Pregnancy outcomes are not improved by administering gonadotropin-releasing hormone at initiation of a 5-day CIDR-Cosynch resynchronization protocol for lactating dairy cows

J. A. Spencer,* K. Carnahan,* B. Shafii,† W. J. Price,† J. Dalton,* and A. Ahmadzadeh*¹

*Department of Animal and Veterinary Sciences, and

†Statistical Programs, College of Agricultural and Life Sciences, University of Idaho, Moscow 83844

ABSTRACT

Using a 5-d controlled internal drug-release (CIDR)-Cosynch resynchronization protocol, the objective of this study was to determine the effect of the initial GnRH injection on pregnancy per artificial insemination (P/AI) to the second artificial insemination in lactating Holstein dairy cows. On 37 ± 3 d (mean $\pm$ standard deviation) after the first artificial insemination, and upon nonpregnancy diagnosis (d 0 of the experiment), lactating cows eligible for a second artificial insemination ($n = 429$) were enrolled in a 5-d CIDR-Cosynch protocol. On d 0, all cows received a CIDR insert and were assigned randomly to receive the initial GnRH injection (GnRH; $n = 226$) of the protocol or no-GnRH ($n = 203$). Blood samples were collected from a sub-group of cows ($n = 184$) on d 0 and analyzed for progesterone (P4) concentration. On d 5, CIDR inserts were removed, and all cows received 1 injection of PGF_{2α}. On d 6 and 7, cows were observed once daily by employees for tail-chalk removal, and cows detected in estrus on d 6 or 7 received artificial insemination that day (EDAI), and did not receive the final GnRH injection. The remaining cows not detected in estrus by d 8 received GnRH and timed artificial insemination (TAI). Pregnancy status was confirmed by transrectal palpation of uterine contents at 37 ± 3 d (mean $\pm$ standard deviation) after the second artificial insemination. Eliminating the initial GnRH injection had no effect on P/AI compared with cows receiving GnRH (27 vs. 21%), respectively. Similarly, method of insemination (EDAI vs. TAI) and its interaction with treatment had no effect on P/AI. Primiparous cows had greater P/AI than multiparous cows (31 vs. 21%). Mean P4 concentrations ($n = 184$) at the initiation of the protocol did not differ between treatments (4.51

± 0.35 ng/mL no-GnRH vs. 3.96 ± 0.34 ng/mL of GnRH). When P4 concentrations were categorized as high (≥ 1 ng/mL) or low (< 1 ng/mL), P/AI tended to be greater for high P4 concentrations ($n = 136$) compared with low ($n = 48$) P4 concentrations (26 vs. 16%, respectively). No differences were observed in the proportion of cows with high or low P4 between treatments. Collectively, these results provide evidence that eliminating the initial GnRH in a 5-d CIDR-Cosynch resynchronization protocol for lactating dairy cows did not reduce P/AI in this study.

Key words: gonadotropin-releasing hormone, resynchronization, dairy cow

INTRODUCTION

It is well documented that approximately 60% of dairy cows fail to conceive to the first AI and will require a second or more inseminations (Fricke et al., 2003; Galvão et al., 2007; Bisinotto et al., 2010). Given that detection of estrus is a challenge on many dairies, and pregnancy per AI (P/AI) to second and greater timed-AI (TAI) is less than first AI (Fricke et al., 2003; Chebel et al., 2006; Sterry et al., 2007), the need to develop or modify resynchronization programs in lactating dairy cows is warranted.

Pregnancy to a TAI protocol is greater if cows respond to the initial GnRH with ovulation of a dominant follicle, complete luteolysis by the time of AI, and exhibit synchrony of ovulation at the end of the protocol (Santos et al., 2010; Bisinotto et al., 2014; Stevenson, 2016). Occurrence of anestrus and anovulation, asynchrony of return to estrus after first AI, and even embryonic loss, however, create a milieu of ovarian structures and hormonal profiles that may reduce the desired physiological responses to exogenous hormones in resynchronization protocols (Lopes et al., 2013; Wijma et al., 2016; Ricci et al., 2017). For example, when GnRH-based synchronization protocols are initiated during random stages of the estrous cycle, 10 to 40% of dairy cows fail to synchronize with ovulation because of

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¹Corresponding author: amin@uidaho.edu

variation in ovarian structures at the time of protocol initiation (Pursley et al., 1995; Vasconcelos et al., 1999; Stevenson, 2016).

