Effects of gonadotropin-releasing hormone at initiation of the 5-d timed artificial insemination (AI) program and timing of induction of ovulation relative to AI on ovarian dynamics and fertility of dairy heifers

F. S. Lima,* H. Ayres,*† M. G. Favoreto,* R. S. Bisinotto,* L. F. Greco,* E. S. Ribeiro,* P. S. Baruselli,† C. A. Risco,‡ W. W. Thatcher,* and J. E. P. Santos*¹

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

†Department of Animal Reproduction, University of São Paulo, São Paulo, Brazil 05508-270

‡Department of Large Animal Clinical Sciences, University of Florida, Gainesville 32610

ABSTRACT

Two experiments evaluated the effects of the first GnRH injection of the 5-d timed artificial insemination (AI) program on ovarian responses and pregnancy per AI (P/AI), and the effect of timing of the final GnRH to induce ovulation relative to AI on P/AI. In experiment 1, 605 Holstein heifers were synchronized for their second insemination and assigned randomly to receive GnRH on study d 0 ($n = 298$) or to remain as untreated controls ($n = 307$). Ovaries were scanned on study d 0 and 5. All heifers received a controlled internal drug-release (CIDR) insert containing progesterone on d 0, a single injection of PGF_{2α} and removal of the CIDR on d 5, and GnRH concurrent with timed AI on d 8. Blood was analyzed for progesterone at AI. Pregnancy was diagnosed on d 32 and 60 after AI. Ovulation on study d 0 was greater for GnRH than control (35.4 vs. 10.6%). Presence of a new corpus luteum (CL) at PGF_{2α} injection was greater for GnRH than for control (43.1 vs. 20.8%), although the proportion of heifers with a CL at PGF_{2α} did not differ between treatments and averaged 87.1%. Progesterone on the day of AI was greater for GnRH than control (0.50 ± 0.07 vs. 0.28 ± 0.07 ng/mL). The proportion of heifers at AI with progesterone <0.5 ng/mL was less for GnRH than for control (73.8 vs. 88.2%). The proportion of heifers in estrus at AI did not differ between treatments and averaged 66.8%. Pregnancy per AI was not affected by treatment at d 32 or 60 (GnRH = 52.5 and 49.8% vs. control = 54.1 and 50.0%), and pregnancy loss averaged 6.0%. Responses to GnRH were not influenced by ovarian status on study d 0. In experiment 2, 1,295 heifers were synchronized for their first insemination and assigned randomly to receive a CIDR on d 0, PGF_{2α} and removal of the CIDR on d 5, and either GnRH 56

h after PGF_{2α} and AI 16 h later (OVS56, $n = 644$) or GnRH concurrent with AI 72 h after PGF_{2α} (COS72; $n = 651$). Estrus at AI was greater for COS72 than for OVS56 (61.4 vs. 47.5). Treatment did not affect P/AI on d 32 in heifers displaying signs of estrus at AI, but COS72 improved P/AI compared with OVS56 (55.0 vs. 47.6%) in those not in estrus at AI. Similarly, P/AI on d 60 did not differ between treatments for heifers displaying estrus, but COS72 improved P/AI compared with OVS56 (53.0 vs. 44.7%) in those not in estrus at AI. Administration of GnRH on the first day of the 5-d timed AI program resulted in low ovulation rate and no improvement in P/AI when heifers received a single PGF_{2α} injection 5 d later. Moreover, extending the proestrus by delaying the final GnRH from 56 to 72 h concurrent with AI benefited fertility of dairy heifers that did not display signs of estrus at insemination following the 5-d timed AI protocol.

Key words: 5-d timed artificial insemination, dairy heifer, gonadotropin releasing hormone, reproduction

INTRODUCTION

The use of timed AI programs in dairy heifers is low compared with that for lactating dairy cows (NAHMS, 2009). Programs to synchronize ovulation of dairy heifers based on GnRH and PGF_{2α} resulted in low pregnancy per AI (P/AI) compared with insemination performed after detection of estrus (Schmitt et al., 1996; Pursley et al., 1997; Rivera et al., 2004). The depressed P/AI for most timed AI programs based on GnRH and PGF_{2α} and the perception by dairy producers that heifers become pregnant easily without the need for intervention justifies the low use of ovulation synchronization protocols for management of reproduction in heifers.

Recently, a 5-d timed AI protocol investigated by Rabaglino et al. (2010a) resulted in P/AI ranging from 52.2 to 61% in dairy heifers in the first 2 inseminations, which resembles the proportion of pregnancy obtained

Received February 6, 2011.

Accepted May 21, 2011.

