Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows

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ABSTRACT

Two experiments evaluated the influence of follicular wave at artificial insemination (AI) on fertility of dairy cows. In experiment 1, data from 5,607 lactating cows enrolled in estrous and ovulation synchronization programs for AI were evaluated. Cows' blood was analyzed for progesterone 7 to 14 d apart, with the second sample collected on the day of the first GnRH (GnRH1) of the synchronization protocol. Cows were classified as cyclic if progesterone was ≥ 1 ng/mL in at least 1 of the 2 samples and as anovular if both samples were < 1 ng/mL. Cyclic cows were categorized as low (CLOW; < 1 ng/mL) or high (CHIGH; ≥ 1 ng/mL) progesterone on the day of GnRH1, which would result in ovulation of the dominant follicle of the first (FW) and second (SW) follicular waves, respectively, at AI. Pregnancy per AI (P/AI) was determined 30 and 53 d after AI. In experiment 2, 220 cyclic Holstein cows received 2 injections of PGF_{2 α} administered 14 d apart. The Ovsynch protocol (d 0 GnRH, d 7 PGF_{2 α} , d 9 GnRH, d 9.5 timed AI) was initiated either 3 or 10 d after the second PGF_{2 α} of the presynchronization to result in insemination to the FW or SW dominant follicles. Blood was analyzed for progesterone and ovaries were scanned to determine ovulatory responses and follicle diameter. Pregnancy was determined on d 32 and 67 after timed AI. In experiment 1, P/AI on d 30 was greater for CHIGH cows than for CLOW and anovular cows (43.0, 31.3, and 29.7%, respectively), but because of pregnancy loss, P/AI on d 53 was lowest for anovular cows. Proportions of cows with short reinsemination intervals differed among groups and were 7.1, 15.7, and 11.9% for CHIGH, CLOW, and anovular cows, respectively. Pregnancy loss was greater for anovular cows than for CLOW cows (15.0 vs. 10.0%) and was intermediate for CHIGH cows (13.5%). In experiment 2, 9.8 and 97.2% of the FW and SW cows, respectively, had progesterone ≥ 1 ng/mL at GnRH1. Concentrations of progesterone at the

GnRH1 and PGF_{2 α} injections of the Ovsynch protocol were greater for SW cows than FW cows. Pregnancy per AI was greater for SW cows than for FW cows (41.7 vs. 30.4%) despite less ovulation to GnRH1 in SW cows than in FW cows (78.7 vs. 88.4%). Collectively, these data indicate that follicular wave of the ovulatory follicle and not cyclic status caused the greatest reduction in P/AI in dairy cows. Whether the culprit is the follicle itself or the hormonal milieu characteristic of the first follicular wave and the early stage of the estrous cycle remains to be elucidated. Synchronization programs that induced ovulation of the FW follicle at AI reduced P/AI in lactating dairy cows, and ovulation of the FW follicle, or development of the ovulatory follicle under low progesterone concentrations, or both, might be mechanisms for reduced fertility in anovular cows.

Key words: anovular, dairy cow, follicular wave, reproduction

INTRODUCTION

Protocols for synchronization of ovulation and timed AI based on GnRH and PGF_{2 α} maximize the proportion of cows inseminated early after the end of the voluntary waiting period and result in satisfactory pregnancy per AI (P/AI) when implemented in commercial dairy herds (Chebel et al., 2006). Optimal P/AI in response to the Ovsynch protocol in lactating dairy cows has been associated with successful ovulation to the first GnRH injection (GnRH1) and the presence of a functional corpus luteum (CL) at the injection of PGF_{2 α} (Vasconcelos et al., 1999; Moreira et al., 2001; Chebel et al., 2006).

The proportion of lactating dairy cows that ovulate to the GnRH1 is greater when it is administered in the presence of a dominant follicle, usually between d 5 and 9 and d 17 and 21 of the estrous cycle (Vasconcelos et al., 1999; Bello et al., 2006; Cerri et al., 2009). However, the proportion of cows with concentrations of progesterone < 1 ng/mL at the injection of PGF_{2 α} and the incidence of ovulation before the final GnRH was higher when the Ovsynch protocol was initiated after d 10 of the estrous cycle (Vasconcelos et al., 1999). Induc-

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tion of ovulation of the dominant follicle of the first follicular wave (**FW**) during early diestrus (i.e., d 5 to 9 of the estrous cycle) with GnRH1 results in recruitment of the second follicular wave (**SW**), which will generate the ovulatory follicle at timed AI. In contrast, when GnRH1 is administered before the acquisition of ovulatory capacity by the FW follicle (d 1 to 4 of the estrous cycle; Sartori et al., 2001) or when ovulation of the SW dominant follicle is induced during proestrus (d 17 to 21 of the estrous cycle), the FW dominant follicle is induced to ovulate at completion of the timed AI protocol before AI. The FW dominant follicle grows under smaller systemic concentrations of progesterone than the SW follicle, which has been associated with changes in the rate of follicle growth and follicular fluid composition (Cerri et al., 2008), increased endometrial synthesis of PGF_{2α} (Shaham-Albalancy et al., 2001), and incidence of short estrous cycles (Cerri et al., 2008). Consequently, cows that received the GnRH1 of the timed AI protocol with progesterone ≥ 1 ng/mL had greater P/AI than cows with progesterone < 1 ng/mL (Cerri et al., 2004; Stevenson et al., 2008).

