Concentrations of luteinizing hormone and ovulatory responses in dairy cows before timed artificial insemination¹

S. L. Pulley,^{*2} D. H. Keisler,[†] and J. S. Stevenson^{*3}

^{*}Department of Animal Sciences and Industry, Kansas State University, Manhattan 66506-0201

[†]Division of Animal Sciences, University of Missouri, Columbia 65211

ABSTRACT

The objective was to determine the incidence of LH surges and ovulatory responses in lactating dairy cows enrolled in a timed artificial insemination (TAI) program. Cows were assigned randomly to 2 presynchronization treatments: (1) Pre10 ($n = 37$): 2 injections of PGF_{2α} (PG; PG-1 and PG-2) 14 d apart (Presynch); or (2) PG-3-G ($n = 33$): one 25-mg injection of PG (Pre-PG) administered 3 d before a 100-μg GnRH injection (Pre-GnRH). Ten days after PG-2 or Pre-PG, all cows were enrolled in a 7-d Ovsynch TAI program [injection of GnRH (GnRH-1) 7 d before PG (PG-3) and GnRH (GnRH-2) administered at either 56 or 72 h after PG-3; TAI at 72 h]. Blood was collected to determine LH at (1) Pre-GnRH: 48 to 80 h after PG-2 and hourly from 72 to 78 h (Pre-GnRH at 72 h); (2) GnRH-1: 0 to 6 h after GnRH-1; and (3) GnRH-2: 48 to 80 h after PG-3 and hourly from 56 to 62 h or 72 to 78 h for cows injected with GnRH-2 at 56 or 72 h after PG-3, respectively. Ovaries were scanned and pregnancy per TAI (P/AI) was diagnosed 31 and 61 d post-TAI by transrectal ultrasonography. The Pre-GnRH injection increased the incidences of LH surges (100 vs. 43%) and ovulation (91 vs. 60%) and subsequent concentrations of progesterone in PG-3-G cows compared with Pre10 cows, respectively. Seven days later, incidence of ovulation (48 to 62%) and occurrence of LH surges (100%) did not differ between treatments after GnRH-1. In contrast, LH concentrations and area under the LH curve of Pre10 cows were greater than that of PG-3-G cows because progesterone was greater in PG-3-G than in Pre10 cows (4.6 ± 0.4 vs. 2.8 ± 0.4 ng/mL), respectively. Concentrations of LH did not differ after GnRH-2 at either 56 or 72 h; however, 1 cow receiving GnRH-2 at 56 h and 3 cows at 72 h had early spontaneous LH surges before GnRH-2. Ovulation was

suppressed overall in 210 blood collection windows in cows with elevated progesterone concentrations. When progesterone was <1 ng/mL after either PG-2 or PG-3 injections, GnRH-induced LH surges occurred in more than 90% of cows, and incidence of ovulation exceeded 80%. Pregnancy per AI tended to differ for PG-3-G (56.7%) compared with Pre10 (37.8%) and for 56 h (54.5%) compared with 72 h (38.2%), with the Pre10–72 h treatment combination producing less than half (22.2%) the pregnancies compared with all other treatment combinations. Furthermore, in these same cows, post-TAI luteal tissue volume tended to be compromised. We conclude that incidences of GnRH-induced LH surges and ovulation are suppressed in cows with elevated progesterone, possibly contributing to some loss in P/AI in TAI programs.

Key words: luteal function, luteinizing hormone, ovulation, presynchronization, progesterone

INTRODUCTION

Synchronization of ovulation to allow for timed artificial insemination (TAI) has become one of the most adopted reproductive technologies by dairy producers (Caraviello et al., 2006; Moeller et al., 2010). The most commonly used TAI programs in the dairy industry are variants of the original Ovsynch protocol [Pursley et al., 1998; injection of GnRH 7 d before and 48 h after PGF_{2α} (PG) with TAI 16 h following the last GnRH injection]. Presynchronizing the estrous cycles of cows improves pregnancy per AI (P/AI) compared with cows starting Ovsynch at random stages of the estrous cycle (Moreira et al., 2001; El-Zarkouny et al., 2004; Navanukraw et al., 2004). Improved P/AI resulting from presynchronization programs before Ovsynch has been associated with synchronizing the majority of estrous cycles to d 5 through 12, which improved ovulation incidence to the first GnRH injection of Ovsynch and resulting P/AI compared with cows treated at random stages of the estrous cycle (Vasconcelos et al., 1999).

A standard PG-presynchronization protocol involves 2 PG injections given 14 d apart, with initiation of the

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²Current address: Arkansas State University, Jonesboro, AR 72467.

³Corresponding author: jss@k-state.edu

Ovsynch protocol 12 d later (14×12 ; Moreira et al., 2001). Other intervals between the second PG Presynch injection and the onset of Ovsynch, 14 d (14×14 ; Navanukraw et al., 2004), 11 d (14×11 ; Galvão et al., 2007), and 10 d (14×10 ; Stevenson et al., 2012), have been investigated. The 14×11 Presynch program increased P/AI compared with Presynch 14×14 (Galvão et al., 2007).

Other presynchronization programs including PG and GnRH (PG-3-G; Peters and Pursley, 2002; Stevenson et al., 2012; Stevenson and Pulley, 2012; or G-6-G; Bello et al., 2006; Ribeiro et al., 2011) and application of a nonbreeding Ovsynch-like treatment before the TAI Ovsynch program (Double Ovsynch; Souza et al., 2008; Ribeiro et al., 2011; Herlihy et al., 2012) has generally increased P/AI compared with Ovsynch alone or with other Presynch-PG programs. Presynchronization programs that include GnRH improved ovulatory responses before Ovsynch (Stevenson et al., 2012), increased the proportion of cows having progesterone concentrations ≥ 1 ng/mL at GnRH-1 (Herlihy et al., 2012; Stevenson et al., 2012; Ayres et al., 2013), increased the number of CL in anovular cows at GnRH-1 (Herlihy et al., 2012; Stevenson et al., 2012), and increased P/AI during summer (Stevenson and Pulley, 2012) compared with current Presynch-PG programs.

Despite wide application of TAI programs, relatively little is known about the characteristics of LH concentrations or incidences of LH surges in association with the PG-induced luteolysis and GnRH injections associated with these TAI programs. Concentrations of LH in response to either 50 or 100 μ g of different GnRH products have been reported (Souza et al., 2009). One product tended to release less LH after both doses and induce fewer ovulations than the other 3 products in diestrous lactating dairy cows treated 7 d after TAI (Souza et al., 2009). Furthermore, this same GnRH product stimulated fewer ovulations and less LH release in beef heifers compared with another GnRH product (Martinez et al., 2003). In virgin heifers and lactating dairy cows treated with 2 injections of PG 11 d apart with GnRH administered 72 h after the last PG injection, LH surges were detected in all females, with 39% being induced by GnRH and 61% occurring spontaneously (Lucy and Stevenson, 1986). It is not known what proportion of LH surges occur before or after GnRH injections in various TAI programs. In addition, the relationship of the characteristics of LH concentrations associated with each GnRH injection and the steroid milieu had not been determined when this study was initiated during autumn 2011.