¹Corresponding author: jepsantos@ufl.edu

with AI after detection of estrus (Kuhn et al., 2006). In fact, additional work by the same investigators evaluating antiluteolytic strategies with 325 heifers synchronized with the 5-d timed AI program observed P/AI of 59.5% on d 45 after insemination (Rabaglino et al., 2010b). Therefore, it is possible to achieve acceptable P/AI in dairy heifers following synchronized ovulation with the 5-d timed AI protocol.

The program comprises an injection of GnRH and insertion of a controlled internal drug-release (CIDR) intravaginal device containing progesterone, followed 5 d later by CIDR removal and an injection of PGF_{2α}, and AI concurrent with a second GnRH injection 72 h after PGF_{2α} (Rabaglino et al., 2010a). Only 23% of the heifers had multiple corpora lutea (CL) 5 d after the injection of GnRH (Rabaglino et al., 2010a), suggesting that ovulation to the initial GnRH was probably low. In fact, heifers receiving a single injection of PGF_{2α} 5 d after GnRH had similar luteolysis and P/AI to those receiving 2 injections given 12 h apart (Rabaglino et al., 2010a). The same was not true when lactating dairy cows were subjected to a similar program with a 5-d interval between GnRH and PGF_{2α} (Santos et al., 2010). Therefore, the low incidence of ovulation induced by the first GnRH combined with more rapid turnover of follicles in heifers (Sirois and Fortune, 1988) might result in little benefit from the initial GnRH in the 5-d timed AI program in dairy heifers.

Altering the timing of the final GnRH to induce ovulation relative to AI in the Ovsynch protocol influences P/AI in lactating dairy cows. Brusveen et al. (2008) reported that GnRH administered 56 h after PGF_{2α} increased P/AI compared with GnRH given concurrent with timed AI at 72 h. In a series of experiments with beef cows subjected to the 5-d timed AI program, extending proestrus from 60 to 72 h was beneficial to fertility (Bridges et al., 2008). In dairy cows subjected to the 5-d timed AI program, P/AI did not differ when the final GnRH was administered either 16 h before or concurrent with AI at 72 h after PGF_{2α} (Bisinotto et al., 2010). Although inducing ovulation 16 h before AI benefits fertility of dairy cows in the standard 7-d timed AI Ovsynch program, it is unclear if a similar benefit would occur in dairy heifers when follicle dominance is reduced such as in the 5-d timed AI.

The hypotheses of the current study were that the first GnRH would result in a low ovulation rate, thereby having little or no effect on fertility of dairy heifers subjected to the 5-d timed AI protocol. A second hypothesis was that administration of the final GnRH concurrent with AI at 72 h after PGF_{2α} would result in similar P/AI as that when GnRH is administered 16 h before AI and AI is performed 72 h after PGF_{2α}. Two experiments with heifers inseminated following the 5-d

timed AI protocol were designed to test our hypotheses. The first experiment evaluated the effect of the first GnRH injection on ovarian responses and P/AI, whereas the second experiment evaluated the effect of timing of the final GnRH to induce ovulation relative to AI on P/AI.

MATERIALS AND METHODS

The University of Florida Institute of Food and Agricultural Sciences Animal Research Committee approved all procedures in this study.

Experiment 1

Heifers, Diets, and Housing. Six hundred and five nulliparous nonpregnant Holstein heifers on d 32 after the first insemination were synchronized to receive second AI. Heifers were from a commercial dairy farm in north central Florida. Heifers averaged 15.3 ± 1.7 mo of age and were enrolled in the study from December 2009 and from March 2010. Heifers were managed on pasture, with access to portable shades and trees, and were fed a TMR once daily that met or exceeded the nutritional requirements of Holstein heifers weighing 360 kg and gaining 0.8 kg/d (NRC, 2001). The diet was based on a mixture of lactating cow rationorts, Bermuda grass silage, wet brewers grain, and a mineral and vitamin supplement. For implementation of synchronization protocols, insemination, blood collection, and pregnancy examination, heifers were handled in an open-sided barn with self-locking stanchions.

Experimental Design and Treatments. Nonpregnant heifers on d 32 after the first AI were blocked according to age and, within each block, allocated randomly to either receive 100 µg of GnRH (gonadorelin hydrochloride; Factrel, Pfizer Animal Health, Madison, NJ) administered i.m. on study d 0 (GnRH group = 298) or to remain as untreated controls (control group = 307). All heifers received a CIDR (Eazi-Breed CIDR Cattle Insert, Pfizer Animal Health) containing 1.38 g of progesterone on study d 0. On study d 5, the CIDR was removed and heifers received an i.m. injection of 25 mg of PGF_{2α} (dinoprost tromethamine; Lutalyse sterile solution, Pfizer Animal Health). On study d 8, an injection of GnRH was administered concurrently with timed AI (Figure 1). Beginning on the day of PGF_{2α} administration, tailheads were painted daily with chalk, and removal of chalk was used as an indication of estrus. Heifers were inseminated by 5 technicians, and semen from 5 Holstein and 6 Jersey sires were used. Technicians and sires were balanced between treatments and later used in the statistical analyses. Heifers were classified according to their age as <15 mo or ≥15 mo of age.

