Effect of an Ovsynch56 protocol initiated at different intervals after insemination with or without a presynchronizing injection of gonadotropin-releasing hormone on fertility in lactating dairy cows

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ABSTRACT

The objectives of this study were to evaluate effects of 2 resynchronization protocols beginning at different intervals after artificial insemination (AI) on the pattern of return to estrus, ovarian responses, and pregnancy per AI (P/AI) to reinsemination. Lactating cows from 2 dairies, located in Texas ($n = 2,233$) and Minnesota ($n = 3,077$), were assigned to 1 of 4 timed AI (TAI) protocols 17 ± 3 d after AI. All cows were examined for pregnancy 31 ± 3 d after previous AI. Cows assigned to early Ovsynch56 (E-OV56) or OV56 received the Ovsynch56 protocol starting 24 or 31 d after AI, respectively. Cows assigned to early GnRH-GnRH-PGF_{2α}-GnRH (E-GGPG) or GGPG received a presynchronizing GnRH injection 17 or 24 d after AI, respectively, 7 d before the start of the Ovsynch56 protocol. Cows observed in estrus after enrollment were inseminated on the same day. Ovaries were examined and blood was sampled for progesterone concentration on the day of first GnRH and PGF_{2α} injection of the Ovsynch56 protocol. Pregnancy was diagnosed at 31 and 66 d after resynchronized AI. On the day of the first GnRH injection of the TAI, a higher percentage of cows on E-GGPG and GGPG protocols had a corpus luteum (E-GGPG = 83.8, GGPG = 91.2, E-OV56 = 80.4, and OV56 = 75.5%) and progesterone concentration >1 ng/mL (E-GGPG = 62.5, GGPG = 76.0, E-OV56 = 53.6, and OV56 = 60.8%) than cows assigned to other protocols. However, the percentage of cows ovulating to the first GnRH injection of TAI was not affected by treatment. Fewer E-GGPG and more OV56 cows were reinseminated in estrus (E-GGPG = 23.7, GGPG = 49.0, E-OV56 = 41.6, and OV56 = 57.6%). Treatment did not affect P/AI at 31 or 66 d for cows

reinseminated in estrus. However, cows reinseminated in estrus had greater P/AI at 31 (40.0 vs. 27.5%) and 66 d (36.0 vs. 23.9%) than cows completing the TAI protocols. Among cows completing the TAI protocols, initiation of GGPG at 24 d after AI increased, whereas initiation of Ovsynch56 at 24 d after AI decreased P/AI at 31 d after reinsemination (E-GGPG = 30.6, GGPG = 28.3.0, E-OV56 = 22.3, and OV56 = 28.7%). Pregnancy per AI did not differ across treatment at 66 d after TAI (E-GGPG = 26.6, GGPG = 24.4, E-OV56 = 20.0, and OV56 = 24.1%). Overall, type of resynchronization protocol and protocol initiation time did not affect P/AI 66 d after reinsemination (E-GGPG = 29.7, GGPG = 30.5, E-OV56 = 26.1, and OV56 = 30.4%). In conclusion, GGPG resynchronization protocols and initiation of resynchronization protocol 24 d after AI reduced the number of cows reinseminated in estrus but neither the timing of initiation of resynchronization nor presynchronization with GnRH affected overall P/AI.

Key words: dairy cow, 2-injection gonadotropin-releasing hormone (GnRH)-prostaglandin 2α -GnRH (GGPG) protocol, resynchronization

INTRODUCTION

Resynchronization of cows with unknown pregnancy status has shown to be inconsistent due to the variability in stage of the estrous cycle at time of pregnancy diagnosis and resynchronization. A common practice for reinsemination of cows of unknown pregnancy status is to synchronize follicle development by initiating the resynchronization protocol 7 d before the nonpregnancy diagnosis with administration of GnRH, and subsequently completing the protocol after nonpregnancy is confirmed (Chebel et al., 2003), which might advance reinsemination of nonpregnant cows. One possible concern with this protocol is that the cows are at random stages of the estrous cycle when presented for resynchronization, which may reduce ovulatory response to the first GnRH injection of the synchronization

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protocol (Vasconcelos et al., 1999), reducing embryo quality (Cerri et al., 2005) and pregnancy per AI (**P/AI**; Chebel et al., 2006). One possible way to alleviate this issue is through presynchronization to increase the number of cows entering the resynchronization protocol between d 5 and 10 of the estrous cycle, which has been shown to be optimal to improve pregnancy rates (Moreira et al., 2000). Silva et al. (2007) showed a significant improvement in P/AI (35.2 vs. 25.6%) and a reduction in embryo loss (7.6 vs. 17.1%) when cows were diagnosed not pregnant and received an injection of PGF_{2α} 12 d before initiation of a resynchronization protocol. Cows presynchronized with PGF_{2α}, however, had a 2-wk delay in reinsemination compared with cows not presynchronized with PGF_{2α}, which is likely to abrogate the improvements in P/AI on 21-d pregnancy rates.