Anovular cows are more likely to ovulate to GnRH1 (Gümen et al., 2003) and, among those that ovulate to GnRH1, the follicle induced to ovulate at the end of the synchronization protocol is the FW dominant follicle. Therefore, cyclic cows that initiate the timed AI protocol when progesterone is < 1 ng/mL and anovular cows are similar in that they both ovulate the FW dominant follicle at AI. It is known that anovular cows have reduced reproductive performance (Cerri et al., 2004; Santos et al., 2009). Therefore, if fertility of anovular cows and that of cyclic cows that ovulate the FW dominant follicle are similar and they are both less than that of cyclic cows ovulating the SW dominant follicle, it is then reasonable to speculate that some of the underlying mechanisms responsible for the reduced P/AI are similar between them, particularly the fact that these cows are induced to ovulate follicles that develop under progesterone concentrations often < 1 ng/mL.

The hypothesis of the present study was that ovulation of the FW dominant follicle at AI reduces fertility in lactating dairy cows compared with ovulation of the SW dominant follicle. It was expected that cows ovulating the FW dominant follicle would have reduced P/AI compared with cows induced to ovulate the SW dominant follicle despite cyclic status at the beginning of the timed AI protocol. In experiment 1, the objectives were to determine the associations of cyclic status and follicular wave at AI with P/AI and risk of pregnancy loss in lactating dairy cows; in experiment 2, the objective was to evaluate the effect of follicular wave on ovulatory responses and P/AI of lactating dairy cows.

MATERIALS AND METHODS

Experiment 1

Data from previously completed experiments (Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004a,b; Chebel et al., 2006; Juchem, 2007; Hillegass et al., 2008; J. E. P. Santos; unpublished data) were collated into a single data set containing a total of 5,607 cows subjected to presynchronized timed AI or estrous synchronization protocols for first postpartum AI. Only cows that did not receive supplemental progesterone during the timed AI or estrous synchronization protocols were used. Detailed description of animals and management are presented in the published papers.

In those studies, the proportion of cows calving in the fall, winter, spring, and summer were 26.7, 52.2, 13.8, and 7.3%, respectively. Inseminations were performed between the months of September and May to avoid the negative effects of heat stress on fertility.

Reproductive Management. Detailed description of reproductive management is presented in the published papers. Briefly, the estrous cycles of cows were presynchronized (Moreira et al., 2001) with 2 injections of 25 mg of PGF_{2α} (Lutalyse, 5 mg/mL of dinoprost tromethamine sterile solution, Pfizer Animal Health, New York, NY) administered 14 d apart, and either 12 or 14 d after the second injection of PGF_{2α} they were enrolled in an estrous or ovulation synchronization protocol. For both synchronizations, the initial portion of the protocols was similar and consisted of a 100-μg injection of GnRH (Cystorelin, 50 μg/mL of gonadorelin diacetate tetrahydrate, Merial Ltd., Iselin, NJ) followed 7 d later by an injection of 25 mg of PGF_{2α}. Cows subjected to insemination upon detection of estrus did not receive any further treatment. These cows were observed for signs of estrus once daily, in the morning, by tail chalking using paint sticks (All-weather Paintstik, La-Co Industries, Chicago, IL), and those observed in estrus based on rubbed chalk were inseminated in the same morning in 3 studies (Cerri et al., 2004; Santos et al., 2004a,b). Cows assigned to timed AI either received an injection of 1 mg of estradiol cypionate (ECP, 2 mg/mL of estradiol cypionate, Pfizer Animal Health) and were inseminated 48 h later (Cerri et al., 2004; Galvão et al., 2004) or received an injection of GnRH 48 to 56 h after PGF_{2α} and were timed AI 12 to 24 h later (Santos et al., 2004a; Chebel et al., 2006; Juchem, 2007). In one study (Hillegass et al., 2008), all cows received GnRH concurrent with AI at either 48 or 72 h after PGF_{2α} to induce ovulation, and half of them were supplemented with estradiol cypionate 24 h after PGF_{2α}.

After the first AI, cows observed to have the tail head paint removed were reinseminated. Cows with an inter-

insemination interval between 5 and 17 d after the first AI were considered to have short inter-AI interval.

Blood Sampling and Progesterone Analysis. Blood was sampled 7 to 14 d apart for analysis of progesterone. The second sample was collected on the day of the GnRH1. The first sample was collected either 7 (Hillegass et al., 2008), 12 (Santos et al., 2004a; J. E. P. Santos; unpublished data), 13 (Chebel et al., 2006), or 14 d earlier (Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004b; Juchem, 2007). Approximately 7 mL of blood was collected by puncture of the median coccygeal vein or artery using evacuated tubes with K₂ EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The samples were immediately placed in ice and were later centrifuged at $2,000 \times g$ for 15 min for separation of plasma. Plasma samples were frozen at -25°C until later analysis by ELISA (Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004a; Chebel et al., 2006; Juchem, 2007; Hillegass et al., 2008; J. E. P. Santos; unpublished data) or RIA (Santos et al., 2004b).