Thus, this study was designed to determine the incidence of spontaneous and predictable GnRH-induced

LH surges (peak LH magnitude, area under the LH secretion curve, and time to peak LH concentration) and subsequent ovulation in lactating dairy cows enrolled in a TAI program preceded by presynchronization of estrous cycles. Ancillary measures of progesterone, estradiol, and ovarian structures were also made to confirm our previous findings in response to the same 2 treatments (Stevenson et al., 2012) as well as improved P/AI in a previous large 4-herd study (Stevenson and Pulley, 2012).

MATERIALS AND METHODS

Cows, Housing, and Diets

The current studies were approved by the Kansas State University Institutional Animal Care and Use Committee. Lactating Holstein cows were enrolled at calving from September 2011 through March 2012 at the Kansas State University Dairy Teaching and Research Center. Cows were considered to be structurally sound and were housed individually in a tiestall barn equipped with feed bunks, automatic waterers, and stall mats covered with wood shavings. Cows were moved to a double-6 herringbone parlor and milked thrice daily. Cows were fed individually ad libitum twice daily at 0630 and 1600 h. A TMR calculated to meet nutrient requirements for lactating dairy cows producing 50 kg of 3.5% milk (NRC, 2001) consisted of alfalfa hay, corn silage, soybean meal, whole cottonseed, corn or milo grain, corn gluten feed, vitamins, and minerals. Cows were evaluated daily for health status by trained farm personnel.

Experimental Design and Treatments

A total of 70 cows were enrolled in 10 weekly clusters according to calving date and were assigned randomly within lactation number (1 vs. ≥ 2) to receive 1 of 2 presynchronization treatments (Figure 1). The first presynchronization treatment (**PG-3-G**; $n = 33$; Stevenson and Pulley, 2012; Stevenson et al., 2012) consisted of a 25-mg injection (i.m.) of PGF_{2 α} (**Pre-PG**; 5 mL Lutalyse Sterile Solution, Zoetis, Florham Park, NJ) 3 d before a 100- μ g (i.m.) injection of GnRH (**Pre-GnRH**; 2 mL Factrel, Zoetis). The second treatment (**Pre10**; $n = 37$) was timed so that the second of 2 (administered 14 d apart) 25-mg injections (i.m.) of PG (**PG-2**; 5 mL Lutalyse Sterile Solution, Zoetis) was administered on the same day as the Pre-PG injection of the PG-3-G treatment (Figure 1). Beginning 10 d after PG-2 (Pre10) or Pre-PG (PG-3-G), all cows were enrolled in a 7-d Ovsynch programs in which they

Figure 1. Experimental design of treatments and blood sampling. At calving, lactating dairy cows were assigned randomly to 1 of 2 presynchronization protocols: PG-3-G [25-mg injection of PGF_{2α} (Pre-PG) and then 100 µg of GnRH (Pre-GnRH) 3 d later, followed by enrollment into the Ovsynch program 7 d later] or Pre10 [25-mg injection of PGF_{2α} (PG-1 and PG-2) administered 14 d apart, with the Ovsynch protocol initiated 10 d later]. The Ovsynch protocol is initiated with an injection of GnRH (GnRH-1) followed 7 d with an injection of PG with a second GnRH injection (GnRH-2) administered at either 56 or 72 h after PG. Blood was collected to determine LH concentrations at (1) Pre-GnRH: every 4 h from 0 to 80 h after PG-2 and hourly from 72 to 78 h (Pre-GnRH injected at 72 h); (2) GnRH-1: hourly from 0 to 6 h after GnRH-1; (3) GnRH-2: every 4 h from 0 to 80 h after PG and either hourly from 56 to 62 h (GnRH-2 injected at 56 h) or hourly from 72 to 80 h (GnRH-2 injected at 72 h). Color version available online.

received (i.m.) 100 µg of GnRH (**GnRH-1**; 2 mL of Factrel, Zoetis), followed in 7 d by 25 mg of PG (**PG-3**; 5 mL Lutalyse Sterile Solution, Zoetis). Approximately one-half of the cows in each presynchronization treatment received the second 100 µg of GnRH (**GnRH-2**; 2 mL of Factrel, Zoetis) at either 56 or 72 h after PG-3. All cows were inseminated at 72 h after PG-3. Therefore, 4 treatment–time combinations were formed at 56 h after PG-3: PG-3-G-56 ($n = 16$); PG-3-G-72 ($n = 17$); Pre10-56 ($n = 19$); and Pre10-72 ($n = 18$). Treatment injections were staggered within cluster so that all cows were inseminated on the same day of the week.

Body condition scores (1 = thin and 5 = fat; Ferguson et al., 1994) were determined and assigned 7 d before the initiation of the TAI Ovsynch program (Figure 1). Cows were at a median of 68 DIM (68.4 ± 0.7 ; mean $\pm$ SE) at TAI. Frozen-thawed semen from multiple sires was used.

Ovarian Structures and Ovulation

Transrectal ovarian scans were conducted in all cows by ultrasonography (7.5-MHz linear-array transducer, Aloka 500V; Corometrics Medical Systems Inc., Wall-

ingford, CT) to record diameter and map location of follicles ≥ 5 mm and any corpus luteum (**CL**) present at Pre-GnRH, GnRH-1, PG-3, GnRH-2, and 6 d post-TAI, when the diameter of all luteal structures was measured (Figure 1). A map of each ovary was drawn with the position and size of all follicles and the location of each CL, which allowed for evaluation of ovulatory responses to Pre-GnRH, GnRH-1, and GnRH-2. Follicle diameter was determined by averaging the width and height of each follicle using the internal electronic calipers of the ultrasound machine. During the ultrasound exam before administration of GnRH-2, the presence of anechoic fluid in the uterine lumen was recorded (bioassay of potential estrus and effects of estradiol).

Pregnancy diagnosis was conducted by transrectal ultrasonography on d 31 and 61 after TAI. A positive pregnancy outcome required the presence of anechoic uterine fluid and a CL ≥ 25 mm in diameter or anechoic uterine fluid and the presence of a viable embryo with a visible heartbeat. Cows that displayed estrus and were re-inseminated before the first pregnancy diagnosis were considered not pregnant to TAI unless later ultrasonography found re-inseminated cows to be pregnant to TAI.

Procedures and Blood Sample Collections

Blood samples were collected by placement of an indwelling jugular catheter or, in the event of jugular catheter failure, by puncture of caudal vessels into evacuated tubes (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ). Cows were fitted with a guide wire-style jugular catheter (Mila International Inc., Erlanger, KY). Under local anesthesia (lidocaine hydrochloride; Agri Laboratories Ltd., St. Joseph, MO), a size-10 scalpel blade was used to make a 0.5-inch incision to initially breach the skin and allow access to the jugular vein. A 45-cm-long guide wire was threaded through the needle. The catheter (20 cm) was then strung onto the wire and guided into the jugular vein. Catheters were flushed twice daily and after each blood collection with a sterile saline solution containing 3.5% sodium citrate to prevent clotting. All blood samples were placed on ice upon collection and stored at 5°C, and then transported to the laboratory and stored at 5°C for approximately 8 to 16 h before serum was harvested by centrifugation at $1,000 \times g$ for 15 min in a refrigerated centrifuge. Blood sera were frozen and stored at -20°C until progesterone, estradiol, and LH concentrations were determined by RIA.