Recently, our group conducted a study to evaluate 3 resynchronization strategies (Dewey et al., 2010). Cows were diagnosed for pregnancy 38 ± 3 d after AI and those diagnosed nonpregnant started the resynchronization protocol on the same day (GnRH injection at nonpregnancy diagnosis, PGF_{2α} injection 7 d later, and timed AI (**TAI**) and GnRH injection 3 d later). However, one-third of the cows received a GnRH injection 1 wk before pregnancy diagnosis (**GGPG**) and another one-third of the cows received a controlled internal drug release (**CIDR**) insert from the day of nonpregnancy diagnosis to the day of PGF_{2α} injection. It was hypothesized that by treating cows with a GnRH injection 7 d before the start of the resynchronization protocol, more cows would ovulate in response to the first GnRH injection of the TAI protocol, and that by treating cows with a CIDR insert during the resynchronization protocol, better synchrony following the TAI protocol would be achieved. In this study, cows receiving GnRH 7 d before the start of the resynchronization protocol were more likely to ovulate in response to the first GnRH injection of the resynchronization protocol [number of corpora lutea (**CL**): control = 0.91, GGPG = 1.19, and CIDR = 0.93] and cows receiving the GnRH injection 7 d before the start of the resynchronization protocol and those receiving a CIDR during the resynchronization protocol had greater P/AI (control = 22.1, GGPG = 31.2, and CIDR = 29.5%; Dewey et al., 2010). These studies indicate that P/AI of resynchronized cows can be improved by presynchronizing cows before the start of the resynchronization protocol. However, this study was conducted with late (38 ± 3 d) nonpregnancy diagnosis only. Although several dairy herds in the United States still diagnosis nonpregnant cows via manual palpation per rectum at later stages of pregnancy (i.e., 35 to 42 d after AI), advancement in ultrasound technologies and use of early pregnancy diagnosis blood

tests are allowing for earlier (31 ± 3 d) nonpregnancy diagnosis to occur, which is gaining in popularity.

The hypotheses of the current experiment were that starting the resynchronization protocol 31 d after AI versus 24 d after AI would increase the percentage of cows reinseminated in estrus, and improve ovarian responses and P/AI of resynchronized cows. Furthermore, we hypothesized that presynchronizing cows with GnRH 7 d before the start of the resynchronization protocol would reduce the percentage of cows reinseminated in estrus, and improve ovarian responses and P/AI of resynchronized cows. The objectives of the current experiment were to compare the effects of administering the GGPG versus the Ovsynch56 resynchronization protocols initiated at different intervals after previous AI on pattern of reinsemination, ovarian responses, and P/AI of lactating cows submitted to an early nonpregnancy diagnosis (31 ± 3 d after previous AI).

MATERIALS AND METHODS

Cows, Housing, and Diet

The experiment was conducted at 2 different dairy farms located in Texas (site 1) and Minnesota (site 2) between February and June 2010. At site 1, primiparous and multiparous Holstein cows were housed in open corrals equipped with metal framework shade at the center of the pen, providing approximately 5 m² of shade per cow and fresh and clean water available ad libitum. At site 2, primiparous and multiparous Jersey cows were housed separately in 4-row freestall cross-ventilated barns (240 headlocks and 260 stalls per pen). Cows were fed the same TMR throughout their lactation and had feed and water ad libitum.

Within each site, cows in all resynchronization protocols had similar diet composition and were fed twice daily as a TMR allowing $\leq 5\%$ feed refusals daily. Diets were formulated to meet or exceed NRC (2001) recommendations for lactating mature cows producing >40 kg/d (site 1) or >35 kg/d (site 2) of 3.5% FCM. In both sites, cows were milked twice daily and yields of milk recorded once per month by the DHIA.

Cows were monitored daily for signs of diseases, in both sites, and if any health issue was observed, cows were moved to intensive care pens and appropriate treatment was performed following dairy protocols. All study procedures involving cows were approved by the Institutional Animal Care and Use Committee of Texas A&M University (College Station) and the University of Minnesota (St. Paul).

