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Effect of gonadorelin dose and an additional gonadorelin treatment 2 days after the initiation of Resynch-25 on ovarian dynamics and fertility of lactating Holstein cows

Iago M. R. Leão,[1,2] Carlos E. C. Consentini,[3] Marcelo S. El Azzi,[1] Everaldo Anta-Galván,[1] Teresita Valdés-Arciniega,[1,2] Lucas Oliveira e Silva,[3] Roberto Sartori,[3] and João Paulo N. Martins[1]*
[1]Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, WI 53706
[2]Department of Animal & Dairy Sciences, University of Wisconsin–Madison, Madison, WI 53706
[3]Department of Animal Sciences, Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba, SP, Brazil 13418-900

ABSTRACT

Our objective was to improve ovulatory response at the initiation of the Resynch-25 protocol by (1) increasing the dose of GnRH from 100 µg to 200 µg; and (2) giving a second GnRH treatment 56 h after the initiation of the protocol. We considered the experimental d 0 the day of the previous service. The experiment consisted of a 2 × 2 factorial design to compare the main effects of GnRH dose (100 vs. 200 µg) and GnRH treatment times (once vs. twice 56 h apart). A total of 2,111 previous services in 1,438 Holstein lactating cows were used. On d 25, cows were assigned to receive either 100 or 200 µg of GnRH only on d 25 or on d 25 and 56 h later (d 27). On d 32, cows diagnosed as nonpregnant (n = 1,076 services) were classified as with or without a corpus luteum (CL). Nonpregnant cows with a CL continued the Resynch-25 protocol receiving $PGF_{2\alpha}$ treatments on d 32 and 33, followed by a GnRH 32 h later and timed AI 16 h after the last GnRH. Blood samples were collected in a subset of cows on d 25, 32, and 34 to assess serum P4 concentrations. In the same subset of cows, transrectal ultrasonographic examinations were performed on d 25, 29, 34 and 36 to assess ovarian parameters and ovulatory response to the GnRH treatments. The overall ovulatory response at the initiation of the protocol, defined as the ovulation between d 25 and 29, was not affected by days of GnRH treatment and averaged 41.9%. On the other hand, nonpregnant cows treated with the higher GnRH dose had a greater ovulatory response at the initiation of the protocol compared with cows treated with the lower dose (48.0% vs. 36.1%). Despite the increase in ovulatory response at the initiation of the protocol, the GnRH dose did not affect fertility of cows submitted to Resynch-25. Furthermore, the second GnRH treatment on d 27 tended to decrease pregnancy per AI on d 32 after AI (39.0% vs. 43.9%), but no effect of days of GnRH treatment was observed in the subsequent pregnancy diagnosis. The absence of a functional CL on d 25 and ovulation at the initiation of the protocol were positively associated with improved fertility. However, the improvement in fertility of cows ovulating at the initiation of the protocol occurred only in cows with a functional CL on d 25. In summary, despite increasing ovulatory response at the initiation of the protocol, the higher dose did not improve fertility. The extra GnRH on d 27 did not increase ovulatory response at the initiation of the protocol and tended to decrease P/AI 32 d after AI of the Resynch-25. In addition, no additive effect of the higher dose and extra GnRH treatment was observed. Despite the lack of overall treatment effect, the data presented in this study suggest that the identification of CL functionality on d 25 may help to optimize the resynchronization strategy used at nonpregnancy diagnosis to potentially increase fertility of cows reinseminated.

Key words: GnRH, resynchronization, timed AI, ovulation

INTRODUCTION

Reducing the interval between inseminations (**IBI**) is one of the most important goals for increasing reproductive efficiency in dairy farms (Overton and Cabrera, 2017). Accurately and effectively identifying nonpregnant cows after previous AI in estrus and reinseminating them at the right time is probably the most efficient way to reduce IBI. Still, some nonpregnant cows will have atypical cycles (Cunha et al., 2022) or will not be detected in estrus before the nonpregnant diagnosis (**NPD**; Ricci et al., 2017) and need to be resynchronized to receive timed AI (**TAI**) in a timely manner. For these

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*Corresponding author: jp.martins@wisc.edu

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situations, programs for resynchronization of ovulation were developed aiming to reduce the IBI and improve reproductive performance of nonpregnant cows (Giordano et al., 2012b; Galvão et al., 2013; Lopes et al., 2013; Wijma et al., 2017). Among these protocols, one of the most used in US dairy farms comprises the initiation of the Ovsynch protocol 25 d after the previous AI, commonly known as Resynch-25 (Silva et al., 2009).

Even though TAI protocols have successfully reduced IBI, the reproductive performance of first service is usually better than the one of subsequent services (DeJarnette et al., 2011). Some factors potentially explain this reduced fertility. First, the proportion of subfertile cows seems to increase along with the service number, decreasing pregnancy per AI as service number increases (Parkinson, 2009). In addition, most of the protocols used for resynchronization of ovulation promote lower synchrony compared with protocols that are used for first service (fertility programs; Souza et al., 2008; Giordano et al., 2012b; Wijma et al., 2018). This occurs because first service protocols are usually composed of a pre-synchronization and a breeding-Ovsynch (Bello et al., 2006; Galvão et al., 2007; Souza et al., 2008), whereas protocols for resynchronization of ovulation usually consist only of a breeding-Ovsynch (Sauls-Hiesterman et al., 2020). Most pre-synchronization strategies used for first service involve the administration of $PGF_{2\alpha}$ and therefore cannot be used before NPD. In addition, implementing a presynchronization strategy for resynchronizing nonpregnant cows may substantially increase IBI. Then, if a satisfactory rate of pregnancy per AI (**P/AI**) is not achieved, it can compromise the overall reproductive performance of the dairy herd (Mendonça et al., 2012).

Some studies have proposed presynchronization strategies using only inducers of ovulation such as human chorionic gonadotrophin (Giordano et al., 2012b) and GnRH (Lopes et al., 2013) 7 d before the initiation of the breeding-Ovsynch for resynchronization of ovulation. Even though a better P/AI was observed for cows that were presynchronized, these strategies either increased IBI (Lopes et al., 2013) or had to start earlier after previous AI (Giordano et al., 2012b), potentially decreasing the proportion of cows expressing estrus before NPD.

One of the presynchronization objectives is to optimize the ovulatory response to the first GnRH of the breeding protocol (**G1**). This leads to improved synchrony by enhancing the responsiveness to all the following treatments of the breeding-Ovsynch (Bello et al., 2006). However, in the absence of a presynchronization strategy, inducing ovulation in a random stage of the estrous cycle is challenging because different factors can impair ovulation upon an exogenous administration of GnRH. One factor is high circulating progesterone (**P4**) concentrations at the time of GnRH administration. Cows with increased levels of circulating P4 have a decreased LH surge and ovulatory response induced by exogenous GnRH compared with cows with low P4 levels (Giordano et al., 2012a; Valdés-Arciniega et al., 2023). Increasing the GnRH dose can partially overcome the negative effect of high P4 on the LH surge (Giordano et al., 2012a). Indeed, a recent study from our group showed that a 200-µg GnRH dose induced more ovulations compared with the 100-µg GnRH dose, when used as the first GnRH of the breeding-Ovsynch, in a Double Ovsynch program for first-service lactating Holstein cows (Valdés-Arciniega et al., 2023).

Another major factor that affects ovulation upon GnRH treatment is the stage of follicle development. At the initiation of a new follicular wave, the immature follicles still do not have LH receptors in the granulosa cells, which is a required characteristic granting ovulatory capability to the follicle (Xu et al., 1995). Follicles without LH receptors in the granulosa will not ovulate, regardless of the occurrence of an induced LH surge. Moreover, the development of LH receptors in the granulosa cells occurs concomitant with the acquisition of dominance by the follicle and is an age-dependent characteristic. Therefore, ovulation will not occur if GnRH is administered in stages of the estrous cycle that a dominant follicle is absent.

Thus, the present study aimed to improve ovulatory response at the start of the resynchronization by overcoming the 2 main factors that impair ovulatory success at the initiation of the Resynch-25 protocol by (1) increasing the dose of GnRH from 100 µg to 200 µg and (2) giving a second GnRH treatment 56 h after the initiation of the protocol. Our first hypothesis was that the higher dose of GnRH would partially overcome the negative effect of high circulating P4 and would increase ovulatory response to G1, improving the overall fertility of cows resynchronized. The second hypothesis was that the second GnRH treatment 56 h after the initiation of the protocol would allow the development of follicles that were immature when the protocol started, increasing the overall ovulatory response and consequently the fertility of the program. Our final hypothesis was that the treatment combining the 200-µg GnRH dose on d 25 and 27 would promote the highest ovulatory response at the beginning of the Resynch-25, leading to the greatest pregnancy per AI (P/AI) when comparing all treatments.