Classification of Cows According to Progesterone Status. Cows were classified as having initiated estrous cycles if progesterone concentration was ≥ 1 ng/mL in at least 1 of the 2 samples, or as anovular when both samples were < 1 ng/mL. Cyclic cows were further classified as having progesterone concentration < 1 ng/mL on the day of GnRH1 (cyclic-low; **CLOW**) or as having ≥ 1 ng/mL on the day of GnRH1 (cyclic-high; **CHIGH**). Therefore, 3 groups of cows were created: anovular, CLOW, and CHIGH. The rationale for such categories was that presynchronized cyclic cows that had low concentration of progesterone on the day of GnRH1 were in either proestrus, estrus, or metestrus and, therefore, when completing the synchronization protocol, were expected to have the FW dominant follicle induced to ovulate. On the other hand, presynchronized cows classified as CHIGH were expected to be in early or mid diestrus and likely ovulated the SW dominant follicle at the end of synchronization protocol. A small proportion of CHIGH cows could also ovulate the SW dominant follicle to GnRH1 if on d 13 of the estrous cycle, which would result in ovulation of the third wave dominant follicle at AI. Similar to FW cows, anovular cows subjected to the synchronization programs based on GnRH and PGF_{2 α} were expected to be inseminated to an ovulation of the FW dominant follicle.

Pregnancy Diagnosis. Cows were examined for pregnancy twice. The first diagnosis was performed by ultrasonography between 27 and 32 d after AI (Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004a,b; Chebel et al., 2006; Juchem, 2007; J. E. P. Santos; unpublished data) or by transrectal palpation at 40 d after AI (Hillegass et al., 2008). Pregnant cows in the first exam were reevaluated for pregnancy 2 (Galvão

et al., 2004; Santos et al., 2004a, b; J. E. P. Santos; unpublished data) or 4 wk later (Cerri et al., 2004; Chebel et al., 2006; Juchem, 2007; Hillegass et al., 2008). Throughout the paper, the first diagnosis is designated as d 30 and the second as d 53 after AI because those were the average intervals from AI to pregnancy diagnosis for all studies combined.

Pregnancy per AI was calculated as the proportion of inseminated cows pregnant at either 30 or 53 d after AI. Pregnancy loss was calculated by dividing the number of cows diagnosed not pregnant on d 53 by the number of cows pregnant on d 30.

Experiment 2

Animals, Housing, and Feeding. Holstein cows ($n = 220$) from a commercial dairy farm were enrolled in this experiment. Cows were housed in free-stall barns and primiparous and multiparous cows were housed separately throughout the experiment. Cows were fed diets formulated by CPM-Dairy software (Cornell-Penn-Miner version 3.0.8; Miner Institute, Chazy, NY) to meet the metabolizable energy and protein, mineral, and vitamin requirements for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% FCM when consuming 26 kg/d of DM (NRC, 2001). All diets were mixed as complete rations and offered twice daily at 0600 and 1600 h to allow for 3 to 5% refusals. Ingredients were alfalfa hay, alfalfa silage, corn silage, steam-flaked corn, citrus pulp, whole cottonseed, solvent-extracted soybean meal, expeller-treated soybean meal, a protein blend of animal-marine byproducts, Ca salts of palm oil, and a mixture of vitamins and minerals.

Treatments. Weekly, cohorts of 8 to 22 lactating dairy cows at 37 ± 3 DIM were blocked by parity (lactation 1 or lactation > 1) and milk yield in the first month postpartum and, within each block, were randomly assigned to 1 of the 2 treatments, insemination to the dominant follicle of the FW (42 primiparous and 70 multiparous) or SW (36 primiparous and 72 multiparous).

The estrous cycles of cows were presynchronized with 2 subcutaneous injections of 25 mg of PGF_{2 α} as dinoprost tromethamine (5 mL of Lutalyse Sterile Solution, Pfizer Animal Health) given 14 d apart, with the injections given at 44 ± 3 and 58 ± 3 DIM for cows in treatment FW and at 37 ± 3 and 51 ± 3 DIM for cows in treatment SW (Figure 1). These schemes for presynchronization were designed such that cows in FW and SW would initiate the timed AI protocol 3 and 10 d after the second PGF_{2 α} , respectively, and they would be inseminated on the same day postpartum. The Ovsynch protocol was initiated at 61 ± 3 DIM

Figure 1. Diagram of activities during experiment 2. BS = blood samples for analysis of progesterone concentration; FW = ovulation of the first wave dominant follicle at AI; GnRH = injection of 100 μ g of gonadorelin diacetate tetrahydrate; PGF_{2 α} = injection of 25 mg of dinoprost as tromethamine salt; SW = ovulation of the second wave dominant follicle at AI; US = ultrasonography of the ovaries.

and consisted of an intramuscular injection of 100 μ g of GnRH (Cystorelin, 50 μ g/mL of gonadorelin diacetate tetrahydrate, Merial Ltd.), followed 7 d later by a subcutaneous injection of 25 mg of PGF_{2 α} on d 68 $\pm$ 3 postpartum and 48 h later by an intramuscular injection of 100 μ g of GnRH on d 70 $\pm$ 3 postpartum, with timed AI performed 12 h after the final GnRH injection of the Ovsynch protocol. The same technician inseminated all cows in the experiment with semen from 5 different sires equally allocated across treatments.