Treatments

Treatments consisted of 2 resynchronization protocols (GGPG and Ovsynch56) initiated at different

Figure 1. Diagram of study activities. OV56 = Ovsynch56 (GnRH injection, PGF_{2α} injection 7 d later, GnRH injection 56 h later, and TAI 16 h later) starting at 31 d after previous AI; E-OV56 = early OV56 (Ovsynch56 starting at 24 d after previous AI); GGPG = presynchronizing injection of GnRH at 24 d after previous AI followed 7 d later by the Ovsynch56 protocol; E-GGPG = early GGPG (presynchronizing injection of GnRH at 31 d after previous AI followed by the Ovsynch56 protocol 7 d later); NON = nonpregnancy diagnosis; GnRH = 100 µg of gonadorelin sterile solution (Cystorelin, 50 µg/mL; Merial Animal Health, Summit, NJ); PGF = 500 µg of prostaglandin (Estrumate, 250 µg/mL of cloprostenol sodium; Merck Animal Health, De Soto, KS); TAI = timed AI; solid black circles = blood sample and ultrasound scan.

times after AI. To be eligible for study enrollment, cows had to have at least 1 AI between 14 and 20 d (17 ± 3 d) before the enrollment date. Cows fulfilling the enrollment criterion were then blocked by parity, number of AI, DIM, and predicted 305-d milk production and were randomly assigned to 1 of 4 resynchronization protocols (Figure 1). Cows assigned to the early GGPG protocol (**E-GGPG**) received a presynchronizing 100-µg GnRH injection (Cystorelin; 50 µg/mL of gonadorelin diacetate tetrahydrate; Merial Animal Health, Duluth, GA) at the time of study enrollment (17 ± 3 d after previous AI) followed by the Ovsynch56 protocol [100 µg of GnRH, followed 7 d later by 500 µg of PGF_{2α} (Estrumate; 250 µg/mL of cloprostenol sodium; Merck Animal Health, De Soto, KS), followed 56 h later by GnRH, and followed 16 h later by TAI] 7 d later, with cows inseminated at fixed time 3 d after nonpregnancy diagnosis. Cows assigned to the GGPG protocol received a presynchronizing GnRH injection 7 d after study enrollment (24 ± 3 d after previous AI) and began the Ovsynch56 protocol 7 d later at the time of nonpregnancy diagnosis (31 ± 3 d after previous AI). Cows assigned to the early Ovsynch56 (**E-OV56**) started the resynchronization protocol Ovsynch56 1 wk after enrollment (24 ± 3 d after previous AI). Finally, cows assigned to the Ovsynch56 protocol (**OV56**) started the Ovsynch56 protocol at nonpregnancy diagnosis (31 ± 3 d after previous AI). Cows were allowed

to be artificially inseminated on detected estrus at any time during the resynchronization protocol. In both sites, estrus detection was performed daily based on the removal of tail chalking (Macmillan et al., 1988) using paint sticks (All-Weather Paintstik, livestock marker; LA-CO Industries Inc., Elk Grove Village, IL) and AI were performed at the same morning as estrus was detected by experienced technicians (Figure 1).

BCS, Ovarian Structures, Blood Collection, and Pregnancy Diagnosis

All cows were scored for body condition using a 5-point scale according to Ferguson et al. (1994) at nonpregnancy diagnosis (2 wk after study enrollment). A subsample of 444 cows (251 multiparous and 193 primiparous) from both sites had their ovaries examined by ultrasonography on the day of the first GnRH injection and again at PGF_{2α} injection of the TAI protocol to determine the response to protocol injections. A real-time ultrasound scanner (Ibex Pro; E. I. Medical Imaging, Loveland, CO) equipped with 7.5-Mhz rectal linear probe was used for nonpregnancy diagnosis. Ovulation to the first GnRH injection of the TAI protocol was characterized by the presence of at least 1 follicle larger than 10 mm at the time of first GnRH injection of the protocol, which was replaced by a corpus luteum (**CL**) at the time of PGF_{2α} injection. Blood samples (10 mL)

were also collected at the same time points from the same subsample of cows by puncture of the coccygeal vein or artery into evacuated tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Immediately after collection, samples were placed on ice and processed at the laboratory within 4 h of collection. Blood tubes were centrifuged at $2,000 \times g$ for 15 min for plasma separation. Plasma was frozen at -25°C and later analyzed for progesterone (P_4) by a solid-phase RIA using a commercial kit (Coat-A-Count; Diagnostic Products Corp., Los Angeles, CA). The assay sensitivity was 0.1 ng/mL and the intra- and interassay coefficients of variation were 5.4 and 7.9%, respectively.

Pregnancy status was diagnosed at 31 ± 3 d after AI by transrectal ultrasonography of the reproductive tract. Observation of embryonic fluid, embryo, and embryonic heartbeat were used as determinants of pregnancy. Pregnancies were reconfirmed at 66 ± 3 d after AI by transrectal palpation of the reproductive tract. Cows diagnosed pregnant at 31 ± 3 d but not pregnant at 66 ± 3 d after AI were considered to have had embryo/fetal loss. Pregnancy rate was defined as the percentage of eligible cows that become pregnant within each week of the experiment. It was calculated on a weekly basis from the study enrollment until the week of fixed-time AI in each treatment.