MATERIALS AND METHODS

All procedures involving animals were previously approved by the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison (IACUC, protocol no. V006207-R01).

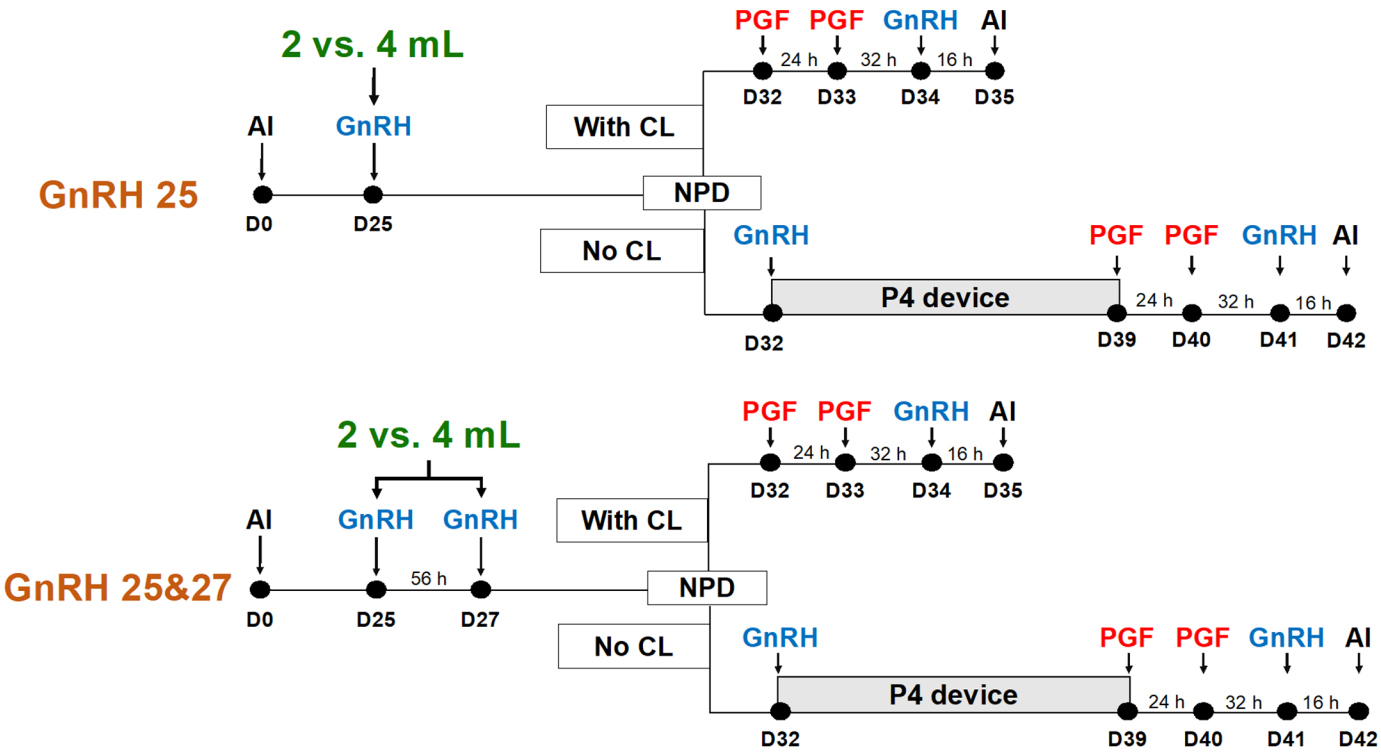

Figure 1. Schematic diagram of the experimental design. Cows were randomly assigned to receive 100 µg or 200 µg, 25 d or 25 and 27 d after the previous AI. A pregnancy diagnosis was performed on d 32 post-AI. Pregnant cows did not receive any further treatment. Nonpregnant cows were checked for the presence of a corpus luteum (CL) by ultrasound during pregnancy diagnosis. Nonpregnant cows without CL were enrolled in an Ovsynch + CIDR protocol (GnRH + CIDR d 32, $PGF_{2\alpha}$ on d 39 and 40 post-AI, GnRH 32 h after the last $PGF_{2\alpha}$, and TAI 16 h later). Nonpregnant cows with a CL finished the Resynch-25 protocol by receiving $PGF_{2\alpha}$ on d 32 and 33 and GnRH 32 h after the last $PGF_{2\alpha}$ treatment, followed by TAI 16 h later. A subset of cows had blood collected on experimental d 25, 32, and 34 and ultrasonographic examination on d 25, 29, 34, and 36.

Animals, Housing, and Farm Reproductive Management

The present experiment was conducted from March to September 2020 on a commercial dairy farm in south central Wisconsin (Evansville, WI). The farm had a herd of ~2,450 milking cows during the experimental period. Lactating cows were either housed in freestalls with natural ventilation and fans over the stalls or freestalls in a cross-ventilated barn, both bedded with recycled sand that was cleaned daily and refilled every 4 d. Cows were grouped into 8 different pens based on their parity (primiparous or multiparous), milk production, and DIM. Cows were fed twice daily a TMR that consisted mainly of corn silage and haylage that was formulated to meet or exceed their nutrient recommendations according to their milk production (NRC, 2001). Cows were milked thrice daily, and the average daily milk yield for the week before treatment was 40.00 ± 0.21 kg/d for primiparous cows and 55.07 ± 0.16 kg/d for multiparous cows. Animals had free access to feed and clean water throughout the day.

All first-service cows were submitted to a Double Ovsynch program (GnRH, 7 d later $PGF_{2\alpha}$, 3 d later GnRH, 7 d later GnRH, 7 d later $PGF_{2\alpha}$, 1 d later $PGF_{2\alpha}$, ~32 h later GnRH, and ~16 h later TAI; Souza et al., 2008). The first service for primiparous and multiparous cows was performed at 82 ± 3 and 72 ± 3 DIM, respectively. The farm relied mostly on TAI, and during the experimental period, only 9.4% of the cows that did not become pregnant in the previous AI were reinseminated after detection of estrus.

Experimental Design

Treatments were administered by trained farm and laboratory personnel. As GnRH, we used gonadorelin hydrochloride (Factrel, Zoetis, Parsippany-Troy Hills, NJ) in the dose of 100 or 200 µg in the first GnRH, according to treatments, and 100 µg for the other GnRH treatments of the protocols used. As for $PGF_{2\alpha}$, we used 25 mg of dinoprost (Lutalyse HighCon, Zoetis, Parsippany-Troy Hills, NJ). The experiment consisted of a 2 × 2 factorial design with GnRH dose (100 vs. 200 µg) being the first factor and GnRH treatment times (1 time vs. 2 times 56 h apart) as the second factor. We used a total of 2,111 previous services (AI, n = 1,931; and em-

bryo transfer [**ET**], n = 180) in 1,438 Holstein lactating cows (510 primiparous and 928 multiparous). After 25 d from the previous AI or 18 d from the previous ET, cows were assigned to receive either 100 or 200 µg of GnRH based on ear tag number (odd or even). At the same time, cows were randomly assigned by the on-farm management software (DairyComp 305, Valley Ag Software) to receive GnRH either only once at the same day (25 d after previous AI or 18 d after ET) or twice, on the same day and 56 h later (25 and 27 d after previous AI or 18 and 20 d after ET; Figure 1).

Pretreatment and Treatment Details

Experimental d 0 was defined as the day of the previous service (day of the previous AI or 7 d before previous ET). Cows used were previously bred 1 to 7 times (1 time, n = 1,221; 2 times, n = 531; 3 times, n = 228; ≥4 times, n = 131), receiving ET (n = 180; primiparous, n = 164; and multiparous, n = 16) or AI (n = 1,931; primiparous, n = 581; and multiparous, n = 1,350). In addition, we used Holstein sires sexed semen (n = 208; primiparous, n = 152; and multiparous, n = 56) and conventional semen (n = 1,450; primiparous, n = 558; and multiparous, n = 892) and Angus sires conventional semen (n = 453; primiparous, n = 35; and multiparous, n = 418). Pregnancy diagnosis was performed by transrectal ultrasonography (Easi-Scan, BCF Technology Ltd.) confirmed by embryo heartbeat 32 d post-AI or 25 d post-ET by farm veterinarians blinded to treatments. Pregnancy was reconfirmed at 46, 88, and 200 d of gestation. Calving records from treated cows were retrieved from the on-farm management software. We considered full-term calving when gestation length was at least 257 d.