Ovarian Ultrasonography. Transrectal ultrasonography of the ovaries was performed using an ultrasound equipped with a 7.5-MHz linear transducer (Sonovet 2000, Universal Medical System, Bedford Hills, NY) on the day of the second PGF_{2 α} of the presynchronization protocol. Initially, 247 cows were evaluated, but only those with a visible CL >18 mm in diameter remained in the experiment (n = 220 cows) to ensure that all cows were cyclic, which was later confirmed by a plasma sample with progesterone $\geq$ 1 ng/mL. The 220 cows that continued the experiment also had their ovaries scanned on the day of GnRH1 and again 7 d later, on the day of the PGF_{2 α} injection of the Ovsynch protocol. Ovarian maps were drawn and diameter and position of the CL and follicles $\geq$ 8 mm in diameter were recorded.

Cows with a follicle $\geq$ 8 mm on the day of GnRH1 injection of the timed AI protocol and with a newly formed CL 7 d later on the ipsilateral ovary were considered to have ovulated in response to GnRH1.

Blood Sampling and Progesterone Analysis. Approximately 7 mL of blood was collected by puncture of the coccygeal artery or vein using evacuated tubes with K₂ EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Samples were collected on the day of the second PGF_{2 α} of the presynchronization protocol, on the day of GnRH1, and again on the day of the PGF_{2 α} of the Ovsynch protocol (Figure 1). Samples were placed in ice upon collection and were transported to the laboratory within 5 h. Blood samples were centrifuged and plasma was separated and frozen at -25°C and later analyzed for progesterone concentration by a validated ELISA (Cerri et al., 2004). Individual samples were analyzed in duplicate and samples with a coefficient of variation >15% were reanalyzed. Sensitivity of the assay was 0.10 ng/mL and the intra- and interassay coefficients of variation were 5.4 and 9.9%, respectively.

BCS and Milk Yield. Cows were scored for body condition (1 = emaciated, 5 = obese) according to Ferguson et al. (1994) on the day of GnRH1. Cows were

milked twice daily and milk yield from the first 3 mo postpartum was recorded for individual cows, and the average milk production in those months was used in the statistical analysis.

Pregnancy Diagnosis and Calculation of Reproductive Responses. Pregnancy was diagnosed by transrectal ultrasonography on d 32 after the timed AI. Presence of an amniotic vesicle with an embryo was used as indicators of pregnancy. Pregnant cows were reexamined for pregnancy 5 wk later, on d 67 of gestation.

Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant at 32 or 67 d after AI by the number of cows receiving AI. The proportion of cows having experienced pregnancy loss was calculated as the number of cows that lost pregnancy between 32 and 67 d after AI divided by the number of cows diagnosed pregnant on d 32 after AI.

Study Design and Statistical Analyses

Experiment 1. Information from individual studies was collated into a single data set for statistical analysis. Data were analyzed by a multivariate logistic regression using the LOGISTIC procedure of SAS (SAS/STAT version 9.2, SAS Institute Inc., Cary, NC). A backward stepwise regression model was used, and explanatory variables were sequentially removed from the model by the Wald statistic criterion if $P > 0.10$. Pregnancy per AI and incidence of pregnancy loss and short inter-AI interval were analyzed with a model that included group (anovular, CLOW, and CHIGH), study (1 to 8), season of calving (fall, winter, spring, and summer), parity (primiparous and multiparous), method of AI (timed AI or synchronized estrus), BCS, milk yield, and interaction between group and method of AI.

Adjusted odds ratio (AOR) and 95% confidence interval were generated by the logistic regression. Comparisons were performed among individual groups (anovular vs. CLOW vs. CHIGH), and an orthogonal comparison was performed to determine the effect of follicular wave (anovular + CLOW vs. CHIGH) on reproductive responses of dairy cows.

Experiment 2. Experiment 2 was completely randomized with blocks. Based on results of experiment 1, it was expected that a 12 percentage units decrease (e.g., 31 vs. 43%) in P/AI would be observed in cows inseminated when the FW dominant follicle was induced to ovulate compared with cows inseminated following induction of ovulation of the SW dominant follicle. One hundred eighty cows were initially planned (90/treatment) to provide enough replicates to detect statistical significance with a 12 percentage unit improvement in P/AI for SW cows ($\alpha = 0.05$, $\beta = 0.90$; one-tailed test).

Because of potential attrition before completion of the study, 220 cows were enrolled.

Binary data were analyzed by logistic regression using the LOGISTIC procedure of SAS (SAS Institute Inc.). The models included the effects of treatment, parity, BCS, and milk yield. Additional covariates were included when relevant. Variables with $P > 0.10$ were sequentially removed from the model by a backward elimination based on the Wald's statistics. Treatment was forced in the final models.

Concentrations of progesterone on the day of GnRH1 and PGF_{2 α} of the Ovsynch protocol were analyzed by ANOVA using the GLM procedure of SAS. For concentration of progesterone on the day GnRH1, the model included the effects of treatment, parity, BCS, milk yield, and interaction of treatment and parity. For concentration of progesterone on the day of the PGF_{2 α} of the Ovsynch, the model also included the effects of ovulation to GnRH1 and interaction between treatment and ovulation to GnRH1.

Diameter of the largest follicle on the PGF_{2 α} was analyzed by ANOVA using the GLM procedure of SAS, and the model included the effects of treatment, parity, BCS, milk yield, ovulation to GnRH1, and interactions between treatment and parity and treatment and ovulation to GnRH1. In both experiments, differences with $P \leq 0.05$ were considered significant, and those with $0.05 < P \leq 0.10$ were considered a tendency.