Statistical Analyses

The experimental design was a randomized complete block design (Kuehl, 1994) in which cows were blocked according to parity, number of AI, DIM, and predicted 305-d milk production. Within each block, cows were randomly assigned weekly to 1 of 4 resynchronization protocols with a 2×2 factorial arrangement, with resynchronization protocol (Ovsynch56 vs. GGPG) and time of initiation of the resynchronization protocol (24 vs. 31 d after AI) as the main factors. A sample size calculation was performed (Minitab Inc., 2000) to estimate the number of experimental units required to detect 7% units difference in P/AI with 95% confidence ($\alpha = 0.05$) and 80% protection against a type II error ($\beta = 0.20$), giving that P/AI ranges from 30 to 40%. Furthermore, the number of experimental units provided enough pregnant cows at 66 ± 3 d after AI to detect statistical significance with a 8% unit difference in pregnancy loss among treatments ($\alpha = 0.05$; $\beta = 0.20$).

Plasma P_4 concentration, a normal distributed data, was transformed into 2 categories (high or low concentration) following a binomial distribution. A concentration higher than 1 ng/mL was defined as high and a concentration less than or equal to 1 ng/mL was defined as low plasma P_4 concentration.

Binomially distributed data such as reinsemination during TAI protocol, P/AI, presence of CL at the first GnRH injection of the TAI protocol, P_4 levels (high or low) at the first GnRH injection of the TAI protocol, and ovulation to the first GnRH injection of the TAI protocol were analyzed by logistic regression using PROC LOGISTIC of SAS (SAS 9.2; SAS Institute, 2008). A backward stepwise regression model was used (Allison, 1999), and the full model included the effects of resynchronization protocol, protocol initiation time, site, parity, BCS, milk yield on previous lactation, number of inseminations at enrollment, and the interactions between treatment and the respective explanatory variables. Variables were continuously removed from the model by the Wald statistic criterion if the significance was at $P > 0.20$. The Akaike information criterion was used to define the best model to be used for each dependent variable analysis. Both protocol and site were forced to remain in each model even if backward elimination suggested to be removed.

The interval between study enrollment and reinsemination was analyzed by the nonparametric method Kruskal-Wallis rank test to identify differences across resynchronization protocols initiated at different times. Median days between study enrollment and subsequent AI was also analyzed by survival analysis using the product limit method of the Kaplan-Meier model (Kaplan and Meier, 1958) by the LIFETEST procedure to illustrate these interval differences across resynchronization programs initiated at different times. Differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were designated as a tendency to differ.

RESULTS

A total of 2,934 lactating dairy cows fulfilled the enrollment criterion (1,368 cows from Texas and 1,606 from Minnesota sites) and were enrolled in the study. The average milk yield at study enrollment was not different across treatments (E-GGPG = 32.3 ± 0.33 , GGPG = 32.1 ± 0.35 , E-OV56 = 32.1 ± 0.36 , and OV56 = 31.7 ± 0.29 kg/d). The BCS at time of non-pregnancy diagnosis did not differ among treatments (E-GGPG = 3.00, GGPG = 2.75, E-OV56 = 3.00, and OV56 = 3.00) and within each treatment, the median BCS for multiparous and primiparous cows were 2.75 and 3.00, respectively.

Estrus Detection and Pattern of Reinsemination

In total, 44.1% of all cows were observed in estrus and inseminated before completing the TAI protocol. Cows assigned to the GGPG protocols were less likely ($P < 0.001$) to be reinseminated in estrus during the

resynchronization protocol than cows assigned to the Ovsynch56 protocols, regardless of protocol's initiation time [GGPG = 37.1% and Ovsynch56 = 50.6%; adjusted odds ratio (AOR) = 0.58; 95% CI = 0.50 to 0.67]. Within each resynchronization protocol (Ovsynch56 and GGPG), cows initiated the resynchronization protocol at 24 d after AI (E-OV56 and E-GGPG) and were less likely ($P < 0.001$) to be identified in estrus before the completion of the TAI protocol (32.7 vs. 53.6%; AOR = 0.42; 95% CI = 0.36 to 0.49) than cows assigned to initiate protocol at 31 d after AI (Figure 2). An interaction between resynchronization protocol and time of protocol initiation was detected in which the resynchronization protocol initiated at 24 d after AI resulted in a more pronounced decrease ($P < 0.01$) in the percentage of reinseminations in estrus during the resynchronization protocol than cows submitted to the Ovsynch56 protocol (Figure 2). The median day interval between study enrollment and subsequent AI was 17 d for cows assigned to the E-GGPG and E-OV56 protocols, whereas the GGPG and OV56 were 21 and 12 d, respectively (Figure 3).