Hormone Concentrations

Concentrations of LH were measured in the following blood sera samples. Beginning with 0 h after Pre-PG or PG-2, and PG-3, blood was collected every 4 h from 48 through 84 h. Additional samples were collected hourly (0 to 6 h) after each GnRH injection (Pre-GnRH or a comparable time for Pre10 cows, GnRH-1, GnRH-2 at either 56 or 72 h; Figure 1). All sera samples for LH concentration were measured in triplicate and analyzed by liquid-phase double-antibody RIA (Atkins et al., 2008). Sera were assayed in triplicate (100 μ L) in 6 assays. Pooled bovine sera assayed in quadruplicate at 25, 40, 60, 100, 175, 200, and 300 μ L averaged 9.8 ± 0.3 ng/mL and paralleled the standard curve. Intra- and interassay CV averaged 4.9 and 5.7%, respectively.

Peak LH concentrations and time to LH peak were determined by examining individual LH plots of each 6-h window of hourly blood collection. To determine if an LH surge occurred, a baseline mean and standard deviation were calculated for the 7 samples (0 through 6 h) after deleting the 2 largest concentrations. An "LH surge" was defined to occur when the largest LH concentration exceeded the mean of the remaining baseline by 2 SD. Surges were then defined as GnRH-induced or having occurred spontaneously before, during, or after the beginning of the 6-h sampling window in the absence of GnRH. Exceptions to the previous procedure

occurred in a few of the 210 LH window plots when a surge was obvious but failed to meet the defined criteria. Area under the curve (AUC) for LH concentration was calculated as the sum of trapezoid areas, in which Cp2 was the LH serum concentration of a sample taken at time 2 (t2) and Cp1 was the LH concentration of a sample taken at time 1 (t1). The following formula was used to calculate the area under the LH curve during the 6-h window after each GnRH injection:

$$\text{AUC} = \Sigma [(Cp1 + Cp2)/2 \times (t2 - t1) + \dots + (Cpn + Cpn)/2 \times (tn + 1 - tn)].$$

Concentrations of progesterone in blood serum were measured in samples collected at 0 and 72 h after PG-2 or Pre-PG and at 0 and 60 h after PG-3 (Figure 1). Additional sera samples were pooled during the 6-h sampling windows at each GnRH injection (Pre-GnRH, GnRH-1, and GnRH-2; Figure 1) by taking 0.1 mL of serum from each sample and combining into a single aliquot, which was then assayed for progesterone concentration. All serum samples for progesterone concentration were measured in duplicate and analyzed by direct quantitative (nonextracted) RIA using Coat-A-Count progesterone kits (cat. no. TKPG; Siemens Medical Solutions Diagnostics, Los Angeles, CA) previously validated for bovine serum (Stevenson et al., 2012). Cows having luteolysis (CL regression) must have had serum progesterone ≥ 1 ng/mL at PG-2 or Pre-PG and <0.5 ng/mL 72 h later or ≥ 1 ng/mL at PG-3 and <0.5 ng/mL 60 h later. Assay sensitivity of the progesterone RIA was 0.047 ± 0.004 ng/mL, and intra- and interassay coefficients of variation for 12 assays were 3.4 and 5.1%, respectively, for a pool of bovine serum that averaged 2.8 ± 0.04 ng/mL.

Concentrations of estradiol were measured in duplicate by RIA (Stevenson, 2011) in samples collected at 0, 24, 36, 48, 60, and 72 h after PG-3 administration (Figure 1). Assay sensitivity was 1.1 ± 0.4 pg/mL, and intra- and interassay coefficients of variation for 2 assays were 2.6 and 5.5%, respectively, for a pool of bovine serum that averaged 6.3 ± 0.3 pg/mL.

Statistical Analyses

The experiment was a completely randomized design with 2 treatments (PG-3-G vs. Pre10), with cows balanced for lactation number (1 vs. ≥ 2) until 56 h after PG-3 when the timing of GnRH-2 occurred at either 56 or 72 h after PG-3. From 56 h, the experimental design became a 2×2 factorial arrangement of 4 treatments (PG-3-G-56, PG-3-G-72, Pre10-56, or Pre10-72); cow was the experimental unit. Unless otherwise specified,

all values were expressed as least squares means ± SEM.

Various binomial characteristics and responses to hormonal injections (GnRH and PG) were analyzed by logistic regression using the LOGISTIC procedure of SAS (SAS Institute Inc., Cary, NC). These responses included the proportions of cows having luteolysis (defined previously), LH surges, uterine fluid, and ovulation (single or >1). The initial model included fixed effects of treatment (PG-3-G vs. Pre10), lactation number (1 vs. ≥2), and BCS (<2.75 vs. ≥2.75). The final model produced by backward stepwise selection of independent variables entered or retained in the model was based on the Wald statistic ($P > 0.10$). In general, for variables assessed 56 h after GnRH-3, the model included permutations of treatment and time of GnRH-3 to form 4 treatments. These models first examined the main effects of treatment and GnRH time. When a significant interaction was detected by ANOVA, the model included all 4 permutations of the main effects.

Analyses of serum LH concentrations, follicle diameter, CL number, LH peak, and area under the LH curve were performed using the MIXED procedure of the SAS program (SAS Institute Inc.). The model included fixed effects of treatment, lactation number, and BCS. The model for the comparisons of area under the curve of LH profiles for GnRH-2 used a model that included treatment, GnRH-2 time (56 vs. 72 h), circulating progesterone concentrations (<0.5 vs. ≥0.5 or <1.0 vs. ≥1.0 ng/mL), circulating estradiol concentration (<2.0 vs. ≥2.0 pg/mL), treatment × time, treatment × progesterone concentration cut-point, treatment × estradiol concentration cut-point, and all 3-way interactions.

Repeated measurements, including serum concentrations of estradiol (0, 24, 36, 48, 60, and 72 h after PG-3) and LH (hourly LH concentrations during 6 h

after GnRH), were analyzed as a split-plot design in the MIXED procedure in SAS. The model included fixed effects of treatment, day, treatment × day, lactation number, and BCS. Cow within treatment was treated as a random variable and was the split-plot error term for testing treatment differences.

Pregnancy outcomes at d 31 and 61 post-TAI and pregnancy losses were analyzed by SAS procedure LOGISTIC using the initial model of treatment, lactation number, their interaction, technician, sire, and BCS.

RESULTS

PG-2

Before PG-2 administration, circulating concentrations of progesterone and proportions of cows having progesterone concentrations ≥1 ng/mL did not differ between treatments (Table 1). The proportion of cows having luteolysis after PG-2 or Pre-PG also did not differ between treatments (Table 1).

Pre-GnRH

Before Pre-GnRH administration, circulating concentrations of progesterone, the proportion of cows with progesterone concentrations <1 ng/mL, the number of CL per cow, the proportion of cows with CL, and the diameters of the largest and second-largest follicles (PG-3-G: 11.2 ± 4.2 vs. Pre10: 11.4 ± 4.4 mm) did not differ between treatments (Table 2).