RESULTS

Experiment 1

The proportion of cows classified as anovular at the beginning of the synchronization protocols was 22.6%. Among the cyclic cows, 14.8 and 85.2% had low and high progesterone at GnRH1, respectively.

Ovulation of the SW dominant follicle reduced ($P < 0.0001$) incidence of short inter-AI interval in nonpregnant cows, and CLOW cows had increased ($P = 0.03$) incidence of short inter-AI interval compared with anovular cows (Table 1). Of the nonpregnant cows, a lesser ($P < 0.0001$) proportion had short inter-AI interval when inseminated in estrus than when receiving timed AI (4.0 vs. 11.1%; AOR = 0.31; 95% CI = 0.20 to 0.50); however, no interaction ($P = 0.16$) between method of insemination and cyclic status or follicular wave was observed for short inter-AI interval.

The overall P/AI on d 30 after first postpartum AI averaged 38.8% and differed according to the follicular wave at insemination (Table 1). Cows that ovulated the SW dominant follicle had greater ($P < 0.001$) P/AI compared with cows that ovulated the FW dominant follicle (43.0 vs. 30.2%; AOR = 4.18; 95% CI = 2.51 to

Table 1. Effect of cyclic status and follicular wave at insemination on fertility responses of dairy cows (experiment 1)

Item, % (no./no.)	Group ¹			P-value
	Anovular	CLOW	CHIGH	
Short AI interval ²	11.9 (106/891) ^b	15.7 (59/376) ^a	7.1 (154/2,165) ^c	<0.001
Pregnant				
d 30	29.7 (377/1,268) ^b	31.3 (171/546) ^b	43.0 (1,630/3,793) ^a	<0.001
d 53	25.1 (318/1,265) ^c	28.1 (153/545) ^b	36.9 (1,392/3,773) ^a	<0.001
Pregnancy loss	15.0 (56/374) ^a	10.0 (17/170) ^{b,e}	13.5 (218/1,610) ^d	0.08

^{a-c}Means in the same row with different superscripts differ ($P < 0.04$).

^{d,e}Means in the same row with different superscripts tend to differ ($P = 0.06$).

¹Anovular = cows with progesterone <1 ng/mL in 2 sequential blood samples collected 7 to 14 d apart, with the second sample collected on the day of the first GnRH of the synchronization protocol. These cows were expected to ovulate the dominant follicle of the first follicular wave. CLOW = cyclic cows but with progesterone <1 ng/mL on the first GnRH of the synchronization protocol. These cows were presumed to ovulate a first wave follicle at AI. CHIGH = cyclic cows with progesterone ≥ 1 ng/mL on the first GnRH of the synchronization protocol. These cows were presumed to ovulate a second wave follicle at AI.

²Nonpregnant cows reinseminated between 5 and 17 d after the first AI.

6.95). In addition, cows ovulating the dominant follicle of the FW had similar ($P = 0.31$) P/AI whether they were previously cyclic or anovular. Pregnancy per AI at d 30 after AI was greater ($P < 0.0001$) for cows inseminated in estrus than for those receiving timed AI, but no interaction ($P = 0.74$) between method of insemination and cyclic status or follicular wave was observed. For anovular cows, P/AI for those inseminated in estrus or receiving timed AI were 38.2 and 26.8%, respectively; for CLOW cows, they were 41.8 and 28.8%, respectively; and for CHIGH cows, they were 50.0 and 39.8%, respectively.

Pregnancy responses on d 53 after AI followed a similar pattern as those observed on d 30 (Table 1). Cows ovulating the SW dominant follicle had greater ($P < 0.001$) P/AI compared with cows that ovulated the FW dominant follicle (36.9 vs. 26.0%; AOR = 2.45; 95% CI = 1.87 to 3.21). In cows induced to ovulate the dominant follicle of the FW, those classified as CLOW had greater (AOR = 1.28; 95% CI = 1.02 to 1.62; $P = 0.04$) P/AI than anovular cows. Cows inseminated in estrus had greater ($P < 0.0001$) P/AI than those receiving timed AI (42.0 vs. 29.9%; AOR = 1.77; 95% CI = 1.52 to 2.06), but no interaction ($P = 0.44$) between method of AI and cyclic status or follicular wave was observed.

Pregnancy loss averaged 13.5% and there was no overall effect ($P = 0.30$) of cows ovulating the FW or SW on losses of pregnancy between 30 and 53 d of gestation (Table 1). Nevertheless, anovular cows had greater (AOR = 2.04; 95% CI = 1.12 to 3.72; $P = 0.02$) and CHIGH cows tended (AOR = 1.71; 95% CI = 1.00 to 2.93; $P = 0.06$) to have greater pregnancy loss than CLOW cows. Cows inseminated in estrus had less ($P = 0.05$) pregnancy loss than those receiving timed AI (10.4 vs. 15.2%), but no interaction ($P = 0.30$) between

method of AI and follicular wave or cyclic status was observed.