Protocol Completion and Ovarian Responses to Protocol

Among all cows enrolled in this study, 55.9% completed their respective resynchronization protocols and were inseminated at fixed time. For these cows, the average milk yield at study enrollment was not different ($P = 0.45$) across treatments (OV56 = 31.7 ± 0.46 , E-OV56 = 32.4 ± 0.49 , GGPG = 32.7 ± 0.48 , and E-GGPG = 32.1 ± 0.42 kg/d) and the median BCS did not differ among treatments (E-GGPG = 3.00, GGPG

= 2.75, E-OV56 = 3.00, and OV56 = 2.75) and within each treatment, the median BCS for multiparous and primiparous cows were 2.75 and 3.00, respectively.

On the day of the first GnRH injection (i.e., 7 d before PGF_{2α} injection) of the resynchronization protocol (d 24 for E-OV56 and E-GGPG; d 31 for OV56 and GGPG), the percentage of cows that had a CL was greater ($P < 0.01$) for cows receiving the GGPG compared with cows assigned to the Ovsynch56 protocols, regardless of initiation time (GGPG = 87.4 and Ovsynch56 = 77.9%, respectively). The percentage of cows with CL on the day of the first GnRH injection of the resynchronization protocol did not differ ($P = 0.40$) between protocols initiated at 24 or 31 d after previous AI (82.0 and 84.1%, respectively). However, an interaction between resynchronization protocol and its initiation time revealed an increased percentage of cows with CL at the time of the first GnRH injection for the Ovsynch56 protocol compared with GGPG when protocols were initiated 24 d after AI.

On the day of the first GnRH injection of the TAI protocol, 64.0% of the cows had P₄ concentrations >1 ng/mL. The percentage of cows with plasma P₄ concentrations >1 ng/mL was greater ($P < 0.01$) for cows assigned to the GGPG protocols compared with cows receiving the Ovsynch56 protocols, regardless of initiation time (GGPG = 69.4 vs. Ovsynch56 = 57.3%). Protocols initiated at 24 d after AI resulted in a decreased ($P = 0.02$) percentage of cows with plasma P₄ concentrations >1 ng/mL at the time of the first GnRH injection of the TAI protocol compared with cows that initiated the resynchronization protocol at 31 d after AI (58.8 and 69.2%, respectively).

At the time of the PGF_{2α} injection, the percentage of cows with plasma P₄ concentration >1 ng/mL and the percentage of cows with at least 1 visible CL was not affected by type of resynchronization method or its initiation time (Table 1). The percentage of cows that ovulated in response to the first GnRH injection of the resynchronization protocol was not different among resynchronization protocol or protocol initiation time (Table 1).

P/AI and Pregnancy Loss

Cows inseminated on detected estrus had increased ($P < 0.01$) P/AI at 31 and 66 d after AI than cows receiving TAI (Figure 4). Risk of pregnancy at 31 and 66 d after reinsemination was not affected by the resynchronization protocol or its initiation time for cows reinseminated on detected estrus ($P > 0.21$). However, for cows receiving TAI, an interaction between resynchronization protocol and protocol initiation time was detected ($P = 0.05$), with risk of pregnancy at 31

Figure 2. Percentage of cows reinseminated on detected estrus. OV56 = Ovsynch56 (GnRH injection, PGF_{2α} injection 7 d later, GnRH injection 56 h later, and TAI 16 h later) starting at 31 d after previous AI; E-OV56 = early OV56 (Ovsynch56 starting at 24 d after previous AI); GGPG = presynchronizing injection of GnRH at 24 d after previous AI followed 7 d later by the Ovsynch56 protocol; E-GGPG = early GGPG (presynchronizing injection of GnRH at 31 d after previous AI followed by the Ovsynch56 protocol 7 d later). Different letters (a–c) indicate statistical differences of $P < 0.01$.

Figure 3. Kaplan-Meier survival analysis illustrating the effect of resynchronization programs on the median day interval between study enrollment and subsequent AI for cows submitted to 2 resynchronization programs starting at 2 different initiation times (E-GGPG = 17, E-OV56 = 17, GGPG = 21, and OV56 = 12 d; $P < 0.001$). OV56 = Ovsynch56 (GnRH injection, PGF_{2α} injection 7 d later, GnRH injection 56 h later, and TAI 16 h later) starting at 31 d after previous AI; E-OV56 = early OV56 (Ovsynch56 starting at 24 d after previous AI); GGPG = presynchronizing injection of GnRH at 24 d after previous AI followed 7 d later by the Ovsynch56 protocol; E-GGPG = early GGPG (presynchronizing injection of GnRH at 31 d after previous AI followed by the Ovsynch56 protocol 7 d later).

d after AI being increased with the GGPG protocol and decreased with the Ovsynch56 protocol when resynchronization was initiated at 24 d after AI (Table 2). When pregnancy was assessed again 66 d after reinsemination, the risk of pregnancy was not affected by the resynchronization protocol, protocol initiation time, or its interaction (Table 2). When data from cows reinseminated on detected estrus and at fixed time was combined (overall P/AI), a tendency for increased P/AI at 31 d after reinsemination was observed for protocols initiated at 24 d after AI ($P = 0.07$) but no longer observed ($P = 0.11$) when pregnancy was assessed at 66 d after reinsemination (Table 2).