Figure 1. Diagram of activities in experiment 1. BS = blood samples for analysis of progesterone concentration; CIDR = controlled internal drug-release containing 1.38 g of progesterone; GnRH = injection of 100 µg of gonadorelin hydrochloride; PGF_{2α} = injection of 25 mg of dinoprost as tromethamine salt; US = ultrasonography of the ovaries.

Ultrasonography of Ovaries. Ovaries of all heifers were scanned using a 5-MHz ultrasound unit (Easi-Scan, BCF Systems, Livingston, UK) on study d 0 and ovarian maps were drawn with the presence and location of CL and follicles ≥ 10 mm. On study d 5, the ovaries of 473 heifers were scanned and the presence and location of CL were recorded.

Blood Sampling and Analysis of Progesterone in Plasma. Blood was sampled from 312 of the 473 heifers evaluated for ovulation to the first GnRH. Blood was sampled on study d 8 by puncture of the median coccygeal vein or artery using evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing K₂ EDTA for plasma separation. Samples were placed immediately on ice until transported to the laboratory. Blood tubes were centrifuged at $2,000 \times g$ for 15 min, and plasma was frozen at -20°C until analysis. Concentration of progesterone in plasma was analyzed in all samples by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.05 ng/mL calculated at 2 SD below the mean counts per min at maximum binding. Samples were analyzed in a single assay. Two known plasma samples containing 1.5 and 2.5 ng/mL of progesterone were included in the assay several times to calculate the intraassay CV, and they were 2.5% for the sample with 1.5 ng/mL and 2.9% for the sample with 2.5 ng/mL.

Evaluation of Ovulation and Progesterone at AI. Ovulation on study d 0 was considered when the heifer had a follicle ≥ 10 mm on d 0 and a new CL was observed on study d 5. Heifers with follicles < 10 mm

on study d 0 but with a new CL on study d 5 were considered to have a new CL, but ovulated before study d 0. The proportion of heifers with a visible CL by ultrasound on the day of PGF_{2α} that had low progesterone at AI was calculated. Three different cut-off values for plasma concentrations of progesterone were used: < 1.0 , < 0.50 , and < 0.25 ng/mL. These values were chosen because traditionally 1 ng/mL has been used to indicate CL regression, and values < 0.50 ng/mL have been implicated as cut-off values that best predict P/AI (Rabaglino et al., 2010a; Santos et al., 2010).

Pregnancy Diagnoses and Evaluation of P/AI and Pregnancy Loss. Pregnancy was diagnosed 32 d after AI by transrectal ultrasound. The presence of an embryo with a heartbeat was the criterion used to determine pregnancy. Heifers diagnosed pregnant were re-examined by transrectal palpation of uterine contents 28 d later, at 60 d of gestation to reconfirm pregnancy and to identify pregnancy loss. Pregnancy per AI was calculated by dividing the number of heifers diagnosed pregnant at 32 or 60 d after AI by the number of heifers receiving AI. Proportion of pregnancy loss was calculated as the number of heifers that lost a pregnancy between 32 and 60 d of gestation divided by the number of heifers diagnosed pregnant on d 32 after AI.

Experiment 2

Heifers, Diets, and Housing. A total of 1,295 nulliparous Holstein, Jersey and crossbreed Holstein-Jersey heifers (15.5 ± 2.6 mo of age) located in 2 farms

Figure 2. Diagram of activities in experiment 2. CIDR = controlled internal drug-release containing 1.38 g of progesterone; GnRH = injection of 100 μg of gonadorelin hydrochloride; PGF_{2α} = injection of 25 mg of dinoprost as tromethamine salt.

in north central Florida were enrolled in the study between January and March 2010. Heifers in both locations were managed on pastures and fed as described in experiment 1. Heifers were moved to an open-sided barn with self-locking stations in farm 1 or to a palpation rail in farm 2 for hormonal treatments, insemination, and pregnancy diagnoses.