Experiment 2

Milk yield during the first 3 mo postpartum was similar ($P = 0.76$) for both treatments and averaged 46.9 ± 0.7 kg/d for multiparous cows and 36.3 ± 0.9 kg/d for primiparous cows. Average BCS did not differ ($P = 0.61$) between treatments, but primiparous cows (2.96 ± 0.04) had greater ($P = 0.01$) BCS compared with multiparous cows (2.83 ± 0.03).

Ovarian Responses and Progesterone Concentration During the Ovsynch. The proportion of cows with high concentration of progesterone on the day of GnRH1 was greater ($P < 0.001$) for SW cows than for FW cows (Table 2). Accordingly, the mean concentration of progesterone on the day of GnRH1 was greater ($P < 0.0001$) for SW cows than for FW cows (Table 2). The ovulatory response to GnRH1 was greater ($P = 0.05$) for FW cows. On the day of the PGF_{2α} injection of the Ovsynch protocol, the proportion of cows with a CL detected by ultrasonography tended to be less ($P = 0.10$) for SW cows than for FW cows, although mean concentration of progesterone was greater ($P = 0.002$) for cows in the SW treatment than for cows in the FW treatment (Table 2). The dominant follicle on the day of the PGF_{2α} injection of the Ovsynch protocol was larger ($P < 0.001$) for FW cows compared with SW cows (Table 2).

P/AI and Pregnancy Loss. Pregnancy per AI on d 32 and 67 after insemination averaged 35.9 and 34.1%, respectively. Cows induced to ovulate the SW had greater P/AI on d 32 and 67 after insemination than those ovulating the FW dominant follicle (Table

Table 2. Effect of follicular wave at insemination on ovarian responses of cyclic dairy cows during the Ovsynch protocol (experiment 2)

Item	Follicular wave ¹		AOR ²	95% CI	P-value
	FW	SW			
Ovulation to GnRH1, ³ % (no./no.)	88.4 (99/112)	78.7 (85/108)	0.47	0.22–1.00	0.05
Corpus luteum at PGF _{2α} , % (no./no.)	96.4 (108/112)	90.7 (98/108)	0.36	0.11–1.20	0.10
Progesterone ≥1 ng/mL, % (no./no.)					
GnRH1	9.8 (11/112)	97.2 (105/108)	322	87–999	0.001
PGF _{2α}	92.0 (103/112)	88.0 (95/108)	0.63	0.26–1.55	0.31
Progesterone, ng/mL					
GnRH1	0.39 ± 0.08	2.55 ± 0.08	—	—	0.001
PGF _{2α}	2.10 ± 0.20	2.92 ± 0.17	—	—	0.002
Follicle at PGF _{2α} , mm	17.9 ± 0.3	15.7 ± 0.2	—	—	0.001

¹Follicular wave of the ovulatory follicle at the timed AI. FW = first wave; SW = second wave.

²AOR = adjusted odds ratio (first wave was the reference for comparison).

³First GnRH of the Ovsynch protocol.

3). In addition, cows that ovulated to GnRH1 of the Ovsynch protocol were more likely ($P < 0.01$) to be diagnosed pregnant on d 32 (39.7 vs. 16.7%; AOR = 3.37; 95% CI = 1.30 to 8.74) and 67 (38.0 vs. 13.9%; AOR = 4.08; 95% CI = 1.47 to 11.28) after AI than cows that did not ovulate to GnRH1. No interaction between treatment and ovulation to GnRH1 was observed for pregnancy on d 32 ($P = 0.63$) and 67 ($P = 0.85$). Cows with high progesterone on the day of the PGF_{2α} of the Ovsynch protocol had greater ($P = 0.02$) P/AI on d 32 after insemination than cows with low progesterone (37.4 vs. 4.6%; AOR = 10.91; 95% CI = 1.40 to 84.91).

Pregnancy loss between d 32 and 67 of gestation was not affected by treatment (Table 3). Similarly, ovulation to GnRH1 did not influence ($P = 0.22$) the risk of cows losing their pregnancy from 32 to 67 d of gestation.

DISCUSSION

Studies have consistently shown that cows that have not resumed ovulation before the first postpartum AI have reduced P/AI and increased risk of pregnancy loss when inseminated following timed AI protocols or following estrus (Gümen et al., 2003; Santos et al., 2004c; Santos et al., 2009). Although the association

between anovulation and impaired reproductive performance is clear, the specific mechanisms by which it reduces fertility remain undefined. Results from experiment 1 showed that P/AI did not differ between anovular cows and cyclic cows that were induced to ovulate the FW dominant follicle before AI. According to Wiltbank et al. (2002), anovular postpartum dairy cows generally have continuous development of follicles that fail to ovulate spontaneously and, in some cases, develop follicular cysts. Nevertheless, the majority of these cows respond to an injection of GnRH through either ovulation or luteinization (Gümen et al., 2003). In that sense, anovular cows that successfully respond to a GnRH-PGF_{2α} based synchronization protocol (i.e., ovulate to GnRH1, regress the newly formed CL in response to the PGF_{2α}, and ovulate after the final GnRH) are expected to ovulate the FW dominant follicle at AI. Because a large proportion of anovular cows are expected to respond to a GnRH-PGF_{2α} based synchronization protocol (Gümen et al., 2003), results observed in experiment 1 indicate that the ovulation of a FW dominant follicle might be one of the components of the poor fertility observed in anovular dairy cows.