On d 25, in the morning, cows were locked in self-locking headlocks at the feedline and had their electronic identification ear tags (Allflex) scanned by a farm employee positioned in front of them using an electronic identification reader and a hand-held computer with Pocket CowCard software (DairyComp 305, Valley Ag Software). After the electronic ear tag was scanned, the hand-held computer showed the GnRH dose that should be administered. Another farm employee then performed the GnRH treatment in the back of the cows using single-dose syringes with 18-gauge, 38-mm needles in the semimembranosus or semitendinosus muscles. Cows assigned to receive the second GnRH treatment 27 d were sorted by an electronic gate on the way back from the milking parlor, in the afternoon, and directed to a management area with self-locking headlocks. Once in this area, the cows' ear tags were visually checked by a person from our laboratory in front of the cows who had a printed list with the cows' identification numbers and the GnRH dose that should be administered to each one. Another laboratory member administered the GnRH treatment on d 27 at the rear of the cows. Cows received the d 27 GnRH administration in the afternoon as the last GnRH of the breeding-Ovsynch.

Post-Treatment Details

Out of the 2,111 previous services, 1,035 (49.0%) were diagnosed pregnant 32 d after previous AI or 25 d after previous ET (primiparous 55.0% [410/745]; multiparous 45.8% [625/1,366]). Cows diagnosed pregnant did not receive any further treatments. Cows diagnosed nonpregnant (n = 1,076 services in 714 cows; n = 335 services in primiparous, and n = 741 services in multiparous) were then classified as having or not having a corpus luteum (**CL**). Cows classified as having a CL continued the Resynch-25 protocol (n = 836 services), receiving a $PGF_{2\alpha}$ treatment at NPD and another on the following day, a GnRH ~32 h after the second $PGF_{2\alpha}$, and then TAI ~16 h later (Figure 1). Of the total Resynch-25 TAI services, 3 used sexed-sorted Holstein semen, 591 used Holstein conventional semen and 242 used conventional beef semen. Thirty-six additional cows were also enrolled in the Resynch-25 but did not receive TAI and were used as embryo recipients 7 d later. Cows classified as not having a CL started an Ovsynch + controlled internal drug release (**CIDR**) protocol (Stevenson et al., 2006) at NPD (n = 104 services). This protocol consisted of a GnRH treatment and the insertion of an intravaginal P4 device (Eazi-Breed CIDR Cattle Inserts, Zoetis, Parsippany-Troy Hills, NJ) at NPD. Seven days later, the CIDR was removed, and 2 $PGF_{2\alpha}$ treatments were administered 1 d apart. Thirty-two hours after the last $PGF_{2\alpha}$, cows received a second GnRH treatment and TAI ~16 h later. Of the cows receiving Ovsynch + CIDR protocol, 9 did not receive TAI or ET, 2 were used as recipients for ET 7 d later, 1 received sexed-sorted Holstein semen, 63 received conventional Holstein semen, and 29 received conventional beef semen. Additionally, 38 cows were designated as "do not breed" at NPD and did not receive reinsemination. These cows were excluded from the analysis performed after NPD. Finally, 62 cows were sold, died, or were diagnosed with any kind of abnormality of the reproductive tract by the veterinarian before or at NPD and did not receive reinsemination. Data from these cows were used until the date of the event that occasioned their exclusion from the trial.

Ultrasonographic Examinations of Ovaries

In a subset of cows, ultrasonographic examinations of the ovaries were performed using a 7.5-MHz linear transducer (Easi-Scan:Go, BCF Technology Ltd.) on d 25, 29, 34, and 36 to assess ovary dynamics as previously

described by El Azzi et al. (2022). Cows receiving the second GnRH on d 27 also had their ovaries examined by ultrasonography on d 27. A mini-iPad generation 4 (Apple Inc., Cupertino, CA) was wirelessly connected to the ultrasound, and videos of the ovaries were recorded each day. Videos were then analyzed later using a computer with a software program for video metrics analysis (Kinovea 0.8.15, Kinovea.org). Only videos from cows diagnosed as nonpregnant on d 32 were analyzed (d 25 [n = 455], 27 [n = 199], 29 [n = 403], 34 [n = 350], and 36 [n = 328]).

Ovulation was determined when one or more large antral follicles disappeared and a corpus hemorrhagicum or luteal structure appeared on the same ovarian location between the examinations on d 25 and 29 (ovulation at the initiation of the protocol) or between d 34 and 36 (ovulation to the final GnRH of the Resynch-25 [**G2**]). In nonpregnant cows receiving GnRH on d 25 and 27, we conducted a second evaluation of ovulation to determine if they ovulated between d 25 and 27 or between d 27 and 29. For measurement of the largest diameter of preovulatory follicle a frozen image of the ultrasonographic videos was used. The ultrasound background gridlines of 10 mm length were used to calibrate software calipers. The preovulatory follicle diameter was then determined as the average between the height and length at a 90° angle of the apparent largest frame.

Blood Sampling and P4 Assays

We performed blood collections in a subset of cows on d 25, 32 and 34 to determine P4 concentrations. Blood collection was performed by the puncture of the coccygeal artery or vein. Vacuum tubes of 9 mL (Vacuette Z serum clot activator, Greiner Bio-One International GmbH, Kremsmünster, Austria) and 20-gauge, 3.8-cm needles (Vacuette Multi-Sample Blood Collection Needle, Greiner Bio-One International GmbH, Kremsmünster, Austria) were used. After being collected, blood samples were kept refrigerated in a cooler with ice for a maximum of 6 h until being transported to the laboratory. For serum separation, blood samples were centrifuged at 2,000 × *g* for 10 min at 4°C. Once separated, serum was harvested and stored in 2-mL microcentrifuge tubes (Premium MCT Graduated Microcentrifuge Tubes, Fisher Scientific, Waltham, MA) in a −20°C freezer until P4 measurement by radioimmunoassay (**RIA**). Only samples from cows diagnosed as nonpregnant on d 32 were used for measurement of serum P4 concentration (d 25 [n = 500], 32 [n = 480], and 34 [n = 478]).

For circulating P4 concentration measurement, a solid-phase RIA kit was used (ImmuChem Coated Tube Progesterone, MP Biomedicals, Costa Mesa, CA). A total of 7 assays were performed. The average assay sensitivity, calculated as 2 SD less than the mean counts per minute of maximum binding, was 0.05 ng/mL. Intra- and interassay coefficients of variation were 8.0% and 17.4%, respectively. Circulating P4 concentrations were only measured in the blood of cows that were diagnosed nonpregnant on d 32. A functional CL presence was considered when P4 concentrations were above or equal to 1.00 ng/mL. Complete luteolysis was considered when serum P4 was ≤0.48 ng/mL on d 34 based on the receiver operating characteristic (**ROC**) analysis described in the "Statistical Analysis" section.

Statistical Analysis

This study used a randomized 2 × 2 factorial design, with each service considered as the experimental unit and treated as an independent event. Cows were eligible for multiple enrollments following each service throughout the experimental period. On the re-enrollment day (d 25), the same treatment assignment criteria used during the initial enrollment were reapplied.

We used the software G*Power 3.1 (version 3.1.9; Faul et al., 2007) to calculate the required sample size. According to an a priori power analysis using the z-test with the logistic regression statistical test, 2-tailed, $\alpha = 0.05$, power of 0.80, and binomial distribution, a total of 771 animals were required to find a 10 percentage point difference in P/AI between 2 treatments (38% vs. 48%).

All continuous variables, such as circulating P4 concentrations and preovulatory follicle diameter, were analyzed by ANOVA using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). To determine if the data met the assumptions of normality and homoscedasticity, the residual option of the MIXED procedure was used. All Studentized residual plots were visually evaluated after fitting the model, and in case the variable did not fit expectations, it was square-root-transformed. For clarity, all continuous variables are presented as means ± SEM that were obtained using the MEANS procedure of SAS.

Binary variables such as proportion of cows with circulating P4 ≥1.00 ng/mL, P/AI, and pregnancy losses were evaluated by logistic regression using the GLIMMIX procedure of SAS. Binary data are presented as proportions followed by the number of occurrences/total number. The proportions were obtained using the MEANS or FREQ procedure of SAS. Variables that did not fit the assumption for a good normal approximation ($n\pi \geq 5$ and $n[1 - \pi] \geq 5$) were analyzed by Fisher's exact test by adding the function exact to the FREQ procedure.