Experimental Design and Treatments. Within farm, nulliparous heifers were blocked by breed and age and, within each block, allocated randomly to 1 of 2 treatments for the first AI. All heifers received a CIDR on study d 0. On study d 5, the CIDR was removed and heifers received an i.m. injection of PGF_{2α}. Heifers in the 5-d timed AI program designated **OVS56** (n = 644) received an injection of GnRH at 56 h after the PGF_{2α} and timed AI was performed 16 h later. Heifers in the 5-d timed AI program designated **COS72** (n = 651) received an injection of GnRH at 72 h after the PGF_{2α}, concurrent with AI. Therefore, heifers in both treatments were inseminated at 72 h after CIDR removal and PGF_{2α}, but in OVS56, induction of ovulation was 16 h before AI (Figure 2). Beginning on the day of PGF_{2α} administration, tailheads were painted daily with chalk, and removal of chalk was used as an indication of estrus. The same 9 technicians inseminated heifers in both farms, and 3 Holstein and 4 Jersey sires were used.

Technicians and sires were balanced between treatments and later used in the statistical analyses. Heifers were classified according to age: <13 mo, between 13 and 15 mo, or ≥15 mo of age.

Pregnancy Diagnoses and Evaluation of Pregnancy Outcomes. Pregnancy diagnoses and calculation of P/AI and pregnancy loss were exactly as described for experiment 1.

Statistical Analyses

Sample sizes were calculated for both studies to allow for sufficient experimental units to detect a difference of 8 percentage units in experiment 1 and 6 percentage units in experiment 2 ($\alpha = 0.05$; $\beta = 0.20$). These differences were based on the expected P/AI of 53% for the second insemination at experiment 1 and 58% for experiment 2. We anticipated that P/AI for the second AI (experiment 1) would range from 45 to 60%. Similarly, it was anticipated that P/AI for the first AI (experiment 2) would range from 50 to 62% based on previous studies with the 5-d timed AI protocol (Rabaglino et al., 2010a,b) and pregnancy results at the study farms. Under these assumptions, a total of 300 experimental units per treatment would be necessary for experiment 1 and 550 experimental units per treatment in experiment 2.

In both experiments, binary responses were analyzed by logistic regression using the LOGISTIC procedure of SAS version 9.2 (SAS/STAT, SAS Institute Inc., Cary, NC). Backward stepwise logistic regression models were used and variables were continuously removed from the models by the Wald statistic criterion when $P > 0.10$. In experiment 1, the models for ovarian responses to treatments, proportion of heifers with low progesterone

Table 1. Ovarian responses (%; no./no. in parentheses) of heifers treated with or without GnRH at the initiation of the 5-d timed AI protocol (experiment 1)

Item	Treatment ¹		AOR ² (95% CI)	P-value
	GnRH	Control		
Study d 0				
Heifers with follicle ≥ 10 mm	90.4 (216/239)	88.6 (210/237)	1.21 (0.67–2.17)	0.53
Corpus luteum (CL) on study d 0	74.1 (177/239)	83.1 (197/237)	0.57 (0.36–0.89)	0.01
Ovulation on study d 0				
All heifers	35.4 (84/237)	10.6 (25/236)	4.49 (2.68–7.51)	<0.01
Heifers with follicles ≥ 10 mm	39.3 (84/214)	12.0 (25/209)	4.82 (2.80–8.30)	<0.01
New CL at PGF _{2α}	43.1 (102/237)	20.8 (49/236)	3.13 (1.91–5.13)	<0.01
CL at PGF _{2α}	88.2 (209/237)	86.0 (203/236)	1.30 (0.75–2.24)	0.35
Proportion of heifers according to progesterone at AI				
<1 ng/mL	90.1 (127/141)	94.9 (129/136)	0.51 (0.20–1.30)	0.16
<0.50 ng/mL	73.8 (104/141)	88.2 (120/136)	0.34 (0.18–0.66)	<0.01
<0.25 ng/mL	41.1 (58/141)	52.9 (72/136)	0.57 (0.35–0.93)	0.03
Progesterone at AI, ng/mL	0.50 $\pm$ 0.07	0.28 $\pm$ 0.07	—	<0.01

¹GnRH = d 0 GnRH and controlled internal drug-release insert (CIDR), d 5 PGF_{2 α} and removal of CIDR, d 8 GnRH and timed AI; Control = d 0 CIDR, d 5 PGF_{2 α} and removal of CIDR, d 8 GnRH and timed AI.

²AOR = adjusted odds ratio. Control is the reference for comparison.

at AI, and estrus at AI included the effects of treatment (GnRH vs. control), age of the heifer, ovarian status on study d 0 (presence or absence of CL), and interaction between treatment and ovarian status. The models for P/AI and pregnancy loss included the effects of treatment, age of the heifer, ovarian status on study d 0, sire, technician, and interaction between treatment and ovarian status. In experiment 2, the model for detection of estrus included the effects of treatment (OVS56 vs. COS72), farm, breed of the heifer, and age of the heifer. The models for P/AI and pregnancy loss included the effects of treatment, farm, breed of the heifer, age of the heifer, sire, technician, and display of signs of estrus at AI. In all analyses in both experiments, treatment was forced in the final model.