Failure of ovulation to the first GnRH injection can reduce the success of TAI protocols and attainment of pregnancy (Vasconcelos et al., 1999; Carvalho et al., 2015; Stevenson, 2016). It is plausible that the variation of ovarian structures and hormonal profiles among cows failing to conceive to a previous AI, and subjected to resynchronization, may result in ovulation failure after the initial GnRH injection, and could contribute to the suboptimal results achieved with current resynchronization methods without presynchronization. In fact, Bisinotto et al. (2010) demonstrated only 35% of dairy cows ovulated in response to the initial GnRH when subjected to an Ovsynch or 5-d controlled-internal drug release (CIDR)-Ovsynch resynchronization protocol.

The requirement of the initial GnRH injection has been examined in CIDR-based TAI protocols. Currently, reported results are inconsistent when beef and dairy cattle are subjected to a 5-d CIDR-Cosynch TAI protocol with or without the initial GnRH. The results of several studies provide evidence that inclusion of the initial GnRH injection in dairy heifers (Colazo and Ambrose, 2011; Lima et al., 2011; Howard et al., 2012) and beef heifers (Howard et al., 2009; Cruppe et al., 2014) does not improve P/AI. In contrast, Lima et al. (2013) and Kasimanickam et al. (2014) reported an improvement in P/AI when the initial GnRH was included in a 5-d CIDR-Cosynch for dairy and beef heifers. The focus of the previous 2 studies, however, was to determine the effect of 1 versus 2 PGF_{2α} injections, and not the role of the initial GnRH injection.

Studies in beef cows (Lamb et al., 2006) and dairy cows (Colazo and Ambrose, 2015) have shown no advantage in P/AI following administration of GnRH at CIDR insertion. To our knowledge only one study has examined the effect of the initial GnRH in a 5-d CIDR TAI protocol in lactating dairy cows. Colazo and Ambrose (2015) observed no difference in P/AI with or without the initial GnRH in a 5-d CIDR-Ovsynch resynchronization protocol in lactating dairy cows. Nevertheless, the previously mentioned study (Colazo and Ambrose, 2015) did not specifically investigate the necessity of the initial GnRH; rather, the focus was on the number of PGF_{2α} injections following CIDR removal in lactating dairy cows.

It remains unclear whether use of GnRH at the time of CIDR insertion in a 5-d CIDR-Cosynch protocol is necessary in lactating dairy cows. More importantly, use of GnRH and its effectiveness at the initiation of this type of resynchronization protocol in dairy cows has not been exclusively examined. Therefore, we hypothesized that similar P/AI would be achieved with

or without the initial GnRH injection for dairy cows subjected to a 5-d CIDR-Cosynch resynchronization protocol with one injection of PGF_{2α} following CIDR removal. The specific objective of this study was to determine the effect of the initial GnRH injection in a 5-d CIDR-Cosynch resynchronization protocol on P/AI for the second insemination in lactating Holstein dairy cows.

MATERIALS AND METHODS

All animal handling procedures and treatment protocols were approved by the Animal Care and Use Committee at the University of Idaho. This study was conducted on a commercial dairy farm in Pasco, Washington, from January through July 2014. The dairy farm milked 5,000 Holstein cows, with an average 150-d ECM production of 35 kg/cow per d. All cows were housed in freestall barns, milked twice daily, and a TMR was fed twice daily (morning and evening) with feed pushups every 6 h. Cows also had ad libitum access to water and >20 h access to TMR. The TMR was formulated to meet or exceed the nutritional requirements for high-producing cows (NRC, 2001), and cows were not supplemented with recombinant bST. The voluntary waiting period was 50 d, and estrous cycles were synchronized for first postpartum AI using a Presynch-Cosynch protocol (PGF_{2α} – 14 d – PGF_{2α} – 11 d – GnRH – 7 d – PGF_{2α} – 72 h – GnRH and AI). Cows detected in estrus after the second PGF_{2α} injection of the Presynch protocol, or after the PGF_{2α} injection of the Cosynch protocol, were inseminated and did not complete the Cosynch protocol. The average DIM for first breeding (DIMFB) was 64 d. The average P/AI for first insemination was 26%.