Patterns of LH concentration during the 6-h blood collection period are shown in Figure 2A. As expected, cows receiving the Pre-GnRH injection had greater ($P < 0.01$) LH concentrations at 1 and 2 h than Pre10 cows receiving no GnRH. The proportion of cows with

Table 1. Progesterone concentrations (LSM ± SEM) and responses to presynchronization injection of PG-2 or Pre-PG

Item	Treatment ¹		P-value
	PG-3-G	Pre10	
Progesterone ² (ng/mL)	3.2 ± 0.5	3.5 ± 0.5	0.657
Response [% (no./no.)]			
Progesterone ≥1 ng/mL ³	72.7 (24/33)	64.9 (24/37)	0.772
Luteolysis ⁴	79.1 (19/24)	87.5 (21/24)	0.872

¹PG-3-G = injection of PGF_{2α} (Pre-PG) administered at the same time as PG-2 followed in 3 d by Pre-GnRH injection, which was administered 7 d before onset of Ovsynch; Pre10 = 2 injections of PGF_{2α} 14 d apart (PG-1 and PG-2) with PG-2 administered 10 d before onset of Ovsynch (see Figure 1).

²Progesterone concentrations before PG-2 or Pre-PG.

³Proportion of cows having progesterone concentrations ≥1 ng/mL before PG-2 or Pre-PG.

⁴Luteolysis was determined by changes in progesterone concentration between PGF-2 (≥1 ng/mL) and 72 h later (<0.5 ng/mL).

Table 2. Ovarian responses at or after Pre-GnRH [injection of PGF_{2α} (Pre-PG) 3 d before injection of GnRH]

Item	Treatment ¹		<i>P</i> -value
	PG-3-G	Pre10	
Progesterone ² (ng/mL)	1.0 ± 0.3 ³	0.6 ± 0.3	0.375
Progesterone <1 ng/mL ⁴ (%)	78.7 (26/33) ⁵	83.8 (31/37)	0.922
Corpora lutea (CL) per cow (no.)	0.5 ± 0.1	0.5 ± 0.1	0.992
Cows with CL (%)	45.5 (15/33)	40.5 (15/37)	0.681
Follicle diameter ⁶ (mm)	14.4 ± 0.6	15.1 ± 0.6	0.444
LH surge ⁷ (%)	100 (33/33)	43.2 (16/37)	0.001
GnRH-induced	69.7 (23/33)	0 (0/37)	0.001
Spontaneous	30.3 (10/33)	43.2 (16/37)	0.001
LH peak ⁸ (ng/mL)	3.5 ± 0.3	2.1 ± 0.3	0.004
Time to LH peak ⁹ (h)	1.2 ± 0.3	2.7 ± 0.4	0.001
Area under LH curve ¹⁰	9.9 ± 0.8	7.8 ± 0.8	0.061
Ovulation ¹¹ (%)	90.9 (30/33)	59.5 (22/37)	0.007
Double ovulation ¹¹ (%)	30.3 (10/33)	13.5 (5/37)	0.180

¹PG-3-G = injection of PGF_{2α} (Pre-PG) administered at the same time as PG-2 followed in 3 d by Pre-GnRH injection, which was administered 7 d before onset of Ovsynch; Pre10 = 2 injections of PGF_{2α} 14 d apart (PG-1 and PG-2) with PG-2 administered 10 d before onset of Ovsynch (see Figure 1).

²Progesterone concentrations at Pre-GnRH.

³Least squares means ± SEM.

⁴Proportion of cows having progesterone concentrations <1 ng/mL at 72 h after Pre-PG or PG-2.

⁵% (no./no.).

⁶Diameter of the largest follicle before Pre-GnRH injection.

⁷Increase in LH concentration greater than 2 SD above baseline during 6 h after Pre-GnRH.

⁸Maximum LH concentration during 6 h after Pre-GnRH.

⁹Time to peak LH concentration during 6 h after Pre-GnRH.

¹⁰Calculated by the trapezoidal method.

¹¹Spontaneous single or multiple ovulation (Pre10) or ovulation in response to Pre-GnRH.

an LH surge after Pre-GnRH was greater ($P < 0.001$) in PG-3-G cows than in Pre10 cows, in which only spontaneous LH surges could occur (Table 2). Although mean LH peak concentration was greater ($P = 0.004$) in PG-3-G cows and time to peak LH occurred earlier ($P = 0.001$) than in Pre10 cows, area under the LH curve only tended ($P = 0.061$) to be greater for PG-3-G than for Pre10 cows. Concentrations of progesterone and the number of CL (0 vs. 1+) had no effect on LH peak concentrations after Pre-GnRH. The proportion of cows ovulating after Pre-GnRH was greater ($P = 0.007$) in PG-3-G than in Pre10 (Table 2).

GnRH-1

The proportion of cows with progesterone concentrations ≥ 1 ng/mL, number of CL per cow, and the proportion of cows with a CL were greater ($P \leq 0.015$) for PG-3-G than for Pre10 cows at GnRH-1 (onset of the Ovsynch protocol; Table 3). Diameters of the largest (Table 3) and second-largest follicles (PG-3-G: 10.7 ± 0.9 vs. Pre10: 9.7 ± 0.9) before GnRH-1 administration did not differ between treatments.

In the face of greater ($P < 0.001$) progesterone concentrations at GnRH-1, concentrations of LH were

lower ($P < 0.01$) at 1, 2, and 3 h after GnRH-1 in PG-3-G cows than in Pre10 cows (Figure 2B). The number of CL per cow did not affect peak LH concentrations; however, cows with progesterone concentrations ≥ 1 ng/mL had decreased ($P = 0.001$) LH peak concentrations after GnRH-1 compared with cows having progesterone concentrations <1.0 ng/mL (2.2 ± 0.2 vs. 4.1 ± 0.4 ng/mL, respectively).

All cows had GnRH-induced LH surges, but LH peak concentration was greater in Pre10 than in PG-3-G cows, whereas time to LH peak did not differ (Table 3). Incidence of single or double ovulation also did not differ between treatments (Table 3).

PG-3

Although the proportion of cows with ≥ 1 CL was greater ($P = 0.053$) in PG-3-G cows than in Pre10 cows before PG-3, none of the other ovarian characteristics assessed before PG-3 differed between treatments (Table 4). Mean concentrations of progesterone were approximately 5 ng/mL, the proportion of cows with progesterone concentrations ≥ 1 ng/mL exceeded 93%, and the number of CL ranged from 1.7 to 1.9 regardless of treatment. No differences were detected in follicle

Figure 2. Pattern of mean ($\pm$ SE) serum LH concentrations during 6 h after Pre-GnRH (A) and GnRH-1 (B) in cows in presynchronization treatments PG-3-G ($n = 33$) or Pre10 ($n = 37$); see Figure 1 for treatment definitions. Pattern of mean serum LH concentrations during 6 h after GnRH-2 (C) according to presynchronization treatments and time of GnRH-2 administration: PG-3-G-56 ($n = 16$), PG-3-G-72 ($n = 17$), Pre10-56 ($n = 19$), or Pre10-72 ($n = 18$). Treatment means within hour having different letters (a, b) differ ($P < 0.05$). Color version available online.

size, estradiol concentrations before PG-3, or incidence of luteolysis after PG-3 between treatments.

GnRH-2

Diameter of the largest follicle at 48 h after PG-3, concentrations of progesterone, and the proportion of cows with progesterone < 1 ng/mL at 60 h did not differ between treatments (Table 5). Of the 70 cows with LH surges, 4 had spontaneous LH surges: 1 PG-3-G-56 cow (64 to 68 h); 2 PG-3-72 cows (68 to 72 h); and 1 Pre10-72 cow (64 to 68 h) after PG-3.