In experiment 1, concentration of progesterone at AI was analyzed by ANOVA using the GLM procedure of SAS, and the model included the effects of treatment, age of the heifer, and ovarian status on study d 0.

RESULTS

Experiment 1

A CL visible by ultrasound was observed in 88.5% of the heifers on study d 0, indicating that the majority of the heifers were cycling. Ovulation on study d 0 and the presence of a new CL at the injection of PGF_{2 α} were both greater ($P < 0.01$) for GnRH than for control heifers (Table 1). Ovulation on study d 0 was greater ($P < 0.01$) for heifers without a CL than for those with CL, and this was observed in both GnRH (59.7 vs. 26.9%) and control (32.5 vs. 6.1%) heifers. Although ovulation rate increased with GnRH, the proportion of heifers with a visible CL on study d 5 did not differ between

treatments and averaged 87.1%. Treatment with GnRH influenced ($P < 0.03$) the proportion of heifers with low progesterone at AI, and the effect was observed when the progesterone cut-off was either 0.50 or 0.25 ng/mL. This difference resulted in GnRH heifers having greater ($P < 0.01$) concentrations of progesterone at AI than control heifers.

Detection of estrus at AI did not differ between treatments and averaged 67.4% (Table 2). Treatment with GnRH on study d 0 did not affect P/AI on either d 32 or 60 after insemination. The response to treatment was not influenced by ovarian status on study d 0. For instance, P/AI on d 32 for heifers with CL on study d 0 were 53.3 and 54.5% for GnRH and control, respectively. For heifers without a CL on study d 0, P/AI were 48.6 and 42.3% for GnRH and control, respectively. Similarly, pregnancy loss between 32 and 60 d of gestation did not differ between treatments and averaged 6.0%.

Experiment 2

As expected, the detection of heifers in estrus on the day before timed AI was similar between treatments (Table 3). However, when GnRH was given 16 h before AI in OVS56, it decreased ($P < 0.001$) the proportion of heifers in estrus on the day of timed AI. Heifers in estrus at timed AI had greater ($P < 0.001$) P/AI than those not detected in estrus (62.0 vs. 50.8%). An interaction ($P = 0.05$) between treatment and detection of signs of estrus at AI was observed for pregnancy on d 32. For heifers in estrus, treatment did not affect P/AI (COS72 = 60.5% vs. OVS56 = 64.1%), but for those not displaying estrus at AI, COS72 tended ($P = 0.07$) to increase P/AI compared with OVS56 (55.0 vs.

Table 2. Effect of the first GnRH injection of the 5-d timed AI protocol on fertility responses (% , no./no. in parentheses) of dairy heifers (experiment 1)

Item	Treatment ¹		AOR ² (95% CI)	P-value
	GnRH	Control		
Estrus at timed AI ³	69.2 (206/298)	64.5 (198/307)	1.23 (0.88–1.73)	0.23
Pregnant ⁴				
Day 32	52.5 (155/295)	54.1 (165/305)	0.94 (0.68–1.30)	0.70
Day 60	49.8 (147/295)	50.0 (150/300)	0.99 (0.72–1.37)	0.97
Pregnancy loss ⁵	5.8 (9/155)	6.2 (10/160)	0.93 (0.37–2.34)	0.87

¹GnRH = d 0 GnRH and controlled internal drug-release insert (CIDR), d 5 PGF_{2α} and removal of the CIDR, d 8 GnRH and timed AI; Control = d 0 CIDR, d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and timed AI.

²AOR = adjusted odds ratio. Control is the reference for comparison.

³Evaluated based on removal of tail chalk on the day of timed AI.

⁴Three GnRH and 2 control heifers left the study before pregnancy diagnosis on d 32.

⁵Five control heifers left the study before reconfirmation of pregnancy on d 60 after AI.

47.6%). On d 60 after AI, heifers receiving COS72 had greater ($P = 0.05$) P/AI than those receiving OVS56, and this effect was observed primarily because for heifers not detected in estrus, those in the COS72 group had greater ($P < 0.05$) P/AI than heifers in the OVS56 (53.0 vs. 44.7%). For heifers detected in estrus, P/AI was not influenced by treatment (COS72 = 57.3 vs. OVS56 = 59.2%). Pregnancy loss between 32 and 60 d of gestation did not differ between treatments and averaged 5.8%.