Circulating P4 concentration at the time of G2 was used to class if cows had undergone complete luteolysis. The threshold of 0.48 ng/mL was determined by the ROC curve analysis using RStudio (version 1.3.1073, RStudio

Team, 2020). The analysis consisted of the determination of the best circulating P4 concentration on d 34 to predict P/AI 32 d after AI. The value of 0.48 presented a specificity of 97.5% and sensitivity of 90.9%, with an area under the curve of 0.49 (95% CI = 0.43–0.54).

The initial models that aimed to evaluate the effect of the different GnRH doses (GnRH dose) and days of GnRH treatment considered the fixed effects of parity (primiparous or multiparous), GnRH dose (100 or 200 µg), GnRH day (only d 25 or d 25 and d 27), and service number (2, 3 or ≥4); the 2-way interactions between GnRH dose and parity and between GnRH Day and parity; and the 3-way interaction between GnRH dose, GnRH day, and parity. The initial models that evaluated the association between the functional CL presence on d 25 and the variables evaluated considered the fixed effects of parity, GnRH dose, GnRH day, presence of a functional CL on d 25 (functional CL cutoff P4 ≥1.00 ng/mL), and service number; the 2-way interactions between functional CL presence with GnRH dose and GnRH day; and the 3-way interaction between functional CL, GnRH dose, and GnRH day. To evaluate the association between ovulation at the beginning of the Resynch-25 protocol and the variables evaluated the initial models considered the fixed effects of parity, ovulatory response at the initiation of the protocol (ovulation or no ovulation), service number, and the 2-way interaction between parity and ovulatory response at the initiation of the protocol. Models that had P/AI as the response variable also considered the technician who performed AI as a fixed effect. Two- and 3-way interactions with $P > 0.20$ were backward eliminated from the model. The GnRH dose × GnRH day interaction was forced in all models that considered these 2 explanatory variables to test our hypothesis. For the analysis of services post-treatment (Resynch-25 and Ovsynch + CIDR) P/AI, pregnancy loss, and calving/AI, we excluded data of services that used sexed semen (n = 4) or for ET (n = 38). All models used a 2-tailed test, and we considered differences when $P \leq 0.05$ and tendencies when $0.05 < P \leq 0.10$.

RESULTS

Effect of GnRH Dose and GnRH Day Treatments on Pretreatment AI

The average parity number was 2.3 and did not differ among treatments ($P = 0.75$). At time of enrollment (d 25 post-AI), service number of the pretreatment AI averaged 1.7 and was similar between treatments ($P = 0.25$). GnRH dose ($P = 0.95$) and GnRH Day ($P = 0.48$) did not affect P/AI 32 d after the pretreatment AI that averaged 49.0%. Primiparous had greater P/AI 32 d after pretreatment AI compared with multiparous ($P < 0.01$; 55.0% vs. 45.8%, respectively). Out of the cows diagnosed nonpregnant on d 32 that continued the Resynch-25, parity (mean = 2.5; $P = 0.73$) and service (mean = 1.7; $P = 0.48$) numbers were similar between all groups.

Effect of GnRH Dose and GnRH Day Treatments on Ovarian Parameters

On average, 87.9% (400/455) of the cows had at least one follicle with diameter ≥9 mm on d 25. A greater proportion of cows receiving 200 µg of GnRH had a follicle ≥9 mm compared with cows receiving 100 µg of GnRH (91.0% [201/221] vs. 85.0% [199/234], respectively; $P = 0.05$). The proportion of cows with at least one follicle with a diameter ≥9 mm on d 25 was similar between cows treated with GnRH on d 25 or d 25 and 27 (89.5% [212/237] vs. 86.2% [188/118], respectively; $P = 0.30$). We did not find an association between the interaction of GnRH dose and GnRH Day and the proportion of cows with at least one follicle ≥9 mm in diameter on d 25 (Table 1).

On average, 46.4% (232/500) of the cows had serum P4 concentration ≥1.00 ng/mL on d 25 after the previous AI. The proportion of cows with P4 ≥1.00 ng/mL on d 25 was not associated with GnRH dose, GnRH day, and GnRH dose × GnRH Day interaction (Table 1). Also, we did not observe any differences in mean circulating P4 concentration for cows with P4 ≥1.00 ng/mL on d 25 between the different groups of GnRH doses and days (Table 1).

Increasing the GnRH dose improved overall ovulatory response, defined as the ovulation between d 25 and 29. Cows treated with 200 µg had a greater ovulatory response compared with cows treated with 100 µg (200 µg: 48.0% [95/198] vs. 100 µg: 36.1% [74/205]; $P = 0.01$). However, when we considered only cows that had at least one follicle with diameter ≥9 mm, we only observed a tendency for 200 µg of GnRH to induce a greater ovulatory response at the initiation of the protocol compared with 100 µg GnRH (200 µg: 51.9% [94/181] vs. 100 µg: 43.0% [74/172]; $P = 0.09$). The overall ovulatory response was not affected by GnRH day treatment (d 25 = 41.2% [82/199], d 25 and 27 = 42.6% [87/204], $P = 0.75$). Additionally, 31.6% of cows treated with GnRH on 25 and 27 ovulated between d 25 and 27, whereas only 11.1% of them ovulated between d 27 and 29. The proportion of cows treated with GnRH on d 25 and 27 that ovulated between d 27 and 29 was not affected by GnRH dose (100 µg: 8.8% [9/102]; 200 µg: 13.4% [13/97], $P = 0.30$). We did not observe any interaction between GnRH dose and GnRH Day in ovulatory response at the initiation of the protocol (Table 1).

Circulating P4 concentration on d 32 was not affected by GnRH dose or GnRH day treatments, and we did not find a GnRH dose × GnRH Day interaction on serum P4

Table 1. Effect of GnRH dose and of a second GnRH treatment 2 d after the initiation of the protocol on ovarian characteristics, ovulatory response, and circulating progesterone (P4) of lactating Holstein cows diagnosed nonpregnant 32 d after previous artificial insemination

	GnRH dose				*P*-value			
	100 µg		200 µg					
Item	Day 25[1]	Day 25 and 27[2]	Day 25	Day 25 and 27	Dose[3]	Day[4]	Dose × day	Parity[5]
Cows with follicle ≥9 mm in diameter on d 25 post-AI, % (n/n)	87.1 (108/124)	82.7 (91/110)	92.0 (104/113)	89.8 (97/108)	0.05	0.30	0.92	0.45
Cows with P4 ≥1.00 ng/mL on d 25 post-AI, % (n/n)	48.9 (68/139)	41.9 (49/117)	46.9 (60/128)	47.4 (55/116)	0.68	0.39	0.37	0.46
P4 of cows with P4 ≥1.00 ng/mL on d 25	5.40 ± 0.29	5.59 ± 0.51	5.70 ± 0.44	6.07 ± 0.47	0.13	0.82	0.76	0.45
Overall ovulatory response to G1,[6] % (n/n)	37.5^{a} (38/101)	34.5^{a} (36/104)	44.9^{ab} (44/98)	51.0^{b} (51/100)	0.02	0.75	0.35	0.59
Ovulatory response to G1 of cows with follicle ≥9 mm in diameter, % (n/n)	43.7^{AB} (38/87)	42.4^{A} (36/85)	48.9^{AB} (44/90)	54.9^{B} (50/91)	0.09	0.61	0.46	0.64
Pre-ovulatory follicle diameter on d 25, mm ± SEM	15.54 ± 0.44	14.54 ± 0.67	14.78 ± 0.40	14.53 ± 0.42	0.48	0.19	0.54	<0.01
Serum P4 concentration on d 32, ng/mL ± SEM	4.14 ± 0.27	4.65 ± 0.28	4.34 ± 0.26	4.60 ± 0.32	0.69	0.22	0.51	0.66
Cows with P4 ≥1.00 ng/mL on d 32, % (n/n)	83.5 (106/127)	86.4 (108/125)	87.2 (102/117)	85.6 (95/111)	0.46	0.80	0.44	0.42
P4 of cows with P4 ≥1.00 ng/mL on d 32 post-AI, ng/mL	4.91 ± 0.27	5.36 ± 0.26	4.94 ± 0.24	5.31 ± 0.32	0.93	0.17	0.61	0.55
Cows enrolled in Ovsynch + CIDR protocol, % (n/n)	11.9 (37/312)	10.2 (23/225)	8.6 (23/268)	11.3 (25/221)	0.59	0.71	0.26	0.27
P/AI on d 32 post-AI, % (n/n)	48.5 (16/33)	50.0 (9/18)	45.0 (9/20)	28.6 (6/21)	0.19	0.55	0.23	0.61
Cows that continued the Resynch-25 protocol								
Cows with P4 ≤0.48 ng/mL on d 34, % (n/n)	88.7 (86/97)	97.2 (104/107)	93.9 (92/98)	95.5 (84/88)	0.29	0.05	0.19	0.66
Cows with P4 ≥1.00 ng/mL on d 32 that had P4 ≤0.48 ng/mL on d 34, % (n/n)	91.4^{a} (74/81)	99.0^{b} (95/96)	96.6^{ab} (86/89)	96.3^{ab} (77/80)	0.85	0.12	0.09	0.47
Ovulatory response to the final GnRH, % (n/n)	83.8 (67/80)	90.1 (82/91)	86.6 (71/82)	81.3 (61/75)	0.49	0.77	0.13	0.55
Proportion of double ovulations, % (n/n)	11.9 (8/67)	8.5 (7/82)	5.6 (4/71)	11.5 (7/61)	0.71	0.44	0.18	0.03
Final pre-ovulatory follicle diameter, mm ± SEM	16.50 ± 0.35	16.20 ± 0.24	16.31 ± 0.32	16.17 ± 0.29	0.75	0.67	0.82	0.39