The risk of pregnancy loss between d 31 and 66 of gestation did not differ between treatments. Pregnancy loss was not influenced by type of protocol (Ovsynch56 or GGPG) or protocol initiation time (24 or 31 d after AI). However, cows reinseminated on detected estrus had a tendency to have fewer pregnancy losses between 31 and 66 d of gestation (AI upon estrus detection = 8.3 vs. TAI = 11.8%; AOR = 0.68; 95% CI = 0.43 to 1.05; $P = 0.08$).

Cows enrolled in the E-GGPG treatment had decreased pregnancy rate from 0 to 7 d after enrollment compared with the other resynchronization protocols,

whereas the OV56 protocol had increased pregnancy rate from 8 to 14 d after enrollment than other protocols. From 15 to 21 d after enrollment, cows in the E-GGPG treatment had increased pregnancy rate and cows in the OV56 and GGPG treatments had decreased pregnancy rate compared with cows in the E-OV56 treatment. Pregnancy rate did not differ between OV56 and GGPG treatments from 22 to 28 d after enrollment (Table 2).

DISCUSSION

Hormonal manipulation of the estrous cycle using GnRH and PGF_{2α} has been widely implemented as resynchronization strategies to decrease the interval between AI in cows that have failed to conceive to a previous AI. Among cows not conceiving, less than 50% display estrus and are reinseminated before nonpregnancy diagnosis (Chebel et al., 2006; Cerri et al., 2009) and few display estrus after receiving the first injection of the resynchronization protocol (Dewey et al., 2010). In this study, 44.1% of all cows were observed in estrus between 17 d after previous AI and the completion of the TAI protocol. Treatment of cows with a presynchronizing GnRH and early initiation of the resynchronization protocol decreased expression of estrus. This decrease

Figure 4. Pregnancy per AI at 31 and 66 d after reinsemination ($P < 0.01$) in cows reinseminated upon estrus detection (black bars) or timed AI (white bars), regardless of resynchronization protocol or its initiation time.

in estrus expression during TAI protocols incorporating a presynchronizing GnRH injection was expected and agrees with previous findings from our laboratory (Bruno et al., 2012; Mendonça et al., 2012; Chebel et al., 2013), with one of the consequences being an increased percentage of cows receiving TAI. Lopes et al. (2013), evaluating similar protocols but initiated at 32 or 39 ± 3 d after AI, identified similar estrus return patterns as observed in the current study when resynchronization with GGPG or Ovsynch56 protocols were compared. However, in the Lopes et al. (2013) study, only 22% of AI was performed upon estrus detection compared with 44.1% observed within the current study. The difference in the number of cows detected in estrus can be attributed to a variety of possible management differences among sites, such as protocol initiation time, type of estrus detection, flooring, level of milk production, and breed, as the proportion of cows AI upon estrus detection for the current study was similar between the study sites. In addition, Giordano et al. (2012) also conducted a study using the GGPG protocol compared with a control with no presynchronization injection or the use of human chorionic gonadotropin 1 wk before the start of the synchronization program. Within the Giordano et al. (2012) study, no difference was observed in fertility between the control (24.0% P/AI) and GGPG (28.5% P/AI) groups but there was an improvement when using human chorionic gonadotropin (31.3% P/AI) to improve fertility. However, estrus detection was not evaluated within their study, which would change the type of population of cows receiving the TAI protocol (i.e., more concentrated population of anovular, cystic, and anestrus cows) and provides an explanation for the difference in results compared with the current study.

The inhibitory effect on estrus expression exerted by GnRH might be associated with the fact that giving an

Table 1. Ovarian responses for 2 resynchronization protocols beginning at different intervals after AI¹

Item ² (%)	Ovsynch56			GGPG		P-value		
	OV56	E-OV56		GGPG	E-GGPG	Protocol	Time	Protocol × time
Day of GnRH injection of TAI								
Presence of CL [% (no./total no.)]								
P ₄ concentration >1 ng/mL [% (no./total no.)]	75.5 (77/102)	80.4 (78/97)		91.2 (114/125)	83.3 (100/120)	<0.01	0.40	0.05
Day of PGF _{2α} injection of TAI	60.8 (62/102)	53.6 (52/97)		76.0 (95/125)	62.5 (75/120)	<0.01	0.02	0.38
Presence of CL [% (no./total no.)]								
P ₄ concentration >1 ng/mL [% (no./total no.)]	93.1 (95/102)	92.8 (90/97)		100.0 (125/125)	90.0 (108/120)	0.94	0.94	0.94
Ovulation to GnRH injection	71.6 (73/102)	77.3 (75/97)		80.8 (101/125)	83.3 (100/120)	0.06	0.30	0.78
	57.8 (59/102)	57.7 (56/97)		61.6 (77/125)	61.7 (74/120)	0.41	0.99	0.98