Experimental Protocol and Treatment

Between 35 to 42 d (37 ± 3 d, mean $\pm$ SD) after the first AI, cows were subjected to weekly pregnancy diagnosis by the herd veterinarian by transrectal palpation of uterine contents. After nonpregnant diagnosis for first service, 476 Holstein cows were initially enrolled into this experiment. Forty-seven cows, however, were eliminated from this study for reasons such as sold/died before pregnancy diagnosis after treatment, or because of a discrepancy in farm records. Therefore, 429 cows completed the study and were used for the analysis on the effect of the initial GnRH in a 5-d CIDR-Cosynch resynchronization on P/AI for second insemination.

On d 0 of the experiment (the day of nonpregnancy diagnosis after first insemination), cows were assigned randomly to receive the initial GnRH (GnRH) injection ($n = 223$, i.m., 100 μ g of gonadorelin hydrochloride;

Factrel, Fort Dodge Animal Health, Fort Dodge, IA), or not to receive the initial GnRH ($n = 206$; no-GnRH) injection of a 5-d CIDR-Cosynch protocol. Treatments were evenly distributed by parity through random assignment within pens containing either primiparous or multiparous cows. Simultaneously on d 0, all cows received a CIDR [1.38 g of progesterone (**P4**); Eazi-Breed CIDR, Zoetis, Florham Park, NJ] insert. On d 5, CIDR inserts were removed and a single dose of $\text{PGF}_{2\alpha}$ (500 μg of cloprostenol sodium, i.m.; Estrumate, Merck Animal Health, Intervet Inc., Summit, NJ) was administered to all cows. From d 5 to 8 of the experiment, detection of estrus was conducted through a combination of visual observation and tail paint removal. Cows detected in estrus were inseminated (**EDAI**; d 6 or 7) and not administered the final GnRH injection. The remaining cows not detected in estrus on d 6 or 7 received a GnRH (100 μg) injection on d 8 and TAI regardless of estrous expression on d 8 (Figure 1). Injections were administered i.m. in the gluteus medius muscle between the ilium and ischium bone, using 18-gauge, 3.8-cm needles. Three technicians performed all inseminations. Pregnancy diagnosis was conducted by the herd veterinarian via transrectal palpation of uterine contents between 35 to 42 d (37 ± 3 d, mean $\pm$ SD) after the second synchronization and AI. All cows identified as

not pregnant to second insemination were enrolled in a 7-d CIDR-Cosynch TAI resynchronization protocol for subsequent inseminations.

Blood Samples and Progesterone Quantification

On d 0, blood samples were collected from a subpopulation ($n = 184$) of cows using a 10-mL evacuated serum collection tube (Vacutainer, Covidien LLC, Mansfield, MA). All blood samples were collected via puncture of the medial caudal vein or artery, at the proximal ventral surface of the tail. The tail blood vessel was punctured using a 20-gauge, 3.8-cm single-use blood collection needle. All samples were placed on ice and transported to the University of Idaho laboratory, and stored at 4°C for 18 to 24 h. Samples were centrifuged at 4°C for 20 min at $1,300 \times g$. Serum was harvested and samples were stored at -20°C until assayed for P4 concentrations. Progesterone concentrations were quantified using a single antibody RIA (Coat-a-Count, Siemens Health Care Diagnostic, Los Angeles, CA). The standard curve ranged from 0.1 to 40 ng/mL, and all standards and samples were run in duplicate. The intraassay coefficient of variation was calculated using a known low, medium, and high P4 reference sample and ranged from 5.4 to 10.2%.