[a,b]Mean values in the same row with different lowercase superscripts differ ($P \leq 0.05$) for the interaction between GnRH dose and days of GnRH treatment.

[A,B]Mean values in the same row with different uppercase superscripts tended to differ (*P*-values >0.05 and ≤0.10) for the interaction between GnRH dose and days of GnRH treatment.

[1]Cows treated with GnRH only once (25 d after previous AI) at the initiation of the Resynch-25 program.

[2]Cows treated with GnRH twice (25 and 27 d after previous AI) at the initiation of the Resynch-25 program.

[3]Fixed effect of GnRH dose (100 or 200 µg of gonadorelin hydrochloride).

[4]Fixed effect of number of GnRH treatments (only on d 25 or on d 25 and 27 after the previous AI).

[5]Fixed effect of parity (primiparous or multiparous).

[6]Ovulation at the initiation of the protocol evaluated on d 25 and 29.

on d 32 (Table 1). In addition, the proportion of cows with functional CL (P4 ≥1.00 ng/mL) was similar between cows treated with the different GnRH dose and GnRH day treatments (Table 1). Similarly, treatment did not affect the proportion of cows diagnosed without a CL by the farm veterinarians on d 32 and enrolled in the Ovsynch + CIDR protocol (Table 1).

Among the cows that continued the Resynch-25 protocol, a greater proportion of cows treated with GnRH on d 25 and 27 underwent complete luteolysis by d 34 (P4 ≤0.48 ng/mL) compared with cows treated with GnRH only on d 25 (96.4% [188/195] vs. 91.3% [178/195], respectively; $P = 0.05$). We also observed a GnRH dose × parity interaction ($P = 0.05$) in the proportion of cows with complete luteolysis by d 34. Although no GnRH dose effect was observed for multiparous cows on luteolysis rate (200 µg: 92.9% [118/127], vs. 100 µg: 94.7% [143/151]; $P = 0.34$), 200 µg of GnRH tended to increase the proportion of primiparous cows that underwent complete luteolysis compared with primiparous cows treated with 100 µg of GnRH (200 µg: 98.3% [58/59], vs. 100 µg: 88.7% [47/53]; $P = 0.09$).

When complete luteolysis on d 34 was evaluated only in cows that had a functional CL on d 32, we did not observe an effect of GnRH day treatment (d 25: 94.1% [160/170], vs. d 25 and 27: 97.7% [172/176]; $P = 0.12$). However, we observed a tendency for interaction between GnRH dose and GnRH Day (Table 1). Of the cows with a functional CL on d 32, a greater proportion of the ones treated with 100 µg of GnRH on d 25 and 27 underwent complete luteolysis compared with cows receiving the same dose only on d 25 (Table 1). Cows with a functional CL on d 32 receiving 100 µg GnRH on d 25 and 27 had a similar complete luteolysis rate to cows receiving 200 µg GnRH only on d 25 or on d 25 and 27 (Table 1). Moreover, when only cows with functional CL were considered, we still observed an interaction between parity and GnRH dose on the proportion of cows with complete luteolysis. As with our observation when all cows were evaluated, a greater proportion of primiparous cows that had a functional CL on d 32 treated with 200 µg of GnRH underwent complete luteolysis by d 34 compared with the ones treated with 100 µg of GnRH (100% [54/54] vs. 89.1% [41/46], respectively; $P = 0.01$). On the other hand, we did not observe an effect of GnRH dose on the proportion of multiparous cows with a functional CL on d 32 that underwent complete luteolysis by d 34 (200 µg: 97.7% [128/131] vs. 100 µg: 94.8% [109/115]; $P = 0.22$).

The GnRH dose, GnRH day, or GnRH dose × GnRH day interaction did not affect ovulatory response to G2, double ovulations to G2, and the diameter of the preovulatory follicle on the day of G2 (Table 1). However, we observed a parity effect on the proportion of cows that double ovulated to G2. A greater proportion of multiparous cows had double ovulations to G2 compared with primiparous cows (11.9% [24/202] vs. 2.5% [2/79], respectively; $P = 0.03$).

Effect of Dose and Number of GnRH Injections on P/AI, Pregnancy Losses, Calving/AI, and Twins

Pregnancy per AI 32 d after insemination of cows that continued the Resynch-25 was not affected by GnRH dose. However, GnRH day tended to affect P/AI 32 d post-AI. Cows treated with GnRH on d 25 and 27 tended ($P = 0.10$) to have a lower P/AI on d 32 post-AI (39.0% [141/362]) compared with cows treated with GnRH only on d 25 (43.9% [208/474]). On d 46, 88, and 200 post-AI, GnRH dose and GnRH day did not affect P/AI (Table 2). The same was observed for calving/AI. Finally, we did not find an effect of GnRH dose, GnRH day, or GnRH dose × GnRH day interaction on pregnancy losses between d 36 and 46, between d 88 and 200, from d 200 until calving, and total pregnancy loss (from d 32 to calving).

Parity and GnRH dose affected the proportion of twin births (Table 2). A smaller proportion of primiparous cows calved twins compared with multiparous cows ($P = 0.04$; 3.5% [03/85] vs. 9.7% [19/195], respectively). In addition, a greater proportion of cows receiving 200 µg of GnRH calved twins compared with cows treated with 100 µg of GnRH ($P = 0.04$; 11.0% [15/136] vs. 4.9 [7/144], respectively). When we evaluated all cows resynchronized together, regardless of whether they were enrolled in the Resynch-25 or in the Ovsynch + CIDR, we observed a similar treatment effect on the fertility parameters evaluated (Table 3).

Association Between Presence of Functional CL on Day 25 Post-AI with Ovarian Parameters and Fertility

A greater proportion of cows with a functional CL (serum P4 ≥1 ng/mL) on d 25 had at least 1 follicle with diameter ≥9 mm compared with cows without functional CL (Table 4). The ovulatory response at the initiation of the protocol was not associated with the presence of a functional CL on d 25 post-AI when considering all cows, but it was when evaluating only cows with at least 1 follicle ≥9 mm (Table 4). A greater proportion of cows without a functional CL on d 25 and with at least 1 follicle ≥9 mm ovulated at the initiation of the protocol compared with cows in the same condition that had a functional CL on d 25 (Table 4). In addition, cows with a functional CL on d 25 tended to have a smaller preovulatory follicle diameter at the initiation of the protocol than cows without functional CL (Table 4).

