows (Kiebofz-Loos et al., 2003). Therefore, it is possible that higher estradiol concentrations during proestrus induce progesterone receptors and inhibit oxytocin receptor activity, thereby preventing the luteolytic cascade to be activated prematurely, which might favor embryonic survival in cows.

High-producing dairy cows have lower concentrations of estradiol than heifers during proestrus despite larger ovulatory follicles (Sartori et al., 2002). As DMI increases, hepatic blood flow also increases (Reynolds et al., 2003), and it has been suggested that changes in DMI influence hepatic clearance of ovarian steroids. In fact, Sangsritavong et al. (2002) demonstrated that increasing DMI increased hepatic blood flow and reduced serum concentrations of estradiol and progesterone. The relationship between increased DMI and reductions in blood concentrations of ovarian steroids has also been demonstrated by others (Vasconcelos et al., 2003). Because higher producing cows have lower concentrations of blood estradiol and because exposure to estradiol during proestrus might reduce short luteal cycles, there is a reasonable possibility that high-producing cows might benefit from supplemental estradiol during proestrus. In beef cows, a small dose of ECP incorporated into the Ovsynch protocol tended to increase pregnancy rates (Ahmadzadeh et al., 2003).

In addition to the uterine effects of estradiol that could explain the improved conception rates in cows receiving ECP, it is possible that higher estradiol during proestrus might have also enhanced sperm transport and fertilization rates. Orihuela et al. (1999) demonstrated that sperm transport in the reproductive tract of rats was improved during proestrus compared with other stages of the estrous cycle. They also demonstrated that exogenous estradiol-17 β facilitated sperm migration into the oviduct and that progesterone antagonized this effect. Therefore, treatment with ECP during proestrus also might have influenced uterine motility and sperm transport through the reproductive tract because of the higher blood concentrations of estradiol-17 β .

Macmillan et al. (2003) indicated that cows subjected to timed AI protocols can experience lower conception rates because follicles of differing maturity can be induced to ovulate, resulting in a less competent CL and a consequent slower rise in plasma progesterone and lower midluteal concentrations. Also, cows subjected to timed AI protocols can experience lower fertility because of the increased prevalence of short estrous cycles

(Macmillan et al., 2003). In the current study, cows were presynchronized with 2 injections of $\text{PGF}_{2\alpha}$ to minimize the occurrence of premature luteolysis during the synchronization protocol and to optimize synchronization of ovulation when the GnRH is given (Moreira et al., 2001). Incidence of short cycles as evaluated by the proportion of nonpregnant cows re-inseminated within 17 d did not differ, although more nonpregnant cows on the Heatsynch treatment were re-inseminated before pregnancy diagnosis on d 30 after the first AI. Nevertheless, Heatsynch cows had higher conception rates to first AI at all times. Increased prevalence of cows re-inseminated with short interval (<18 d) might be related to lack of synchronization of ovulation at the first AI. Only 80 to 90% of the cows subjected to induced ovulation with GnRH or ECP synchronize ovulation (Pursley et al., 1997; Pancarci et al., 2002; Galvão et al., accepted). Galvão et al. (accepted) demonstrated that 14% of the cows subjected to the Heatsynch protocol did not ovulate after treatment with ECP. This finding was associated with cows that were anovulatory before the synchronization protocol and did not display estrus after ECP treatment.

Insemination following a timed AI protocol with ECP did not affect late embryonic survival in dairy cows. It has been suggested that lactating dairy cows inseminated at a fixed time might experience higher late embryonic losses (Cartmill et al., 2001; Lucy, 2001). However, recent studies have not observed differences in late embryonic losses in lactating dairy cows inseminated following timed AI or following an induced or spontaneous estrus (Chebel et al., 2004; Santos et al., 2004a). Recently, Santos et al. (2004b) indicated that lactating dairy cows inseminated following timed AI protocols had similar late embryonic losses (11.2%) compared with cows inseminated following estrus detection (12.7%) in several published studies. However, when beef cows were induced to ovulate small follicles (≤ 11 mm) with an injection of GnRH at the time of AI, late embryonic losses were increased (Perry et al., 2003). Because cows in the current study were presynchronized, it is likely that those that responded to the presynchronization would have a recently recruited, well-developed dominant follicle at the time of induced ovulation in the Heatsynch protocol.

Although cows enrolled in the Heatsynch protocol had better reproductive performance in the current study, those that did not display signs of estrus at timed AI had lower conception rates than cows observed in estrus. Pancarci et al. (2002) previously reported that cows not in estrus 48 h after the ECP treatment had low conception rates. Cyclicity affected the proportion of cows displaying estrus in both treatments, and ECP increased observed estrous behavior in anovulatory

cows. Cyclic cows had higher conception rates than anovular cows (Moreira et al., 2001; Galvão et al., accepted; Santos et al., 2004a), and, in the Heatsynch protocol, more cyclic than anovular cows were detected in estrus (Galvão et al., accepted). Furthermore, ovulation rate in cows subjected to the Heatsynch regimen was increased when they were observed in estrus (Galvão et al., accepted). Therefore, it is suggested that conception rates were higher in cows displaying estrus during the Heatsynch because it was associated with cyclicity and increased ovulation rate.