Figure 1. Schematic diagram of experimental procedures and treatment administration. Lactating Holstein dairy cows eligible for the second AI were enrolled in a 5-d controlled-internal drug release (CIDR)-Cosynch and assigned randomly to either the no-GnRH treatment ($n = 203$) or treatment with an initial GnRH injection ($n = 226$). The gray box at the bottom of the figure indicates the day of the experiment with d 0 being the initiation of the resynchronization protocol. GnRH = injection of gonadorelin hydrochloride containing 100 μg ; CIDR = controlled internal drug-release containing 1.38 g of progesterone; $\text{PGF}_{2\alpha}$ = injection containing 500 μg of cloprostenol sodium; BS = blood sample for analysis of progesterone concentrations; EDAI = estrus detection and AI without the second GnRH injection; TAI = timed AI.

Table 1. Least squares means $\pm$ SEM for ECM yield, DIM at various end points, parity, and progesterone concentrations at the time of treatment for GnRH (n = 226) and no-GnRH (n = 203) treatments

Variable	Treatment ¹		P-value
	GnRH	no-GnRH	
Milk yield ² (kg)	41 $\pm$ 1	41 $\pm$ 1	0.66
DIM, first breeding (d)	66 $\pm$ 1	66 $\pm$ 1	0.95
DIM, second breeding (d)	112 $\pm$ 1	111 $\pm$ 1	0.87
Parity	2.6 $\pm$ 0.1	2.7 $\pm$ 0.1	0.66
Progesterone ³ (ng/mL)	3.96 $\pm$ 0.34	4.51 $\pm$ 0.35	0.27
Progesterone ⁴ (%)	28 (no. = 27/95)	24 (no. = 21/89)	0.46

¹Treatments at the initiation of a 5-d controlled-internal drug release (CIDR)-Cosynch resynchronization were either administered or not administered the initial GnRH injection.

²150-d ECM yield.

³Mean progesterone concentrations are from a subsample of 184 cows.

⁴Proportion of cows with progesterone <1 ng/mL from a subsample of 184 cows.

Statistical Analysis

As determined by the herd owner, this study had access to a total of 500 cows. With this constraint, power analyses, assuming a binomial response distribution across a presumed range of 21 to 31% P/AI, indicated that approximately 237 animals per treatment would be required to reach a 70% power for detecting a treatment difference of 10% in P/AI at $\alpha = 0.05$. The power analyses were performed using PASS software (Power Analysis and Sample Size; Hsieh et al., 1998).

Analysis of variance was used to compare 150-d ECM, DIMFB, DIM at second breeding (**DIMSB**), parity, and P4 concentrations at initiation of experiment (d 0), and the model included the fixed effect of treatment.

Logistic regression was used to assess treatment effects on P/AI. The model included effects for treatment (GnRH vs. no-GnRH), method of insemination (EDAI = d 6 or 7, TAI = d 8 of experimental protocol), technician (3 technicians), parity (primiparous vs. multiparous), and all 2-way interactions with treatment. The effects of P4 concentrations (d 0) levels were categorized as low (P4 < 1 ng/mL) or high (P4 $\geq$ 1 ng/mL) from a subpopulation of 184 cows on P/AI and analyzed using logistic regression. The model included the effects of P4 category, treatment (GnRH vs. no-GnRH), and their interaction. Finally, the effect of treatment on proportion of cows with low or high P4 concentrations within the same subset of 184 animals was analyzed using a generalized linear model, assuming a binomial distribution with a logit link function. All statistical computations were carried out using SAS version 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

No differences were detected between the GnRH and no-GnRH treated cows for 150-d ECM ($P = 0.66$),

DIMFB ($P = 0.95$), DIMSB ($P = 0.87$), and parity ($P = 0.66$; Table 1). Mean serum P4 concentrations did not differ ($P = 0.27$) between GnRH (n = 95) and no-GnRH treated cows (n = 89; Table 1). The proportion of cows with low P4 concentrations (<1 ng/mL) at enrollment (nonpregnancy diagnosis to first insemination) did not differ ($P = 0.46$) between treatments (28% GnRH vs. 24% no-GnRH; Table 1).