After GnRH-2 administration, LH concentrations increased ($P < 0.001$) to peaks at 1 to 2 h before steadily decreasing to basal concentrations by 6 h (Figure 2C). Neither treatment nor time had any detectable effects on LH concentrations during the 6-h sampling period after GnRH-2. Furthermore, none of the other LH characteristics (LH peak concentration, time to LH peak, or area under the curve) differed between treatments (Table 5). Tendencies were detected for time to LH peak between treatments ($P = 0.114$; earlier peaks for Pre10 than PG-3-G cows) and between times ($P = 0.124$; earlier peaks for cows injected at 72 than 56 h after PG-3).

Ovulatory incidence did not differ among treatment-time combinations; only 7 of 70 cows did not ovulate, and 7 of 64 cows ovulated > 1 follicle (Table 5).

Concentrations of estradiol did not differ among treatments until 72 h after PG-3 (Figure 3), when concentrations were greater ($P < 0.05$) in PG-3-G than in Pre10 cows. The presence of uterine fluid during the ultrasound examination at 48 h after PG-3 was not influenced by treatment (PG-3-G = 27.3% vs. Pre10 = 43.2%). Cows with estradiol concentration ≥ 2.0 pg/mL at 48 h after PG-3, however, had a greater ($P = 0.04$) incidence of uterine fluid than cows with estradiol concentrations ≤ 2.0 pg/mL [87.0% (20/23) vs. 17.4% (4/23)], respectively.

Ovulatory Response and Progesterone

Ovulatory responses to GnRH-induced or spontaneous LH surges in the presence of a functional or regressing CL are summarized in Table 6. Overall incidence of ovulation was 73.3% and did not differ between treatments. Of opportunities to detect LH surges in the 37 Pre10 cows (no Pre-GnRH injection was given), LH surges were not detected in 21 cows, but 9 of the 21 cows ovulated. In the remaining 16 cows, 13 of 16 cows ovulated. During the Pre-GnRH period, more cows ovulated in which an LH surge was detected compared with no detected LH surge (87.8 vs. 42.9%; $P = 0.042$). Incidence of ovulation did not

Table 3. Ovarian responses at or after GnRH-1 (100 µg of GnRH to initiate a 7-d Ovsynch program)

Item	Treatment ¹		P-value
	PG-3-G	Pre10	
Progesterone ² (ng/mL)	4.6 ± 0.4 ³	2.8 ± 0.4	<0.001
Progesterone ≥1 ng/mL ⁴ (%)	100 (33/33) ⁵	78.4 (29/37)	0.015
Corpora lutea (CL) per cow (no.)	1.5 ± 0.1	0.9 ± 0.1	0.005
Cows with CL (%)	97.0 (32/33)	70.3 (26/37)	0.003
Follicle diameter ⁶ (mm)	13.9 ± 0.7	13.4 ± 0.7	0.991
LH surge ⁷ (%)	100 (33/33) ⁸	100 (37/37)	—
GnRH-induced	100 (33/33)	100 (37/37)	—
Spontaneous	0 (0/33)	0 (0/37)	—
LH peak ⁸ (ng/mL)	2.1 ± 0.2	2.8 ± 0.2	0.039
Time to LH peak ⁹ (h)	1.3 ± 0.1 ⁷	1.4 ± 0.1	0.575
Area under LH curve ¹⁰	7.4 ± 0.7	10.0 ± 0.7	0.008
Ovulation ¹¹ (%)	48.5 (16/33)	62.2 (23/37)	0.315
Double ovulation ¹¹ (%)	6.7 (2/33)	20.0 (6/37)	0.238

¹PG-3-G = injection of PGF_{2α} (Pre-PG) administered at the same time as PG-2 followed in 3 d by Pre-GnRH injection, which was administered 7 d before onset of Ovsynch; Pre10 = 2 injections of PGF_{2α} 14 d apart (PG-1 and PG-2) with PG-2 administered 10 d before onset of Ovsynch (see Figure 1).

²Progesterone concentrations at GnRH-1.

³Least squares means ± SEM.

⁴Proportion of cows having progesterone concentrations ≥1 ng/mL at GnRH-1.

⁵% (no./no.).

⁶Diameter of the largest follicle before GnRH-2 injection.

⁷Increase in LH concentration greater than 2 SD above baseline during 6 h after GnRH-1.

⁸Maximum LH concentration during 6 h after GnRH-1.

⁹Time to peak LH concentration during 6 h after GnRH-1.

¹⁰Calculated by the trapezoidal method.

¹¹Single or double ovulation in response to GnRH-1.

differ between treatments after GnRH-1 or GnRH-2. During 210 blood collection periods, cows with elevated progesterone (3.3 to 4.5 ± 0.2 ng/mL) had reduced ($P < 0.001$) incidence of ovulation—to nearly 68% of that in cows with low concentrations of progesterone (0.2 ± 0.1 ng/mL; Table 6).

Table 4. Ovarian responses at or after PG-3 injection (25 mg of PGF_{2α})

Item	Treatment ¹		P-value
	PG-3-G	Pre10	
Progesterone ² (ng/mL)	5.1 ± 0.4 ³	4.6 ± 0.4	0.442
Progesterone ⁴ (%)	93.9 (31/33) ⁵	94.6 (35/37)	0.726
Corpora lutea (CL) per cow	1.9 ± 0.2	1.7 ± 0.1	0.329
Cows with CL (%)	100 (33/33)	89.2 (33/37)	0.053
Follicle diameter ⁶ (mm)	12.4 ± 0.6	12.2 ± 0.6	0.774
Estradiol ⁷ (pg/mL)	1.5 ± 0.6	2.4 ± 0.6	0.303
Luteolysis ⁸ (%)	96.7 (30/31)	91.4 (32/35)	0.369

¹PG-3-G = injection of PGF_{2α} (Pre-PG) administered at the same time as PG-2 followed in 3 d by Pre-GnRH injection, which was administered 7 d before onset of Ovsynch; Pre10 = 2 injections of PGF_{2α} 14 d apart (PG-1 and PG-2) with PG-2 administered 10 d before onset of Ovsynch (see Figure 1).

²Progesterone concentrations at PG-3 administration.

³Least squares means ± SEM.

⁴Proportion of cows having progesterone concentrations ≥1 ng/mL before PG-3.

⁵% (no./no.).

⁶Diameter of the largest follicle at PG-3.

⁷Estradiol concentrations at PG-3.

⁸Progesterone (≥1 ng/mL) at PG-3 decreased to <0.5 ng/mL by 60 h after PG-3.

Figure 3. Least squares mean ($\pm$ SE) serum estradiol concentrations for PG-3-G ($n = 33$) or Pre10 ($n = 37$) cows treated with GnRH-2 at 56 or 72 h after PG; see Figure 1 for treatment definitions. Different letters (a, b) indicate that least squares treatment means differed ($P < 0.05$) at 72 h. Color version available online.

Postinsemination Events

Neither number of post-TAI CL per cow nor total luteal tissue volume at 6 d post-TAI differed among treatment-time combinations. A tendency ($P = 0.096$) for a treatment $\times$ time interaction was detected, in

which total luteal tissue volume in Pre10–72 cows was 53 to 70% of that assessed in the other treatments (Pre10–72 = 3.5 ± 1.1 cm³; Pre10–56 = 6.6 ± 1.0 cm³; PG-3-G-72 = 5.8 ± 1.1 cm³; and PG-3-G-56 = 5.0 ± 1.21 cm³).