The size of the largest follicle 48 h after the $\text{PGF}_{2\alpha}$ treatment influenced the display of behavioral estrus. As cows had larger follicles, detection of estrus increased, and this effect was observed for both Selectsynch and Heatsynch cows. Larger follicles produced greater amounts of estradiol, as indicated by higher concentrations in blood (Inskeep, 2002). Thus, it is possible that estrous response in the Heatsynch protocol was not solely influenced by estradiol concentrations as a result of ECP injection, but also dependent upon ovarian estradiol production.

Progesterone class affected conception and pregnancy rates. As expected, cows classified as HHL had the highest conception and pregnancy rates. These are cyclic cows that responded to the presynchronization as they had high progesterone at the injections of GnRH and $\text{PGF}_{2\alpha}$ (Moreira et al., 2001). Furthermore, these cows had their CL regressed within 48 h of the $\text{PGF}_{2\alpha}$ treatment, and if in the Heatsynch treatment, they were timed inseminated under low progesterone, which favors conception rates.

Other factors were associated with reproductive performance of dairy cows. As BCS at AI increased, conception and pregnancy rates also increased. This relationship has been demonstrated before, and in part is related to increased cyclicity before first AI (Santos et al., 2004a). Also, in the current study, milk production was associated with reproductive performance, and cows with production below the mean milk yield had higher conception and pregnancy rates than those with production above the mean milk yield. Lucy (2001) described factors associated with impaired reproductive performance in the modern dairy cow and demonstrated evidence that the increase in rolling herd average in the last 30 yr for dairies served by DHIA was associated with reduced conception rates, which have declined from 55% to approximately 40% during the same period. Recently, Chebel et al. (2004) found no association between milk production and conception rates or pregnancy losses in high-producing dairy cows in 3 dairy herds, but Peters and Pursley (2002) observed that cows with production above the mean milk yield had higher

pregnancy rates to the Ovsynch protocol than cows with production below the mean milk yield.

Milk production during the study period was virtually the same for cows in the Heatsynch and the Selectsynch protocols, except during the month of ECP treatment. Reduction in milk yield when ECP was given could have been the result of the greater display of behavioral estrus reducing feed intake and milk production. However, milk production was not lower for cows expressing signs of estrus, and no interaction between treatment and estrus was observed for milk yield. Another hypothesis concerns the effect of estradiol on the mammary gland, because an injection of 1 mg of ECP increases basal estradiol concentrations for 2 to 3 d (Lopes et al., 2000). Although estradiol is necessary for initiation and maintenance of lactation, during galactopoiesis, high concentrations of estradiol can temporarily decrease milk yield (Akers, 2002). Nevertheless, production of milk for cows in both treatments was virtually the same during the first 6 mo in lactation.

CONCLUSIONS

High-producing lactating dairy cows subjected to a presynchronized timed AI protocol utilizing ECP to induce ovulation had increased proportions in estrus, as well as higher conception and pregnancy rates compared with cows inseminated following detected estrus induced by a presynchronized Selectsynch protocol. The improvements in fertility responses for cows subjected to the Heatsynch protocol were observed regardless of parity, BCS, and dairy site, increasing the external validity of the data. Responses to the Heatsynch protocol were improved when lactating cows were observed in estrus after ECP treatment, which was associated with cyclic status. More cyclic cows exhibited estrus in both treatments, and cyclic cows had higher conception and pregnancy rates. Neither treatment nor cyclic status affected late embryonic losses up to 58 d of gestation. Conception rates during the second postpartum AI, when cows were inseminated either upon detection of spontaneous estrus or following resynchronization with the Ovsynch protocol, were similar for cows previously subjected to the Heatsynch or Selectsynch protocols.

When cows were classified according to progesterone concentrations above or below 1.0 ng/mL when injected with GnRH and with PGF_{2α}, as well as 48 h later, those that responded to the presynchronization and underwent luteolysis (HHL) had the highest conception and pregnancy rates. Size of the largest follicle 48 h after the PGF_{2α} affected estrus response but did not alter conception or pregnancy rates. Treatment with ECP to induce ovulation had no effect on daily milk yield during the first 6 mo postpartum but reduced milk production

in the month of treatment, and this effect was not associated with the display of behavioral estrus.

Because cows inseminated following the Heatsynch protocol had higher conception rates, it is possible that supplemental estradiol as ECP during proestrus enhances fertility of high-producing dairy cows.

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