¹OV56 = Ovsynch56 (GnRH injection, PGF_{2α} injection 7 d later, GnRH injection 56 h later, and TAI 16 h later) starting at 31 d after previous AI; E-OV56 = early OV56 (Ovsynch56 starting at 24 d after previous AI); GGPG = presynchronizing injection of GnRH at 24 d after previous AI followed 7 d later by the Ovsynch56 protocol; E-GGPG = early GGPG (presynchronizing injection of GnRH at 31 d after previous AI followed by the Ovsynch56 protocol 7 d later).

²TAI = timed AI; CL = corpus luteum; P₄ = plasma progesterone.

Table 2. Pregnancy per AI in cows inseminated on detected estrus and at fixed time¹

Item ² [% (no./total no.)]	Ovsynch56		GGPG		P-value		
	OV56	E-OV56	GGPG	E-GGPG	Protocol	Time	Protocol × time
31 d after AI							
ED	39.0 (191/490)	39.3 (105/266)	40.5 (150/370)	42.9 (65/153)	0.38	0.65	0.73
TAI	28.7 (103/359)	22.3 (86/386)	28.3 (108/382)	30.6 (151/493)	0.07	0.27	0.05
Overall	34.6 (294/849)	29.3 (191/652)	34.3 (258/752)	33.3 (216/646)	0.28	0.07	0.20
66 d after AI							
ED	35.1 (170/485)	34.5 (92/264)	36.6 (133/363)	39.5 (60/152)	0.22	0.63	0.51
TAI	24.1 (86/357)	20.0 (77/384)	24.4 (92/376)	26.6 (131/492)	0.11	0.53	0.16
Overall	30.4 (256/842)	26.1 (169/648)	30.5 (225/739)	29.7 (191/644)	0.30	0.11	0.30
Pregnancy loss ³	10.8 (31/287)	9.8 (18/187)	8.1 (20/245)	11.0 (23/214)	0.67	0.60	0.32
Pregnancy rate ⁴							
0 to 7 d after enrollment	12.7 (107/842) ^a	11.7 (76/648) ^a	14.6 (108/739) ^a	6.5 (42/644) ^b	—	—	<0.01
8 to 14 d after enrollment	9.4 (50/533) ^a	3.5 (15/426) ^b	3.9 (18/456) ^b	3.4 (18/527) ^b	—	—	<0.01
15 to 21 d after enrollment	3.0 (12/396) ^c	19.8 (78/393) ^b	2.5 (10/400) ^c	27.0 (131/485) ^a	—	—	<0.01
22 to 28 d after enrollment	24.1 (87/361)	—	25.2 (89/353)	—	—	—	0.73

^{a-c}Values within a row with different superscripts differ ($P < 0.10$).

¹OV56 = Ovsynch56 (GnRH injection, PGF_{2α} injection 7 d later, GnRH injection 56 h later, and TAI 16 h later) starting at 31 d after previous AI; E-OV56 = early OV56 (Ovsynch56 starting at 24 d after previous AI); GGPG = presynchronizing injection of GnRH at 24 d after previous AI followed 7 d later by the Ovsynch56 protocol; E-GGPG = early GGPG (presynchronizing injection of GnRH at 31 d after previous AI followed by the Ovsynch56 protocol 7 d later).

²ED = estrus detection; TAI = timed AI.

³Pregnancy loss between 31 and 66 d after AI.

⁴Pregnancy rate at 66 d after AI.

injection of 100 µg of GnRH 7 d before the resynchronization protocol initiation induced ovulation of follicles ≥ 10 mm in diameter, resulting in the formation of a new CL (Ryan et al. 1991). Moreover, GnRH can directly affect follicle development by downregulating the expression of aromatase in granulosa cells, reducing the secretion of estradiol by the dominant follicle (Fitzpatrick et al., 1997), thereby inhibiting estrus expression.