Although the study was not designed to detect differences in P/AI, these same cows (Pre10–72) with numerically reduced post-AI luteal tissue volume tended ($P = 0.15$) to have fewer pregnancies at 31 d post-AI than cows from other treatment-time combinations (Table 7). The 4 of 18 Pre10–72 cows that conceived each had a GnRH-induced LH surge and ovulated. Treatment differences at d 61 post-TAI followed a pattern similar to that at d 31. Pregnancy loss did not differ among treatment-time combinations, with only 1 pregnancy loss in the PG-3-G-72 treatment.

DISCUSSION

This study was designed to determine the incidence of spontaneous and predictable GnRH-induced LH surges and subsequent ovulation in lactating dairy cows enrolled in a TAI program preceded by presynchronization of estrous cycles. This objective was met by applying 2 presynchronization treatments, one of which (Pre10) should provide the greatest potential for ovulation in

Table 5. Incidence of LH surges and ovulation after GnRH-2

Item	Treatment ¹ (T)				P-value		
	PG-3-G		Pre10				
	56 h	72 h	56 h	72 h	T	Time	T × time
Progesterone ² (ng/mL)	0.3 ± 0.2 ³		0.4 ± 0.2		0.686	—	—
Progesterone ⁴ (%)	97.0 (32/33) ⁵		91.9 (34/37)		0.362	—	—
Follicle size ⁶ (mm)	13.6 ± 0.5		13.5 ± 0.5		0.774	—	—
LH surge ⁷ (%)	100 (16/16)	100 (17/17)	100 (19/19)	100 (18/18)	—	—	—
GnRH-induced	93.7 (15/16)	88.2 (15/17)	100 (19/19)	88.9 (17/18)	0.682	0.240	0.783
Spontaneous	6.3 (1/16)	11.8 (2/17)	0 (0/19)	5.6 (1/18)	0.682	0.240	0.783
LH peak ⁸ (ng/mL)	4.3 ± 0.5	4.9 ± 0.5	5.0 ± 0.5	4.8 ± 0.5	0.619	0.649	0.384
Time to LH peak ⁹ (h)	1.7 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	0.114	0.124	0.608
Area under LH curve ¹⁰	14.9 ± 1.8	17.8 ± 1.7	16.1 ± 1.7	14.8 ± 1.7	0.603	0.624	0.200
Ovulation ¹¹ (%)	87.5 (14/16)	82.3 (14/17)	100 (19/19)	88.9 (16/18)	0.969	0.968	0.971
Double ovulation ¹¹ (%)	12.5 (2/16)	11.8 (2/17)	5.3 (1/19)	11.1 (2/18)	0.673	0.797	0.489

¹PG-3-G = injection of PGF_{2α} (Pre-PG) administered at the same time as PG-2 followed in 3 d by Pre-GnRH injection, which was administered 7 d before onset of Ovsynch; Pre10 = 2 injections of PGF_{2α} 14 d apart (PG-1 and PG-2) with PG-2 administered 10 d before onset of Ovsynch. Cows were administered GnRH-2 at either 56 or 72 h after PG (see Figure 1).

²Progesterone concentrations at 60 h post-PG administration.

³Least squares means $\pm$ SEM.

⁴Proportion of cows having progesterone concentrations <0.5 ng/mL at 60 h post-PG-3.

⁵% (no./no.).

⁶Determined at 48 h after PG-3.

⁷Increase in LH concentration greater than 2 SD above baseline.

⁸Maximum LH concentration during 6 h after GnRH-2.

⁹Time to peak LH concentration during 6 h after GnRH-2.

¹⁰Calculated by the trapezoidal method.

¹¹Single or multiple ovulation after GnRH-2.

Table 6. Ovulatory response to GnRH-induced or spontaneous LH surge¹ depending on concentrations of progesterone and presynchronization treatment

Item	Treatment					
	PG-3-G ²		Pre10 ³			
	Progesterone ⁴ (ng/mL)	Ovulation ⁵ (%)	Progesterone ⁴ (ng/mL)	Ovulation ⁵ (%)		Ovulation total (%)
				No surge	Surge	
Pre-GnRH ⁶						
	3.9 ± 0.4	85.7 (6/7)	2.6 ± 0.4	20.0 (1/5)	100 (1/1)	61.5 (8/13)
	0.3 ± 0.2	92.3 (24/26)	0.2 ± 0.2	50.0 (8/16)	80.0 (12/15)	77.2 (44/57)
	Total	90.9 (30/33)	—	42.9 (9/21)	81.2 (13/16)	74.3 (52/70)
GnRH-1 ⁷						
	4.6 ± 0.4	48.5 (16/33)	3.5 ± 0.2	—	60.7(17/28)	54.1 (33/61)
	—	0 (0/0)	—	—	66.7 (6/9)	66.7 (6/9)
	Total	48.5 (16/33)	—	—	62.2 (23/37)	55.7 (39/70)
GnRH-2 ⁸						
	5.3	0 (0/1)	3.1 ± 0.2	—	100 (3/3)	75.0 (3/4)
	0.2 ± 0.1	87.5 (28/32)	0.2 ± 0.1	—	94.1 (32/34)	90.9 (60/66)
	Total	84.8 (28/33)	—	—	94.6 (35/37)	90.0 (63/70)
Overall response						
	4.5 ± 0.2 ^a	53.7 (22/41)	3.3 ± 0.2 ^a	20.0 (1/5)	65.6 (21/32)	56.4 ^a (44/78)
	0.2 ± 0.1 ^b	89.7 (52/58)	0.2 ± 0.1 ^b	50.0 (8/16)	86.2 (50/58)	83.3 ^b (110/132)
	Total	74.7 (74/99)	—	42.9 (9/21)	78.9 (71/90)	73.3 (154/210)

^{a,b}Means within a column with different superscripts differ ($P < 0.01$).

¹Increase in LH concentration (GnRH-induced or spontaneously occurring before, during, or after GnRH injection) greater than 2 SD above baseline during 6 h after GnRH.

²PG-3-G = injection of PGF_{2α} administered at the same time as PG-2 followed in 3 d by Pre-GnRH injection, which was administered 7 d before onset of Ovsynch (see Figure 1). LH surges were detected in all PG-3-G cows.

³Pre10 = 2 injections of PGF_{2α} 14 d apart (PG-1 and PG-2) with PG-2 administered 10 d before onset of Ovsynch-56 (see Figure 1).

⁴Concentration of progesterone at the time of GnRH injection.

⁵Percentage of cows ovulating at least 1 follicle in response to LH surge.

⁶Pre-GnRH injection given 3 d after Pre-PG or no injection in Pre10 cows.

⁷GnRH-1 injection given at the onset of Ovsynch.