Cyclic status and follicular wave before AI were characterized in experiment 1 based on the concentration of progesterone in samples collected on the day of GnRH1

Table 3. Effect of follicular wave at insemination following the Ovsynch protocol on fertility responses of cyclic dairy cows (experiment 2)

Item, % (no./no.)	Follicular wave ¹		AOR ²	95% CI	P-value
	FW	SW			
Pregnant					
d 32	30.4 (34/112)	41.7 (45/108)	1.85	1.04–3.30	0.04
d 67	27.7 (31/112)	40.7 (44/108)	2.06	1.15–3.70	0.02
Pregnancy loss	8.8 (3/34)	2.2 (1/45)	0.20	0.02–2.18	0.19

¹Follicular wave of the ovulatory follicle at the timed AI. FW = first wave; SW = second wave.

²AOR = adjusted odds ratio (first wave was the reference for comparison).

and 7 to 14 d earlier. For cyclic cows with progesterone ≥ 1 ng/mL on the day of GnRH1, it was assumed that these cows were in diestrus, which would likely result in the ovulation of the SW dominant follicle in lactating dairy cows after a GnRH-PGF_{2 α} based synchronization protocol. On the other hand, cyclic cows with progesterone < 1 ng/mL at GnRH1 would likely be in proestrus, estrus, or metestrus and they would likely ovulate the FW dominant follicle at AI. The exception would be cows in metestrus, around d 3 of the cycle, that might ovulate to GnRH1 and recruit a SW dominant follicle to ovulate at the end of the synchronization program. Nevertheless, this proportion is expected to be very low (Vasconcelos et al., 1999; Cerri et al., 2009) and unlikely to change the interpretation of the results of this study. In fact, when cows were on d 3 of the estrous cycle, ovulation to GnRH1 and recruitment of the SW was observed in only 7.1% of them (Cerri et al., 2009). In addition, the small proportion of cows in diestrus at GnRH1 in FW cows in experiment 2 reinforces the criteria used in the first experiment to stage the estrous cycle and determine the follicular wave at AI. Because cyclic cows initiating the synchronization protocol with progesterone < 1 ng/mL in experiment 1 had reduced P/AI, but similar to that of anovular cows, experiment 2 was designed to specifically test the hypothesis that ovulation of the FW dominant follicle impairs P/AI in lactating dairy cows. Results from experiment 2 confirm the findings of experiment 1: P/AI was greater for cows induced to ovulate the SW dominant follicle at the end of the protocol.

A major difference between FW and SW dominant follicles is the hormonal milieu on which they develop. The FW dominant follicle emerges early in cycle and grows under low and increasing concentrations of progesterone, whereas the SW grows during diestrus concurrent with a mature CL. Progesterone exerts a negative feedback on the pulsatility of LH, and the latter stimulates the growth and steroidogenesis of dominant follicles (Evans et al., 1997). In accordance, cows induced to ovulate the FW dominant follicle had lower concentration of progesterone from the GnRH1 to the PGF_{2 α} of the Ovsynch protocol, resulting in a larger preovulatory follicle in experiment 2. Previous studies have reported that the ovulation of larger follicles with increased steroidogenic capacity resulted in larger CL, greater concentration of progesterone during the subsequent diestrus, and increased P/AI (Vasconcelos et al., 2001; Lopes et al., 2007). Despite evidence of a greater steroidogenic capacity in luteinized theca cells from FW dominant follicles, no difference in progesterone production was found in luteinized granulosa cells originated from FW and SW dominant follicles or in concentration of progesterone of cows ovulating FW or

SW dominant follicles (Wolfenson et al., 1999). Therefore, although the FW ovulatory follicle was larger at AI than the SW follicle, it is unlikely that progesterone concentrations in the subsequent estrous cycle differed in experiment 2.

Low concentration of progesterone during the development of the ovulatory follicle reduced the concentration of total IGF-1 in the follicular fluid of the preovulatory follicle (Cerri et al., 2008). Insulin-like growth factor-1 increases ovarian responsiveness to gonadotropins, steroidogenic capacity, and proliferation of granulosa cells and protects the oocyte and granulosa cells from apoptosis (Quirk et al., 2000; Wasieleski and Bogacki, 2007; Velazquez et al., 2008). The FW dominant follicle develops under lower concentrations of progesterone than the SW dominant follicle. Low concentrations of progesterone might result in increased LH pulsatility, which can disrupt oocyte quality (Revah and Butler, 1996), thereby influencing fertility.

Superstimulated lactating dairy cows used for embryo collection had improved embryo quality when FSH treatment was initiated at the emergence of the second follicular wave, or at the first follicular wave with supplemental progesterone compared with the first follicular wave without progesterone (Rivera et al., 2009). Denicol et al. (2009) observed a tendency for increased P/AI on d 38 after timed insemination in cows induced to ovulate the FW dominant follicle supplemented with progesterone during the development of the ovulatory follicle compared with unsupplemented cows. Progesterone concentrations during the development of the ovulatory follicle were approximately 1 and 3 ng/mL for unsupplemented and supplemented cows, respectively (Denicol et al., 2009). Collectively, these data suggest that induction of ovulation of the FW dominant follicle impairs fertility of dairy cows because of the low concentrations of progesterone during the development of the ovulatory follicle, which might compromise oocyte and embryo quality.