The P/AI to second AI did not differ ($P = 0.54$) between the no-GnRH and GnRH treatments (27 vs. 21%; Figure 2). Pregnancy per AI for cows inseminated either EDAI or TAI did not differ (28 vs. 23%; $P = 0.51$; Figure 2). No interaction between treatment and method of insemination ($P = 0.80$) or parity ($P = 0.81$) was detected. For cows detected in estrus on d 6 or 7, the P/AI for the GnRH (n = 25) and no-GnRH (n = 25) treatments were 28%. In contrast, parity did affect ($P < 0.01$) P/AI, as primiparous cows had greater P/AI than multiparous cows (31 vs. 21%; Figure 2). Technician had an effect on P/AI ($P < 0.05$); however, no treatment by technician interaction occurred ($P = 0.89$).

Pregnancy per AI did not differ between treatment ($P = 0.54$) nor treatment by serum P4 categories (high vs. low; $P = 0.18$). In contrast, P/AI for cows with high P4 (n = 136) at initiation of resynchronization tended ($P = 0.09$) to be greater (26%) than cows with low P4 (16%; n = 48), regardless of treatment.

DISCUSSION

We observed no difference in P/AI between lactating Holstein cows given the initial GnRH or not given the initial GnRH in a 5-d CIDR-Cosynch resynchronization protocol for second insemination. The number of cows used in the current study limited our ability to detect a 6-percentage point difference in P/AI. Nevertheless,

Figure 2. Comparison of pregnancy per AI (P/AI) for cows receiving no-GnRH versus initial GnRH treatment, method of insemination [estrus detection and AI (EDAI) = d 6 or 7 vs. timed AI (TAI) = d 8], and parity (primiparous vs. multiparous). ^{a,b}Bars within a category with different letters differ ($P < 0.05$).

our findings are similar to other studies conducted in dairy heifers (Colazo and Ambrose, 2011; Lima et al., 2011; Howard et al., 2012), reporting that eliminating the initial GnRH injection did not alter P/AI compared with its administration in 5-d CIDR protocol. In fact, our study provides supporting evidence to that of Colazo and Ambrose (2015), who also demonstrated no difference in P/AI with or without the initial GnRH in a 5-d CIDR-Ovsynch resynchronization protocol for lactating dairy cows. Colazo and Ambrose (2015) investigated the effects of the initial GnRH with 2 PGF_{2α} injections (12 h apart) versus no-GnRH and 1 PGF_{2α} injection in a 5-d CIDR-Ovsynch protocol on P/AI for first and subsequent synchronizations. In their study, P/AI for cows subjected to treatments for resynchronization did not differ (Colazo and Ambrose, 2015) even though the number of PGF_{2α} injections differed between treatments. It is possible that cows that do not receive the initial GnRH injection may have a reduced risk of developing new luteal tissue. This in turn may not necessitate the use of 2 PGF_{2α} injections for complete luteolysis by the time of AI; however, this requires further investigation.

Studies have shown that ovulation to the initial GnRH can enhance pregnancy risk (Bello et al., 2006; Chebel et al., 2006; Galvão and Santos, 2010); however, we did not detect a difference in P/AI between cows resynchronized with or without the initial GnRH. This

may be related to the variation in the size of the dominant follicle, as well as P4 milieu among cows during resynchronization resulting in an inadequate response to the initial GnRH with ovulation (Vasconcelos et al., 1999; Bisinotto et al., 2010). Given that return to estrus may occur between d 18 and 24 after AI, and non-pregnancy diagnosis and initiation of resynchronization may occur between 28 to 42 d after first AI, the initial GnRH injection would be given between d 3 and 18 of the estrous cycle (Pursley et al., 1997; Chenault et al., 2003; Bisinotto et al., 2010). Furthermore, 1 in 5 cows will not return to estrus by d 32 after first insemination (Ricci et al., 2017), providing even greater evidence of the variability in ovarian structures and physiological status among cows at the time of resynchronization. Some cows may lack a dominant follicle that is responsive to the initial GnRH, or have a corpus luteum (CL) and elevated P4 concentrations >3 ng/mL that will limit the GnRH-induced LH release and ovulatory response (Giordano et al., 2012; Stevenson and Pulley, 2016; Stevenson, 2016). Other cows may have low P4 concentrations, lack a CL, and respond to the initial GnRH with ovulation, but still have low fertility (Denicol et al., 2012; Bisinotto et al., 2015). Use of presynchronization before resynchronization can optimize the hormonal milieu, and improve the ovulatory response to the initial GnRH of a resynchronization protocol; however, studies have shown that an improvement in