⁸GnRH-2 injection given at either 56 or 72 h after PG-3 (see Figure 1).

response to GnRH-1 of Ovsynch when PG-Presynch programs precede Ovsynch (Vasconcelos et al., 1999; Galvão et al., 2007). Perhaps equally as important as

increasing ovulation response to GnRH of Ovsynch is producing greater concentrations of progesterone at the onset of Ovsynch, which also resulted in greater fertil-

Table 7. Pregnancy per AI after presynchronization with PG-3-G or Pre10 and GnRH-2 administration at 56 or 72 h after PG-3

Item	Treatment ¹			
	PG-3-G		Pre10	
	56 h	72 h	56 h	72 h
Pregnancy per AI [% (no./no.)]				
At 31 d ²	57.1 (8/14)	56.3 (9/16)	52.6 (10/19)	22.2 (4/18)
At 61 d ³	57.1 (8/14)	50.0 (8/16)	52.6 (10/19)	22.2 (4/18)
Pregnancy loss ⁴ (%)	0 (0/8)	11.1 (1/9)	0 (0/10)	0 (0/4)

¹PG-3-G = injection of PGF_{2α} (Pre-PG) administered at the same time as PG-2 followed in 3 d by Pre-GnRH injection, which was administered 7 d before onset of Ovsynch; Pre10 = 2 injections of PGF_{2α} 14 d apart (PG-1 and PG-2) with PG-2 administered 10 d before onset of Ovsynch. Cows were administered GnRH-2 at either 56 or 72 h after PG (see Figure 1). Three PG-3-G cows were culled before pregnancy diagnosis.

²Treatment ($P = 0.072$), time ($P = 0.192$), and treatment × time ($P = 0.153$).

³Treatment ($P = 0.104$), time ($P = 0.115$), and treatment × time ($P = 0.230$).

⁴Pregnancy losses were calculated between the 2 pregnancy diagnoses.

ity (Stevenson et al., 2012; Ayres et al., 2013). Thus, PG-3-G was chosen as the second presynchronization treatment (Stevenson et al., 2012).

Improving the ovulatory response to GnRH-1 results in better embryo quality (Cerri et al., 2009) and increased P/AI (Chebel et al., 2006; Stevenson et al., 2007; Rutigliano et al., 2008). The PG-3-G treatment was chosen because of its potential to stimulate ovulation in cows before commencement of a TAI protocol (Bello et al., 2006; Souza et al., 2008), particularly in anovular cows (Stevenson et al., 2012). The novel findings of the present study are the characteristics of LH release associated with each GnRH injection in a presynchronization-Ovsynch program and subsequently documented ovulation in 210 possible cases.

Incidence of spontaneous LH surges was greater in Pre10 than in PG-3-G cows receiving the Pre-GnRH injection. Consequently, Pre10-treated cows had lesser LH peak concentrations during the 6-h window 72 h after PG-2 compared with the induced LH surge peak concentrations in PG-3-G treated cows. Spontaneous LH surges in control cows occurred from 52 to 104 h after PG injection, whereas in cows administered GnRH at 72 h after PG, induced LH surges occurred between 74.2 and 74.5 h (Lucy and Stevenson, 1986). The findings of current study confirm this earlier report.

Incidence of ovulation after Pre-GnRH was 91% for PG-3-G and 60% for Pre10 cows. Previous studies reported ovulation incidence of 80 and 85%, which is similar to the 91% of the current study, following the Pre-GnRH injection in the same (Stevenson et al., 2012) or similar treatments (G-6-G; GnRH injection was administered 2 d after a PG injection; Bello et al., 2006). Seven days after the Pre-GnRH injection, progesterone concentrations were greater in PG-3-G than in Pre10 cows because more PG-3-G cows ovulated in response to Pre-GnRH.

At GnRH-1, the proportion of cows with progesterone concentrations ≥ 1 ng/mL, the number of CL per cow, and the proportion of cows having a CL were greater in PG-3-G cows than in Pre10 cows, confirming our earlier findings utilizing the same treatment and control (Stevenson et al., 2012). Progesterone concentrations > 3 ng/mL in cows presynchronized with Double Ovsynch 7 d after GnRH-induced ovulation (Souza et al., 2008; Giordano et al., 2012) or Presynch-Ovsynch (Moreira et al., 2001; Stevenson et al., 2012) have been reported in several studies, and the current study corroborates these previous results. More PG-3-G cows had progesterone concentrations ≥ 1 ng/mL (100 vs. 80%) compared with Pre10 cows, which is similar to our previous report for the same treatments (90.5 vs. 76.2%; Stevenson et al., 2012). Likewise, the number of CL per cow and the proportion of cows with a CL

were greater in PG-3-G cows than in Pre10 cows in the current study and in our recent report (Stevenson et al., 2012).

All cows regardless of treatment had induced LH surges after GnRH-1. Time to peak LH concentration was 0.7 h earlier for PG-3-G compared with Pre10 cows, but LH peak concentration and area under the LH curve was greater in Pre10 than in PG-3-G cows. Ovulation incidence did not differ between treatments, ranging from 48 to 62%, which is consistent with our previous findings (Stevenson et al., 2012) and is similar to another report of ovulation incidence of 55% 7 d after a prior GnRH injection (Souza et al., 2009).

Giordano et al. (2012) reported that peak LH concentrations and the area under the LH curve were greater in 12 cows with low (< 1 ng/mL) progesterone than in 12 cows with high (> 1 ng/mL) progesterone concentrations. In the present study, progesterone concentrations, proportions of cows with progesterone ≥ 1 ng/mL, number of CL per cow, and proportions of cows with a CL were less in Pre10 cows than in PG-3-G cows, which explains why peak LH concentrations were greater in Pre10 than in PG-3-G cows after GnRH-1. Not only were the concentration of LH and the incidence of LH surges reduced in the presence of greater progesterone concentrations, but the incidence of ovulation was also suppressed. The pituitary gland exhibits attenuation in its response to exogenous GnRH treatment in the presence of high circulating concentrations of progesterone (Schoenemann et al., 1985). Similar findings have been reported in beef heifers (Dias et al., 2010) and beef cows (Cline, 2002; Colazo et al., 2008). Area under the LH curve was greater in Pre10 cows with lower progesterone concentrations in the present study than in PG-3-G cows having greater progesterone concentrations. Area under the LH curve (39.9 ± 4.5 ng²) after using the same gonadorelin product 7 d after a previous GnRH injection in lactating dairy cows (Souza et al., 2009) was reported to be greater than that detected in the current study after GnRH-2 (7.62 ± 1.97 to 10.56 ± 4.94 ng²). Despite the lesser LH concentrations secreted in response to GnRH-1 than those previously reported, follicle diameter and ovulation rates after GnRH-1 administration did not differ between treatments, and incidence of double ovulation was similar.

Immediately before PG-3, progesterone and estradiol concentrations, the proportion of cows with progesterone concentrations ≥ 1 ng/mL, the number of CL per cow, and follicle diameter did not differ between treatments. In our previous study of these same presynchronization treatments, we reported that PG-3-G tended to increase the proportion of cows with progesterone concentrations ≥ 1 ng/mL and increased the number of CL per cow (Stevenson et al., 2012). In the present

study, with fewer cows, we were unable to detect any of the previously reported differences at PG-3, except for the increased proportion of cows with a CL in PG-3-G cows.

To control timing of the LH surge and ovulation, the final GnRH injection should be given before a spontaneous LH surge for TAI to result in pregnancy outcomes similar to breeding based on detected estrus (Peters and Pursley, 2002). Studies have indicated that the earliest a spontaneous LH surge occurs is 36 to 48 h following PG-induced luteal regression (Twagiramungu et al., 1992; Walker et al., 1996). In the present study, only 1 cow had a spontaneous surge before GnRH was given at 56 h, whereas 3 cows had spontaneous LH surges before GnRH was administered at 72 h, likely producing less synchrony between AI and ovulation. No other LH characteristics differed between treatment-time combinations after GnRH-2, including incidence of ovulation.