It is also important to mention that the rate at which cows were reinseminated was not only affected by the presynchronizing GnRH injection but it was also affected by the time of protocol initiation, with a remarkable decreased estrus expression among cows receiving the presynchronizing GnRH at 17 d after AI (E-GGPG) and increased estrus expression in cows starting the TAI protocol without presynchronization and at 31 d after AI (OV56). The plausible explanation for the negative effect on estrus expression may be due to the fact that cows submitted to the E-GGPG protocol received 2 injections of GnRH before the nonpregnancy diagnosis (i.e., 17 and 24 d post-AI), whereas cows submitted to the OV56 did not receive any presynchronization and received TAI 10 d after nonpregnancy diagnosis, increasing the period for cows to be observed in estrus.

The increased percentage of cows with CL and P₄ concentrations >1 ng/mL at the initiation of the resynchronization protocols also indicates a possible increase in ovulation in response to the presynchronizing GnRH injection compared with those protocols without pre-

synchronization. These findings are in agreement with other studies in which the effect of the presynchronizing GnRH injection on ovarian structures was evaluated (Dewey et al., 2010; Alkar et al., 2011; Bruno et al., 2012) and have been responsible for improvements in fertility. The effect of P₄ on the follicle, embryo, and fetus have been demonstrated by others (Mann and Lamm, 2001; Mann et al., 2003), with increased fertility being reported when elevated concentrations of P₄ were observed during the estrous cycle preceding AI (Folman et al., 1973). Most recently, Denicol et al. (2012) demonstrated an increased likelihood for pregnancy when a synchronized follicular wave developed under high P₄ concentrations. This improvement in fertility, due to elevated concentrations of P₄, has been associated with improvements in oocyte (Pfeifer et al., 2009) and embryo quality (Rivera et al., 2011).

Progesterone concentration at the time of the PGF_{2α} injection of the resynchronization protocol was not different among treatments, which may be a consequence of the lack of difference in the percentage of cows that ovulated in response to the first GnRH injection of the resynchronization protocol. This finding was unexpected and differs from other experiments (Dewey et al., 2010; Giordano et al., 2012; Lopes et al. 2013). We speculate that the divergence in outcomes within these studies could be due to the timing of resynchronization initiation. Resynchronization protocols in the Dewey et al. (2010) study started on d 39 and within the Lopes

et al. (2013) study, the protocols began on d 32 or 39 after previous AI. However, within the current study, resynchronization protocols began at either 24 or 31 d after previous AI, which could place cows at different stages of the follicular wave at the time of the GnRH injection. Cows reinseminated on detected estrus had increased P/AI compared with cows reinseminated at fixed time, regardless of resynchronization treatment. Pregnancy per AI did not differ among treatments when all reinseminations were analyzed together. This increase in fertility of cows reinseminated on detected estrus has been reported by others (Kasimanickam et al., 2005; Bruno et al., 2012) and is likely to be the result of efficient and accurate estrus detection.

A tendency ($P = 0.07$) for increased P/AI at 31 d after AI was observed for cows receiving the GGPG compared with cows receiving the Ovsynch56 protocol. Moreover, an interaction between type of resynchronization protocol and its initiation time was identified ($P = 0.05$) in which initiation of the Ovsynch56 protocol at 24 d after AI decreased, whereas initiation of the GGPG resynchronization protocol at 24 d after AI increased P/AI at 31 d after AI compared with cows initiating the protocols at 31 d after AI. However, no difference was observed at 66 d after reinsemination. Different than our results, Dewey et al. (2010) reported an improvement in P/AI when a GnRH injection was administered 7 d before the initiation of the TAI and related this improvement in P/AI to the increased percentage of cows ovulating to the first GnRH injection of the TAI protocol, which was not observed in the current study. Similar to our results, Alkar et al. (2011) also reported no improvement in fertility when a presynchronized GnRH injection was administered 7 d before the TAI protocol initiation. The reasons are still unclear for these differences in responses in fertility to protocols in which a GnRH injection was used for presynchronization. This variation in responses to the presynchronizing GnRH injection may be associated with different stages of the follicular wave at the time when a presynchronizing GnRH injection is administered, as the interval between previous AI and GnRH injection for all 3 studies was different. Also, different from other studies, within the current study, cows were allowed to be artificially inseminated within the protocol when signs of estrus were observed, leading to a large percentage of cows submitted to TAI being subfertile cows, which can affect the outcome results.

CONCLUSIONS

Pregnancy per AI of dairy cows subjected to Ovsynch56 or GGPG resynchronization programs was not affected by type of protocol or by intervals

between previous AI and protocol initiation time. However, the percentage of cows AI on detected estrus was reduced when the resynchronization protocol started earlier and also when a presynchronizing GnRH injection was administered 7 d before the resynchronization protocol initiation. Furthermore, cows reinseminated on detected estrus had increased P/AI compared with cows submitted to TAI. Dairy farms with good estrus detection should prioritize resynchronization programs that allow more cows to be reinseminated on detected estrus, as higher fertility and reduced cost and labor are associated with these programs, thereby reducing the interval between AI.

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