ovulatory response to the initial GnRH does not always improve P/AI (Lopes et al., 2013; Carvalho et al., 2015; Pulley and Stevenson, 2015). In fact, Stevenson (2016) reported that ovulatory response to the initial GnRH injection did not account for significant variation in pregnancy risk, whereas ovulatory response to the final GnRH injection, P4 concentrations preceding PGF_{2α}, and successful luteolysis demonstrated higher predictability on pregnancy outcomes (Wijma et al., 2017, 2018). Thus, the importance of the initial GnRH on improving P/AI in resynchronization protocols for lactating dairy cows is questionable.

In this study, the majority of cows (75.1%) had high P4 concentrations (≥ 1 ng/mL) at the time of nonpregnancy diagnosis and initiation of resynchronization, occurring on average 37 ± 3 d (mean $\pm$ SD) after first AI. These findings are similar to other studies, which have shown greater than 60% of cows have a CL and high P4 (≥ 1 ng/mL) at the time of resynchronization on d 33 (Santos et al., 2016), and d 32 or 39 (Bilby et al., 2013; Lopes et al., 2013) after previous AI. In the present study, a tendency for greater P/AI was detected for cows with $P4 \geq 1$ ng/mL, at the initiation of resynchronization, than for cows with low P4. Likewise, other studies have shown greater P/AI when P4 is ≥ 1 ng/mL at the initiation of resynchronization (Bisinotto et al., 2010; Pulley and Stevenson, 2015; Santos et al., 2016). Mean P4 concentrations at initiation of resynchronization were high in both GnRH and no-GnRH treatments (3.96 ± 0.34 ng/mL and 4.51 ± 0.35 ng/mL, respectively). This may have influenced the ineffectiveness of the initial GnRH injection to induce ovulation. Studies have shown when P4 concentrations are high (Giordano et al., 2012), and a CL is present (Pulley et al., 2015; Stevenson and Pulley, 2016), the negative feedback of P4 will inhibit the LH surge, and subsequent ovulation. Insertion of a CIDR results in immediate increase in blood P4 concentrations (Galvão et al., 2004; Stevenson et al., 2008) that may further reduce the effectiveness of the initial GnRH injection to cause ovulation. Taken together, these factors may have contributed to the lack of difference observed in P/AI between the GnRH and no-GnRH treatments in this study.

A small number of cows (12%) were detected in estrus and inseminated before TAI in this study (d 6 and 7 of the experiment). These findings are similar to other studies in which less than 15% of cows express estrus following CIDR removal and before TAI (Pulley and Stevenson, 2015; Santos et al., 2016). In this study, no differences in P/AI were detected between EDAI or TAI. Studies have shown P/AI to be greater when cows are in estrus at AI in TAI synchronization protocols (El-Zarkouny and Stevenson, 2004; Stevenson and Pul-

ley, 2012); however, estrus at TAI in this study was not recorded. As expected, P/AI were greater in primiparous compared with multiparous cows, consistent with other reports (Stevenson et al., 2007; Bisinotto et al., 2010; Stevenson and Pulley, 2012) that show primiparous dairy cows have greater fertility compared with multiparous cows.

In this study the initial GnRH injection in a 5-d CIDR-Cosynch resynchronization protocol did not have an advantageous effect on P/AI. If the initial GnRH injection, under these circumstances, is not needed, it could potentially save producers \$2.50 to \$3.50 per cow on resynchronization costs (Stevenson, 2012). Further research, however, should be conducted using multiple locations and a larger number of cows to definitively evaluate the necessity of the initial GnRH injection in a 5-d CIDR-Cosynch resynchronization protocol for lactating dairy cows.

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