In the current study, LH peak concentrations in response to GnRH treatments ranged from 2.19 to 5.24 ng/mL. These peak concentrations are similar to those reported by Lucy and Stevenson (1986), with LH peak concentration ranging from 0.6 to 6.7 ng/mL in dairy cows. In contrast, Souza et al. (2009) reported LH peak concentration in response to 50 µg of GnRH of 9.6 ng/mL and 21.6 ng/mL after 100 µg of GnRH in lactating dairy cows. Most of these differences are likely related to using different LH standards, GnRH products (Souza et al., 2009), and frequencies of blood sample collection.

At 72 h, estradiol concentrations were greater for PG-3-G cows than Pre10 cows. Previously reported maximal estradiol concentrations before ovulation in lactating dairy cows were 7.3 ± 0.8 pg/mL (Sartori et al., 2004) and 7.9 ± 0.8 pg/mL (Wiltbank et al., 2006). Cows receiving GnRH-2 at 72 h had an additional 16 h for follicular maturation and estradiol production and, because most cows had not been exposed to an LH surge, estradiol production increased unattenuated (Wiltbank and Pursley, 2014). In response to the LH surge, the original source of elevated estradiol, the preovulatory follicle begins to undergo changes that result in reduced estradiol production, breakdown of the basement membrane, and reinitiation of meiosis in the oocyte before ovulation occurs after a delay of approximately 28 h in the cow (Wiltbank and Pursley, 2014). Additional increases in concentrations of estradiol in plasma resulted in more cows in estrus at AI (Hillegass et al., 2008), which has been linked with improved fertility of cows in TAI programs (Santos et al., 2010). Cows with estradiol concentrations ≥ 2 pg/mL also had an increased presence of uterine fluid at 48 h after PG-3 compared with cows having lesser

estradiol concentrations, perhaps facilitating improved uterine and oviductal transport of sperm (Hawk, 1987).

Pregnancy per AI at 31 d postinsemination was numerically lower in cows receiving the Pre10-72 treatment compared with other treatment-time permutations. We previously reported that P/AI was numerically greater in PG-3-G (40%) cows than in Pre10 (33.3%) cows at d 32 when GnRH was given 56 h after PG (Stevenson et al., 2012). Although consistent in direction, both studies lacked sufficient power to detect differences in P/AI. In the current study, P/AI tended to differ for PG-3-G (56.7%) compared with Pre10 (37.8%) and for 56 h (54.5%) compared with 72 h (38.2%), with P/AI in the Pre10-72 h treatment combination producing less than half (22.2%) the pregnancies compared with all other treatment combinations. Furthermore, in these same cows, post-TAI luteal tissue volume tended to be compromised. In another study investigating the same presynchronization treatments in 4 commercial dairy herds, we reported that P/AI was numerically increased in 1,286 PG-3-G-treated cows (41.2%) compared with 1,247 Pre10 cows (35%) at d 32 to 38 (Stevenson and Pulley, 2012). During the summer, cows presynchronized with PG-3-G had greater P/AI than Pre10 cows, but the results did not differ during non-summer seasons (Stevenson and Pulley, 2012).

Adaptations to the Ovsynch protocol, such as the timing of the final GnRH administration at 56 h after the PG injection to optimize the timing of AI in relation to ovulation, which occurs approximately 24 to 32 h after GnRH, may explain the improved P/AI in the present study as well as in other reports (Pursley et al., 1998; Brusveen et al., 2008; Wiltbank and Pursley, 2014). Cows receiving the combination of Pre10 and GnRH-2 administered at 72 h had increased incidence of spontaneous LH surges before GnRH-2. Insemination near or after ovulation may provide insufficient time for sperm capacitation and transport in the reproductive tract, resulting in aged oocytes before fertilization (Hunter and Wilmut, 1983; Wilmut and Hunter, 1984; Hawk, 1987). A 4-h interval between AI and anticipated ovulation may be insufficient for sperm transport and before postovulatory oocyte aging (Brusveen et al., 2008). Despite these reports, many dairies utilize the Cosynch-72 protocol, in which the final GnRH injection is given concurrently with TAI at 72 h after PG because it eliminates one cow-handling period and facilitates once-daily restraint of cows for administration of hormone injections for TAI (Sterry et al., 2007).

Studies have compared Cosynch to Ovsynch with conflicting P/AI results (DeJarnette and Marshall, 2003; Portaluppi and Stevenson, 2005; Cornwell et al., 2006; Brusveen et al., 2008). Reports by Brusveen et

al. (2008) of increased first-service and repeat-service P/AI in cows receiving Ovsynch-56 compared with Cosynch-72 are consistent with the present study. Brusveen et al. (2008) attributed the differences in P/AI to the timing of the final GnRH injection because time of AI did not differ among treatments. Another study reported no difference in P/AI at 30 d in grazing dairy cows treated with either Double Ovsynch or Presynch-10 when assigned to a 5-d Cosynch with the final GnRH and TAI administered at either 58 or 72 h (Ribeiro et al., 2012). Delayed interval between the second GnRH injection and AI is likely to optimize P/AI in ovulation-synchronization protocols (DeJarnette and Marshall, 2003). The PG-3-G treatment seems to be more flexible in terms of GnRH administration timing, because P/AI did not differ regardless of when GnRH administration occurred (56 or 72 h) in the present study.

In summary, presynchronization using the PG-3-G protocol increased the proportion of cows having LH surges and ovulation incidence after Pre-GnRH. Incidence of LH surges after GnRH-1 did not differ because all cows had an LH surge regardless of treatment; however, Pre10 cows had greater LH peak concentrations and area under the curve for LH compared with PG-3-G in the face of lower progesterone concentrations than was detected in PG-3-G cows. Overall, greater progesterone concentrations reduced the incidence of LH surges and ovulation. At GnRH-2, treatments did not differ in ovulation incidence, incidence of induced LH surges, LH peak concentrations, or area under the LH curve. Although 100% of cows had either spontaneous or GnRH-induced LH surges after GnRH-2, 10% of those cows failed to ovulate even though all but one cow had concentrations of progesterone averaging 0.2 ng/mL. Documenting the cause of this ovulation failure warrants further study. Administration of GnRH-2 at 72 h tended to decrease time to peak LH concentration and increase incidence of spontaneous LH surges compared with GnRH at 56 h. Pregnancy per AI at 31 d postinsemination seemed to be lower in cows receiving the Pre10-72 treatment compared with those receiving Pre10-56, PG-3-G-56, or PG-3-G-72 treatments.

CONCLUSIONS

We conclude that PG-3-G increased progesterone concentrations and the number of CL before GnRH-1 (onset of Ovsynch), thus decreasing LH peak concentrations. Concentrations of LH, incidence of LH surges, and ovulation were suppressed when progesterone was elevated. Delaying GnRH-2 administration to 72 h after PG-3 may increase the incidence of spontaneous LH surges and reduce P/AI in cows receiving Pre10-72

treatment compared with Pre10-56, PG-3-G-56, or PG-3-G-72. The PG-3-G treatment may be more forgiving for the timing of GnRH administration before TAI because P/AI was similar regardless of when GnRH administration occurred (56 or 72 h) in the present study.

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