DISCUSSION

Optimization of the 5-d timed AI program to synchronize ovulation of dairy heifers allows producers to incorporate timed insemination when needed with acceptable fertility. Earlier work with the Ovsynch program in dairy heifers resulted in low P/AI (Pursley et al., 1997), and it was suggested that timed AI should not be used in dairy heifers. The 5-d timed AI program initially described by Bridges et al. (2008)

and investigated for dairy heifers by Rabaglino et al. (2010a,b) resulted in P/AI that resemble those of heifers inseminated following estrus and usually better than results previously obtained with heifers subjected to the standard Ovsynch protocol and some of its variations (Pursley et al., 1997; Rivera et al., 2004). In fact, results from Rabaglino et al. (2010a,b) and those from the current experiments are close to the 57% P/AI reported for Holstein heifers in the United States (Kuhn et al., 2006).

An interesting aspect of the 5-d timed AI program evaluated by Rabaglino et al. (2010a) was the low incidence of heifers with multiple CL 5 d after the injection of GnRH, suggesting a poor ovulatory response. In lactating dairy cows subjected to ovulation synchronization programs such as Ovsynch and Cosynch, ovulation to the first GnRH is variable according to day of the cycle when it is administered (Vasconcelos et al., 1999). It is optimized when GnRH is administered on d 6 of the estrous cycle (Bello et al., 2006). In dairy heifers, ovulation to the first GnRH usually is

Table 3. Effect of time of administration of the final GnRH of the 5-d timed AI protocol relative to insemination on fertility responses (% , no./no. in parentheses) of dairy heifers (experiment 2)

Item	Treatment ¹		AOR ² (95% CI)	P-value
	OVS56	COS72		
Estrus ³				
Day before timed AI	46.7 (301/644)	43.2 (281/651)	1.16 (0.93–1.45)	0.19
Day of timed AI	47.5 (306/644)	61.4 (400/651)	0.52 (0.45–0.70)	<0.001
Pregnant				
Day 32	55.4 (357/644)	58.4 (380/651)	0.75 (0.42–1.08)	0.08
Day 60	51.6 (332/644)	55.6 (362/651)	0.72 (0.39–1.04)	0.05
Pregnancy loss	7.0 (25/357)	4.7 (18/380)	1.52 (0.81–2.83)	0.19

¹COS72 = d 0 controlled internal drug-release insert (CIDR), d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and timed AI; OVS56 = d 0 GnRH and CIDR, d 5 PGF_{2α} and removal of CIDR, d 7.3 GnRH, d 8 timed AI.

²AOR = adjusted odds ratio. COS72 is the reference for comparison.

³Evaluated based on removal of tail chalk on the day before and day of timed AI.

less than that observed for lactating dairy cows, even when the estrous cycle is presynchronized (Stevenson et al., 2008). When given at random stages of the estrous cycle, GnRH resulted in only 35.4% ovulation in experiment 1, and only 26.9% of the heifers with a CL ovulated in response to administration of GnRH. Because a large proportion of heifers had CL on study d 0, the low ovulatory response to GnRH did not influence the proportion of heifers with visible luteal tissue by ultrasound on study d 5. Nevertheless, treatment with GnRH reduced the proportion of heifers with progesterone concentrations <0.50 ng/mL. The traditional cut-off for luteolysis commonly cited in the literature has been 1 ng/mL, but on the day of insemination, P/AI is optimized when progesterone concentrations are <0.50 ng/mL. In fact, in many cases with both dairy heifers and lactating dairy cows, the optimized cut-off value for progesterone to predict pregnancy was <0.30 ng/mL (Rabaglino et al., 2010a; Santos et al., 2010). Therefore, although ovulation rate was low, it was sufficient to compromise the proportion of heifers with low progesterone at AI when a single injection of PGF_{2 α} is administered 5 d later. In lactating dairy and beef cows, compromised luteolysis reduces P/AI in the 5-d timed AI program, thereby requiring 2 sequential treatments with PGF_{2 α} (Kasimanickam et al., 2009; Santos et al., 2010). However, in dairy heifers, an additional treatment with PGF_{2 α} did not further improve P/AI (Rabaglino et al., 2010a).

In the current study, administration of GnRH on study d 0 did not benefit P/AI of dairy heifers, and this response was observed regardless of the presence or absence of a CL on study d 0. In lactating dairy cows, ovulation to the initial GnRH of the timed AI protocol is critical to improve P/AI (Vasconcelos et al., 1999; Bello et al., 2006; Santos et al., 2010), but the same has not been observed in dairy heifers (Stevenson et al., 2008). Stevenson et al. (2008) administered GnRH 6 d before initiation of the timed AI protocol to presynchronize the estrous cycle of dairy heifers. Although they were able to increase ovulation to the first GnRH of the timed AI program in presynchronized heifers, ovulation did not influence P/AI or pregnancy loss. Therefore, it is possible that in dairy heifers, typically having 3 waves of follicle development (Sirois and Fortune, 1988), ovulation and recruitment of a new wave has less effect on fertility in timed AI protocols because of the typically shorter period of ovulatory follicle dominance than in lactating cows. Also, it is possible that the benefit of GnRH inducing ovulation was mitigated by the single injection of PGF_{2 α} that resulted in a smaller proportion of cows with progesterone <0.5 ng/mL at AI. Nevertheless, when a single injection of PGF_{2 α} is used, the results of this study indicate that

the initial GnRH is not required to optimize P/AI in dairy heifers subjected to the 5-d timed AI protocol.