Table 2. Effect of GnRH dose and of a second GnRH treatment 2 d after the initiation of the protocol on pregnancy per AI (P/AI), calving rate, pregnancy losses, and twin calving of lactating Holstein cows submitted to the Resynch-25 program for resynchronization of ovulation

	GnRH dose				*P*-value			
	100 µg		200 µg					
Item	Day 25[1]	Day 25 and 27[2]	Day 25	Day 25 and 27	Dose[3]	Day 27[4]	Dose × day 27	Parity[5]
P/AI on 32 d post-AI, % (n/n)	45.5 (115/253)	40.1 (73/182)	42.1 (93/221)	37.8 (68/180)	0.85	0.10	0.67	0.35
P/AI on 46 d post-AI, % (n/n)	42.5 (107/252)	36.8 (67/182)	38.5 (85/221)	36.1 (65/180)	0.84	0.27	0.68	0.27
P/AI on 88 d post-AI, % (n/n)	41.0 (103/251)	33.9 (61/180)	37.7 (83/220)	36.1 (65/180)	0.47	0.22	0.44	0.47
P/AI on 200 d post-AI, % (n/n)	39.5 (98/248)	32.4 (57/176)	36.4 (78/217)	35.0 (63/180)	0.47	0.22	0.43	0.22
Calving/AI, % (n/n)	37.2 (90/242)	31.4 (55/175)	35.2 (75/213)	34.1 (61/179)	0.39	0.30	0.53	0.12
Pregnancy loss between 32 and 46 d post-AI, % (n/n)	6.1 (7/114)	8.2 (6/73)	8.6 (8/93)	4.4 (3/68)	0.68	0.60	0.19	0.56
Pregnancy loss between 46 and 88 d post-AI, % (n/n)	2.8^{AB} (3/106)	6.2^{B} (4/65)	1.2^{A} (1/84)	0.0^{A} (0/65)	0.97	0.98	0.98	0.09
Pregnancy loss between 88 and 200 d post-AI, % (n/n)	2.0 (2/100)	0.0 (0/57)	1.3 (1/80)	3.1 (2/65)	0.99	0.99	0.99	0.99
Pregnancy loss between 200 d post-AI and calving, % (n/n)	2.2 (2/92)	1.8 (1/56)	0.0 (0/75)	1.6 (1/62)	0.98	0.98	0.98	0.72
Pregnancy loss between 32 d post-AI and calving, % (n/n)	13.5 (14/104)	16.7 (11/66)	11.8 (10/85)	9.0 (6/67)	0.17	0.98	0.32	0.60
Twin calving, % (n/n)	5.6^{a} (5/90)	3.6^{a} (2/55)	8.0^{ab} (6/75)	14.8^{b} (9/61)	0.04	0.91	0.27	0.04

^{a,b}Mean values in the same row with different lowercase superscripts differ ($P \leq 0.05$) for the interaction between GnRH dose and days of GnRH treatment.

^{A,B}Mean values in the same row with different uppercase superscripts tended to differ (*P*-values >0.05 and ≤0.10) for the interaction between GnRH dose and days of GnRH treatment.

[1]Cows treated with GnRH only once (25 d after previous AI) at the initiation of the Resynch-25 program.

[2]Cows treated with GnRH twice (25 and 27 d after previous AI) at the initiation of the Resynch-25 program.

[3]Fixed effect of GnRH dose (100 or 200 µg of gonadorelin hydrochloride).

[4]Fixed effect of number of GnRH treatments (only on d 25 or on d 25 and 27 after the previous AI).

[5]Fixed effect of parity (primiparous or multiparous).

Table 3. Effect of GnRH dose and of a second GnRH treatment 2 d after the initiation of the protocol on pregnancy per AI (P/AI), calving rate, pregnancy losses, and twin calving of lactating Holstein cows submitted to resynchronization of ovulation strategies post-treatment combined (Resynch-25 and Ovsynch + CIDR)

	GnRH dose							
	100 µg		200 µg		*P*-value			
Item	Day 25[1]	Day 25 and 27[2]	Day 25	Day 25 and 27	Dose[3]	Day 27[4]	Dose × day 27	Parity[5]
P/AI on 32 d post-AI, % (n/n)	46.0 (131/285)	41.2 (82/199)	42.5 (102/240)	36.8 (74/201)	0.90	0.06	0.96	0.47
P/AI on 46 d post-AI, % (n/n)	42.8 (121/283)	37.2 (74/199)	38.9 (93/239)	35.3 (71/201)	0.98	0.19	0.77	0.23
P/AI on 88 d post-AI, % (n/n)	41.1 (116/282)	34.0 (67/197)	38.0 (90/237)	35.3 (71/201)	0.62	0.16	0.48	0.36
P/AI on 200 d post-AI, % (n/n)	39.8 (111/279)	32.1 (62/193)	36.8 (86/234)	33.3 (66/198)	0.66	0.10	0.52	0.13
Calving/AI, % (n/n)	37.5^{B} (102/272)	31.3^{A} (60/192)	35.7^{AB} (82/230)	32.5^{AB} (64/197)	0.54	0.14	0.65	0.06
Pregnancy loss between 32 and 46 d post-AI, % (n/n)	6.2 (8/129)	9.8 (8/82)	8.8 (9/102)	4.1 (3/74)	0.58	0.63	0.11	0.27
Pregnancy loss between 46 and 88 d post-AI, % (n/n)	3.3^{AB} (4/120)	6.9^{B} (5/72)	1.1^{A} (1/91)	0.0^{A} (0/71)	0.97	0.98	0.97	0.27
Pregnancy loss between 88 and 200 d post-AI, % (n/n)	1.8 (2/113)	1.6 (1/63)	1.1 (1/87)	2.9 (2/68)	0.84	0.48	0.50	0.98
Pregnancy loss between 200 d post-AI and calving, % (n/n)	1.9 (2/104)	1.6 (1/61)	0.0 (0/82)	1.5 (1/65)	0.98	0.98	0.98	0.71
Pregnancy loss between 32 d post-AI and calving, % (n/n)	13.6^{ab} (16/118)	20.0^{b} (15/75)	11.8^{ab} (11/93)	8.6^{a} (6/70)	0.09	0.78	0.16	0.25
Twin calving, % (n/n)	5.9^{a} (6/102)	3.3^{aA} (2/60)	11.0^{abB} (9/82)	15.6^{b} (10/64)	0.01	0.78	0.31	0.04

^{a,b}Mean values in the same row with different lowercase superscripts differ ($P \leq 0.05$) for the interaction between GnRH dose and days of GnRH treatment.

^{A,B}Mean values in the same row with different uppercase superscripts tended to differ (*P*-values >0.05 and ≤0.10) for the interaction between GnRH dose and days of GnRH treatment.

[1]Cows treated with GnRH only once (25 d after previous AI) at the initiation of the Resynch-25 program.

[2]Cows treated with GnRH twice (25 and 27 d after previous AI) at the initiation of the Resynch-25 program.

[3]Fixed effect of GnRH dose (100 or 200 µg of gonadorelin hydrochloride).

[4]Fixed effect of number of GnRH treatments (only on d 25 or on d 25 and 27 after the previous AI).

[5]Fixed effect of parity (primiparous or multiparous).

Functional CL presence on d 25 was associated with circulating P4 concentrations on d 32. Cows with a functional CL on d 25 had greater circulating P4 concentration on d 32 compared with cows without functional CL on d 25, regardless of treatment (Table 4). However, a greater proportion of cows without a functional CL on d 25 had a functional CL on d 32 compared with cows with a functional CL on d 25 (Table 4). Furthermore, the proportion of cows with active CL on d 32 was smaller for cows with functional CL on d 25 that were treated with GnRH only on d 25 compared with both groups of cows without a functional CL on d 25 (Figure 2). Nevertheless, the proportion of cows with functional CL on d 25 treated with GnRH on d 25 and 27 that also had a functional CL on d 32 was similar compared with cows without functional CL on d 25 receiving GnRH on the same days (Figure 2). Both GnRH day groups of cows without a functional CL on d 25 had a similar proportion of cows with a functional CL on d 32 (Figure 2).

The presence of a functional CL on d 25 was also associated with the overall proportion of cows with P4 ≤0.48 ng/mL on d 34 (day of G2) in cows that continued the Resynch-25 (Table 4). However, the same difference was not observed when only cows that had a functional CL on d 32 were considered. Moreover, cows with functional CL on d 25 treated with GnRH on d 25 and 27 had similar proportion of cows with P4 ≤0.48 ng/mL on d 34 compared with cows without functional CL on d 25 (Figure 2). On the other hand, cows with functional CL on d 25 treated with GnRH only on d 25 had the lowest proportion of cows with P4 ≤0.48 ng/mL on d 34 compared with the other 3 groups (Figure 2). Cows with functional CL on d 25 treated with GnRH only on d 25 also had the lowest luteolysis rate (cows with functional CL on d 32 and P4 ≤0.48 ng/mL on d 34; functional CL on d 25 and GnRH on d 25 = 88.3% [53/60]; functional CL on d 25 and GnRH on d 25 and 27 = 98.6% [70/71]; no functional CL on d 25 and GnRH on d 25 = 97.7% [86/88]; no functional CL on d 25 and GnRH on d 25 and 27 = 96.8% [91/94]; $P = 0.03$).