Anovulation and consequent long-term lack of exposure to progesterone has been associated with shortened luteal lifespan after the first postpartum ovulation and reduced P/AI in dairy and beef cattle (Crowe, 2008). Nevertheless, recent data suggest that insufficient concentration of progesterone during the development of the ovulatory follicle and not necessarily throughout the entire postpartum period is associated with poor fertility. In fact, supplementation with progesterone 20 to 15 d before ovulation was unable to extend the interestrus interval (Kyle et al., 1992). Conversely, the increase in the concentration of 13,14-dihydro-15-keto PGF_{2 α} in response to an injection of oxytocin on d 15 or 16 of the estrous cycle was higher for cyclic cows with low compared with cows with high concentrations

of progesterone during the development of the ovulatory follicle (Shaham-Albalancy et al., 2001; Cerri et al., 2008). In fact, cows ovulating the SW dominant follicle that developed under low concentrations of progesterone had increased incidence of short luteal phase (25%) compared with cows developing the ovulatory follicle under high (0%) concentrations of progesterone (Cerri et al., 2008). In experiment 1, CLOW cows had increased incidence of short inter-AI interval compared with CHIGH cows, suggesting either lack of synchronization or potentially premature luteolysis.

The endometrial expression of oxytocin receptors is prevented by the action of progesterone until mid diestrus, when progesterone receptors are downregulated in the luminal epithelium and superficial glands and the binding of oxytocin to its receptors triggers the luteolytic cascade (McCracken et al., 1999). It has been proposed that the exposure to progesterone before ovulation programs the endometrial dynamics of progesterone and oxytocin receptors in the subsequent cycle and, consequently, has an effect on interestrus interval. Postpartum beef cows supplemented with a progestogen (i.e., norgestomet) during the development of the ovulatory follicle had increased endometrial expression of receptors for progesterone and reduced expression of receptors for oxytocin at d 5 of the subsequent cycle compared with untreated controls (Zollers et al., 1993). This is likely to be the cause of premature endometrial responsiveness to oxytocin in cows not exposed to progesterone in the preceding estrous cycle (Zollers et al., 1989). Sá Filho et al. (2009) reported smaller abundance of mRNA for oxytocin receptors on d -2, 0, and 5 relative to ovulation in postpartum beef cows pretreated with progesterone, but no changes in expression of progesterone receptor mRNA were found during the same period. Therefore, it is plausible that inadequate exposure to progesterone during the development of the ovulatory follicle might enhance the responsiveness of the endometrium to oxytocin and result in premature luteolysis in more cows ovulating the FW compared with the SW dominant follicle.

The success of AI programs is related to cyclic status and the stage of the estrous cycle at which synchronization protocols are initiated (Vasconcelos et al., 1999; Santos et al., 2004c; Cerri et al., 2009). Increased ovulation to GnRH1 combined with the presence of a functional CL at the induced luteolysis and ovulation to the final GnRH were achieved when the protocol was initiated from d 5 to 10 of the estrous cycle (Vasconcelos et al., 1999; Bello et al., 2006) and have been linked with improved embryo quality (Cerri et al., 2009) and increased P/AI (Vasconcelos et al., 1999; Moreira et al., 2001; Chebel et al., 2006). Conversely, in experiment 2 of the present study the proportion of cows that

ovulated to GnRH1 was greater and the proportion of cows with a functional CL at the PGF_{2α} of the Ovsynch protocol tended to be greater in FW cows than SW cows; however, these same cows had reduced P/AI on d 32 and 67 after AI. It is known that anovular cows and cows in proestrus are more likely to ovulate to GnRH1 (Gümen et al., 2003), but the growth of the FW dominant follicle under reduced concentrations of progesterone and the induced ovulation of the FW dominant follicle at AI likely negate the benefits of high ovulation to GnRH1 on P/AI. Because anovular cows subjected to GnRH-PGF_{2α} based synchronization protocols ovulated the FW dominant follicle at AI, it is suggested that reduced P/AI in these cows is caused by the ovulation of the FW dominant follicle that develops under low concentrations of progesterone and not by complete lack of previous exposure to progesterone.

CONCLUSIONS

The follicular wave of the ovulatory follicle affected fertility of dairy cows submitted to synchronization protocols. Pregnancy per AI increased in cows that ovulated the SW dominant follicle compared with cows induced to ovulate the dominant follicle of the FW. Furthermore, the reproductive responses evaluated for cows that ovulated the FW dominant follicle and for anovular cows were similar. Collectively, these data indicate that follicular wave of the ovulatory follicle and not cyclic status had the greatest effect reducing P/AI of dairy cows. Nevertheless, in experiment 1, both follicle wave and, to a lesser extent, cyclic status influenced P/AI on d 53 after insemination. Whether the culprit for reduced fertility in cows ovulating the FW dominant follicle is the follicle itself or the hormonal milieu characteristic of the FW and the early stage of the estrous cycle remains to be elucidated. These data indicate that some of the underlying mechanisms by which delayed resumption of ovulation reduces fertility are associated with the ovulation of the FW dominant follicle and the related changes in the hormonal milieu during growth of the ovulatory follicle. Furthermore, they also indicate that synchronization programs for lactating dairy cows should be designed to result in ovulation of the SW dominant follicle at AI or a follicle that develops under high concentrations of progesterone to optimize fertility of lactating dairy cows.

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