Because of the lack of benefit from GnRH on fertility of dairy heifers, experiment 2 was designed to evaluate whether induction of ovulation 16 h before AI would improve P/AI of dairy heifers in the 5-d timed AI protocol without the GnRH on study d 0. Administering GnRH to induce ovulation concurrent with AI, in general, was beneficial to P/AI of dairy heifers compared with GnRH 16 h before AI. However, the benefit was observed only in heifers that did not display signs of estrus on the day of AI. Bisinotto et al. (2010) observed that for lactating dairy cows subjected to the 5-d timed AI program, induction of ovulation before AI was not beneficial to fertility. When cows undergo timed AI protocols with 7 d between the first GnRH and PGF_{2 α} , administration of the final GnRH 16 h before AI benefits P/AI (Brusveen et al., 2008). This is thought to be mediated by improved synchrony between sufficient numbers of spermatozoa capable of fertilization in the oviduct and the presence of a viable oocyte (Saacke, 2008). In fact, results from Brusveen et al. (2008) agree with those of Dransfield et al. (1998) in which the highest P/AI was achieved when insemination was performed 4 to 16 h after the onset of estrus.

When cows are subjected to the 5-d timed AI program, the period of ovulatory follicle development is reduced by approximately 2 d compared with the more traditional 7-d programs (Santos et al., 2010). This reduction results in ovulatory follicles of smaller diameter, reduced concentration of estradiol in plasma, and a smaller proportion of cows in estrus at AI compared with cows in the conventional 7-d program (Santos et al., 2010). In dairy heifers, these parameters have not been fully characterized. In experiment 2, delaying the administration of the GnRH to 72 h increased the proportion of heifers in estrus at AI, which is a sign of increased exposure to endogenous estradiol. In a series of experiments with beef cows subjected to the 5-d program, extending proestrus from 60 to 72 h was beneficial to fertility (Bridges et al., 2008). It would be expected that induction of ovulation 16 h before AI might be beneficial to the fertility of dairy heifers because of the potentially better synchrony between the moment of ovulation and the availability of capacitated spermatozoa in the oviduct for fertilization of the oocyte (Saacke, 2008). Nevertheless, 45% of the heifers were in estrus in the afternoon before the scheduled AI. Furthermore, when heifers were subjected to COS72, more than 61% were in estrus at the moment of timed AI. Heifers in estrus receiving COS72 likely already had a spontaneous LH surge when insemination was performed, which would diminish the benefit of administering GnRH 16 h before AI. The fact that delaying

the administration of GnRH to the moment of AI improved P/AI of heifers not in estrus suggests that the additional period of proestrus was beneficial to fertility. The improvement in fertility might have been mediated by additional exposure to estradiol and growth of the ovulatory follicle. The additional proestrus might be particularly important in a program of reduced period of follicle dominance to allow for sufficient preovulatory follicle growth and production of estradiol as suggested by Bisinotto et al. (2010). Thus, these results suggest that the prolonged proestrus in COS72 benefits fertility of dairy heifers in the 5-d program, counterbalancing the potentially better synchrony between ovulation and insemination obtained by OVS56.

CONCLUSIONS

Fertility of dairy heifers subjected to the 5-d timed AI protocol was not affected by administration of the first GnRH on study d 0. The lack of benefit is attributed to the low ovulation rate to the initial GnRH and the reduced proportion of heifers with low progesterone at AI when receiving a single PGF_{2α} treatment 5 d later. Timing of induction of ovulation with GnRH relative to AI influenced P/AI of heifers not displaying estrus at AI, and it was usually better when heifers received the ovulatory stimulus concurrent with AI at 72 h after PGF_{2α} than 16 h before timed AI. Therefore, when heifers are subjected to the 5-d timed AI program with a single treatment of PGF_{2α}, we suggest that the initial GnRH is not necessary and the period of proestrus should be 72 h with administration of GnRH to induce ovulation concurrent with timed AI.

ACKNOWLEDGMENTS

The authors thank the owner and staff of Alliance Dairies (Trenton, NJ) for the use of their heifers and facilities. The assistance of Nilo Francisco of Alliance Dairies is greatly appreciated. Our appreciation is extended to Roger Saltman of Pfizer Animal Health (Madison, NJ) for providing the Factrel, Lutalyse, and CIDR inserts for this study.