The presence of a functional CL on d 25 was also associated with a tendency to decrease the ovulatory response to G2 (Table 4). Additionally, we observed a tendency for interaction between functional CL on d 25 and GnRH day at the initiation of the protocol on ovulatory response to G2. Cows with a functional CL on d 25 treated with GnRH on d 25 and 27 had similar ovulatory response to G2 compared with cows without functional CL on d 25, regardless of GnRH day treatment, and tended to have greater ovulatory response to G2 compared with cows with a functional CL on d 25 treated with GnRH only on d 25 (Figure 2). The presence of a functional CL on d 25 was not associated with the proportion of double ovulations to G2 (Table 4). However, average preovulatory follicle size at G2 was greater for cows without a functional CL on d 25 compared with cows with functional CL on the same day (Table 4).

Pregnancies per AI on d 32, 46, 88, and 200 post-AI, calving/AI, and total pregnancy loss were associated with the presence of a functional CL on d 25 (Table 4). Cows with a functional CL on d 25 had lower P/AI on d 32, 46, 88, and 200 post-AI, as well as lower calving/AI and greater total pregnancy loss compared with cows without a functional CL on d 25 (Table 4). We did not observe an association between functional CL presence on d 25 and twin calving (Table 4). We also did not observe any interaction between functional CL presence on d 25 and treatments on the fertility parameters evaluated (Table 4).

Association Between Ovulatory Response at the Initiation of the Resynch-25 Protocol with Ovarian Parameters and Fertility

No differences in serum P4 on d 25 were observed between cows that ovulate and did not ovulate between d 25 and 29 (Table 5). The proportion of cows with a functional CL on d 25 was also not different among the groups (Table 5). However, a greater proportion of cows that ovulated at the initiation of the protocol had at least one follicle ≥9 mm in diameter compared with cows that did not ovulate (Table 5).

Ovulatory response at the initiation of the protocol was not associated with differences in serum P4 concentrations on d 32 (Table 5). However, a greater proportion of cows that ovulated had a functional CL on d 32 compared with cows that did not ovulate (Table 5). In accordance, a greater proportion of cows that did not ovulate was visually detected without a CL by the farm veterinarians and assigned to the Ovsynch + CIDR protocol compared with cows that did ovulate (Table 5). Furthermore, when considering only the serum P4 of cows that had a functional CL, ovulation at the initiation of the protocol was associated with a greater circulating P4 on d 32 (Table 5).

For cows that continued the Resynch-25 protocol, we found no association between ovulation at the initiation of the protocol and the proportion of cows with P4 ≤0.48 ng/mL on d 34 (Table 5). However, a greater proportion of cows that ovulated at the initiation of the protocol also ovulated in response to G2 compared with cows that did not ovulate (Table 5). Additionally, cows that ovulated at the initiation of the protocol had a greater incidence of double ovulations after G2 and had a smaller preovulatory follicle diameter than cows that did not ovulate (Table 5).

Finally, ovulatory response at the initiation of the protocol was associated with an enhanced P/AI on d 32, 46, 88, and 200 post-AI, and calving/AI (Table 5). However, this better fertility was observed only in cows with functional CL on d 25, and we did not detect an association

Table 4. Association of the presence or absence of a functional corpus luteum (CL) 25 d after previous AI with ovulatory response to G1,[1] circulating progesterone (P4) concentrations, pregnancies per AI (P/AI), and pregnancy losses, and its association with GnRH dose,[2] and number of GnRH treatments[3] at the initiation of the Resynch-25 program for lactating Holstein cows

	Functional CL presence on d 25		*P*-value			
Item	Yes	No	CL[4]	CL × GnRH day	CL × GnRH dose	CL × GnRH dose × GnRH day
P4 concentration on d 25, ng/mL ± SEM	5.68 ± 0.21	0.17 ± 0.01	<0.01	0.96	0.54	0.26
Cows with follicle ≥9 mm in diameter on d 25 post-AI, % (n/n)	95.7 (198/207)	81.1 (193/238)	<0.01	0.35	0.33	0.30
Overall ovulatory response to G1, % (n/n)	40.6 (78/192)	44.8 (100/223)	0.43	0.49	0.87	0.49
Ovulatory response to G1 of cows with follicle ≥9 mm, % (n/n)	42.4 (78/184)	55.6 (100/180)	0.02	0.46	0.51	0.66
Pre-ovulatory follicle diameter on d 25, mm ± SEM	14.46 ± 0.31	15.35 ± 0.33	0.06	0.18	0.45	0.42
Cows with P4 ≥1.00 ng/mL on d 32, % (n/n)	79.8 (162/203)	90.9 (209/230)	<0.01	0.12	0.39	0.53
P4 of cows with P4 ≥1.00 ng/mL on d 32 post-AI, % (n/n)	6.52 ± 0.25	4.08 ± 0.12	<0.01	0.77	0.90	0.43
Cows enrolled in Ovsynch + CIDR protocol, % (n/n)	11.3 (24/213)	9.9 (26/263)	0.97	0.17	0.62	0.79
Cows enrolled in Resynch-25						
Cows with P4 ≤0.48 ng/mL on d 34, % (n/n)	89.6 (138/154)	97.0 (193/199)	0.05	0.12	0.79	0.88
Cows with P4 ≥1.00 ng/mL on d 32 that had P4 ≤0.48 ng/mL on d 34, % (n/n)	93.9 (123/131)	97.3 (177/182)	0.60	0.06	0.98	0.99
Ovulatory response to the final GnRH, % (n/n)	80.5 (107/133)	89.3 (158/177)	0.07	0.09	0.12	0.78
Proportion of double ovulations, % (n/n)	13.1 (14/107)	7.0 (11/158)	0.13	0.33	0.69	0.70
Final pre-ovulatory follicle diameter, mm ± SEM	15.25 ± 0.20	16.86 ± 0.20	<0.01	0.30	0.72	0.68
P/AI 32 d post-AI, % (n/n)	35.9 (60/167)	47.3 (105/222)	0.03	0.79	0.52	0.79
P/AI 46 d post-AI, % (n/n)	32.3 (54/167)	45.0 (100/222)	0.02	0.47	0.63	0.79
P/AI 88 d post-AI, % (n/n)	31.3 (52/166)	44.3 (98/221)	0.01	0.25	0.44	0.55
P/AI 200 d post-AI, % (n/n)	29.1 (48/165)	42.6 (92/216)	<0.01	0.38	0.63	0.55
Calving rate, % (n/n)	28.5 (47/165)	42.1 (90/214)	<0.01	0.38	0.56	0.63
Pregnancy loss between 32 and 46 d post-AI, % (n/n)	10.0 (6/60)	4.8 (5/105)	0.19	0.98	0.98	0.98
Pregnancy loss between 32 d post-AI and calving, % (n/n)	19.0 (11/58)	7.2 (7/97)	0.03	0.54	0.70	0.71
Twin calving, % (n/n)	10.6 (5/47)	4.4 (4/90)	0.56	0.12	0.99	0.98

[1]Ovulation at the initiation of the protocol evaluated on d 25 and 29.

[2]Cows were treated with 2 different GnRH doses (100 or 200 µg of gonadorelin hydrochloride).

[3]Cows were treated in 2 different frequencies (only on d 25 or on d 25 and 27 following the previous AI).

[4]Fixed effect of the presence of a functional CL on d 25.