REFERENCES

- Bello, N. M., J. P. Steibel, and J. R. Pursley. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. *J. Dairy Sci.* 89:3413–3424.
- Bisinotto, R. S., E. S. Ribeiro, L. T. Martins, R. S. Marsola, L. F. Greco, M. G. Favoreto, C. A. Risco, W. W. Thatcher, and J. E. P. Santos. 2010. Effect of interval between induction of ovulation and AI and supplemental progesterone for resynchronization on fertility of dairy cows subjected to a 5-d timed AI program. *J. Dairy Sci.* 93:5798–5808.
- Bridges, G. A., L. A. Helser, D. E. Grum, M. L. Mussard, and M. L. Day. 2008. Decreasing the interval between GnRH and PGF_{2α} from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. *Theriogenology* 69:843–851.
- Brusveen, D. J., A. P. Cunha, C. D. Silva, P. M. Cunha, R. A. Sterry, E. P. B. Silva, J. N. Guenther, and M. C. Wiltbank. 2008. Altering the time of the second gonadotropin-releasing hormone injection and artificial insemination (AI) during Ovsynch affects pregnancies per AI in lactating dairy cows. *J. Dairy Sci.* 91:1044–1052.
- Dransfield, M. B. G., R. L. Nebel, R. E. Pearson, and L. D. Warnick. 1998. Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. *J. Dairy Sci.* 81:1874–1882.
- Kasimanickam, R., M. L. Day, J. S. Rudolph, J. B. Hall, and W. D. Whittier. 2009. Two doses of prostaglandin improve pregnancy rates to timed-AI in a 5-day progesterone-based synchronization protocol in beef cows. *Theriogenology* 71:762–767.
- Kuhn, M. T., J. L. Hutchison, and G. R. Wiggans. 2006. Characterization of Holstein heifer fertility in the United States. *J. Dairy Sci.* 89:4907–4920.
- NAHMS. 2009. Dairy 2007, Part IV: Reference of Dairy Cattle Health and Management Practices in the United States, 2007. #N494.0209. Centers for Epidemiology and Animal Health, USDA:APHIS:VS, Fort Collins, CO.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
- Pursley, J. R., M. C. Wiltbank, J. S. Stevenson, J. S. Ottobre, H. A. Garverick, and L. L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus. *J. Dairy Sci.* 80:295–300.
- Rabaglino, M. B., C. A. Risco, M.-J. Thatcher, I. H. Kim, J. E. P. Santos, and W. W. Thatcher. 2010a. Application of one injection of prostaglandin F_{2α} in the five-day Co-Synch + CIDR protocol for estrous synchronization and resynchronization of dairy heifers. *J. Dairy Sci.* 93:1050–1058.
- Rabaglino, M. B., C. A. Risco, M.-J. Thatcher, F. Lima, J. E. P. Santos, and W. W. Thatcher. 2010b. Use of a five-day progesterone-based timed AI protocol to determine if flunixin meglumine improves pregnancy per timed AI in dairy heifers. *Theriogenology* 73:1311–1318.
- Rivera, H., H. Lopez, and P. M. Fricke. 2004. Fertility of Holstein dairy heifers after synchronization of ovulation and timed AI after removed tail chalk. *J. Dairy Sci.* 87:2051–2061.
- Saacke, R. G. 2008. Insemination factors related to timed AI in cattle. *Theriogenology* 70:479–484.
- Santos, J. E. P., C. D. Narciso, F. Rivera, W. W. Thatcher, and R. C. Chebel. 2010. Effect of reducing the period of follicle dominance in a timed AI protocol on reproduction of dairy cows. *J. Dairy Sci.* 93:2976–2988.
- Schmitt, E. J., P. T. Diaz, M. Drost, and W. W. Thatcher. 1996. Use of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin for timed insemination in cattle. *J. Anim. Sci.* 74:1084–1091.
- Sirois, J., and J. E. Fortune. 1988. Ovarian follicular dynamics during the estrous cycle monitored by real-time ultrasonography. *Biol. Reprod.* 39:308–317.
- Stevenson, J. L., J. C. Dalton, J. E. P. Santos, R. Sartori, A. Ahmadzadeh, and R. C. Chebel. 2008. Effect of synchronization protocols on follicular development and estradiol and progesterone concentrations of dairy heifers. *J. Dairy Sci.* 91:3045–3056.
- Vasconcelos, J. L., R. W. Silcox, G. J. Rosa, J. R. Pursley, and M. C. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. *Theriogenology* 52:1067–1078.