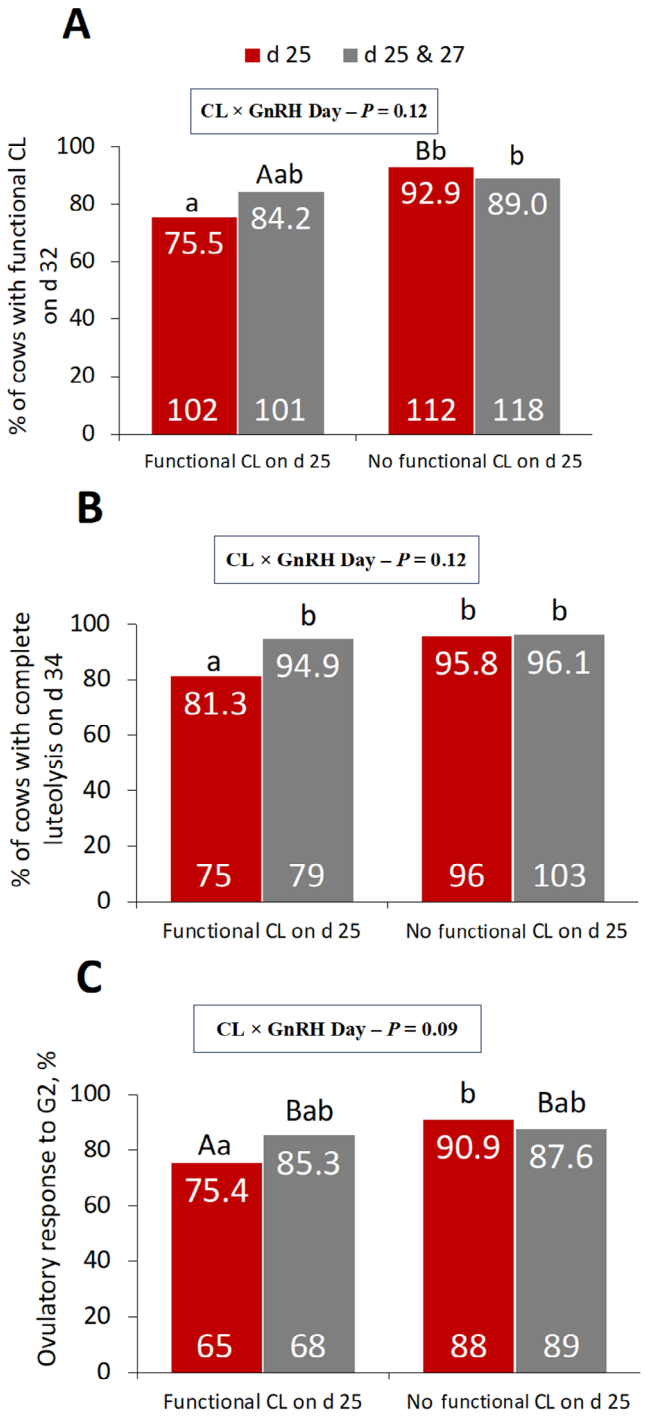

Figure 2. Effect of the interaction between the presence or absence of a functional corpus luteum (CL) 25 d after previous AI and days of GnRH treatment (GnRH on d 25 or GnRH on d 25 and 27 after previous AI) on (A) proportion of cows with an active CL (circulating P4 ≥1.00 ng/mL) on d 32, (B) proportion of cows undergoing complete luteolysis (circulating P4 <0.48 ng/mL) by d 34, and (C) ovulatory response after the last GnRH treatment of the protocol. Different lowercase letters (a,b) denote differences ($P \leq 0.05$) between columns, obtained by pairwise comparisons. Different uppercase letters (A,B) denote tendencies (P-values >0.05 and ≤0.10) between columns, obtained by pairwise comparisons. Numbers at the bottom of the columns indicate the service count for each group.

of ovulating at the initiation of the Resynch-25 and better P/AI or calving/AI for cows without a functional CL on d 25 (Figure 3). We did not find associations between ovulatory response at the initiation of the protocol and pregnancy losses or twin births (Table 5).

DISCUSSION

The present study determined the effect of a 200-µg dose of GnRH and an additional GnRH treatment 56 h after the beginning of the Resynch-25 program on ovulatory response at the initiation of the protocol, ovarian parameters, and fertility of lactating Holstein dairy cows submitted to a Resynch-25 program. Differently from what we hypothesized, the extra GnRH treatment on d 27 post-AI did not increase the ovulatory response between d 25 and 29. Our initial hypothesis was that giving 56 extra hours for follicle development would increase ovulatory response by increasing the chances of the follicle to acquire dominance and ovulatory capacity. Nonetheless, only 11.1% of cows receiving the extra GnRH injection ovulated between d 27 and 29. Although a high proportion of nonpregnant cows had at least one follicle ≥9 mm in diameter on d 25 (87.9%), presence of a large follicle at time of the first GnRH of the breeding-Ovsynch is not the best indicative of its ovulatory capability (Martins et al., 2023). On a specific day after AI, cows have follicles in random stages of development, and on d 25 after previous AI, some cows are expected not to have a dominant follicle (Ricci et al., 2017). The acquisition of ovulatory capability by the follicle occurs upon the development of LH receptors in the granulosa cells and is an age-dependent characteristic, usually occurring 5 d after the initiation of the follicular wave (Xu et al., 1995). Therefore, cows that had just started a new follicular wave on d 25 most likely did not have a dominant follicle on d 27 and would not ovulate even with the additional GnRH treatment on d 27. Only nonpregnant cows that were around d 3 to 5 of the follicular wave 25 d post-AI would likely benefit from this extra GnRH treatment. This very specific group of animals apparently represents a low proportion of the total of nonpregnant cows, leading to a small range for improvement and potentially explaining the lack of effect of the additional GnRH on d 27 after previous AI on improving ovulatory response at the initiation of the Resynch-25 program.

Our second hypothesis was that a larger GnRH dose would overcome the negative effects of high circulating P4 concentrations on the magnitude of the LH surge induced by a GnRH treatment (Giordano et al., 2012a). As we hypothesized, the greater GnRH dose increased ovulatory response at the initiation of the protocol when all cows were included and tended to increase when only cows with at least one follicle ≥9 mm in diameter were

Table 5. Effect of ovulatory response to G1[1] on ovarian characteristics, ovulatory response, circulating progesterone (P4) concentrations, pregnancies per AI (P/AI), and pregnancy losses in lactating Holstein cows diagnosed nonpregnant 32 d after previous AI

	Ovulatory response to G1		*P*-value		
Item	Ovulation	No ovulation	Ov25[2]	Parity[3]	Ov25 × parity
Serum P4 concentration on d 25, ng/mL ± SEM	2.25 ± 0.22	2.93 ± 0.23	0.12	0.20	0.40
Cows with P4 ≥1.00 ng/mL on d 25, % (n/n)	43.8 (78/178)	48.1 (114/237)	0.43	0.39	0.88
Cows with follicle ≥9 mm in diameter on d 25 post-AI, % (n/n)	99.5 (183/184)[4]	78.8 (183/240)	<0.01	0.53	0.97
Serum P4 concentration on d 32, ng/mL ± SEM	4.46 ± 0.25	4.69 ± 0.21	0.31	0.89	0.05
Cows with P4 ≥1.00 ng/mL on d 32, % (n/n)	93.9 (138/147)	81.5 (176/216)	<0.01	0.07	0.45
Serum P4 concentration on d 32 of cows with P4 ≥1.00 ng/mL on d 32, ng/mL ± SEM	4.72 ± 0.25	5.71 ± 0.19	<0.01	0.12	0.17
Cows enrolled in the Ovsynch + CIDR protocol, % (n/n)	4.8 (7/165)	12.4 (27/218)	0.01	0.57	0.86
Cows enrolled in Resynch-25					
Cows with P4 ≤0.48 ng/mL on d 34, % (n/n)	94.7 (124/131)	93.1 (161/173)	0.58	0.76	0.49
Ovulatory response to the final GnRH, % (n/n)	92.4 (110/119)	82.5 (127/154)	0.01	0.53	0.08
Proportion of double ovulations, % (n/n)	14.5 (16/110)	4.7 (6/127)	0.01	0.3	0.98
Final pre-ovulatory follicle diameter, mm ± SEM	15.84 ± 0.21	16.36 ± 0.23	0.05	0.64	0.18
P/AI on 32 d post-AI, % (n/n)	47.8 (75/157)	37.1 (69/186)	0.03	0.97	0.96
P/AI on 46 d post-AI, % (n/n)	45.9 (72/157)	33.9 (63/186)	0.03	0.80	0.91
P/AI on 88 d post-AI, % (n/n)	44.9 (70/156)	33.9 (63/186)	0.05	0.97	0.85
P/AI on 200 d post-AI, % (n/n)	42.9 (67/156)	31.1 (56/180)	0.04	0.62	0.75
Calving rate, % (n/n)	41.6 (64/154)	31.1 (56/180)	0.05	0.54	0.86
Pregnancy loss between 32 and 46 d post-AI, % (n/n)	4.0 (3/75)	8.7 (6/69)	0.26	0.53	0.98
Pregnancy loss between 32 d post-AI and calving, % (n/n)	11.1 (8/72)	11.1 (7/63)	0.99	0.41	0.79
Twin calving, % (n/n)	7.8 (5/64)	3.6 (2/56)	0.36	0.97	0.99

[1]Ovulation at the initiation of the protocol evaluated on d 25 and 29.

[2]Fixed effect of ovulatory response at the initiation of the protocol (ovulation or no ovulation).

[3]Fixed effect of parity (multiparous or primiparous).

[4]One cow ovulated a follicle with a diameter of 8.75 mm.

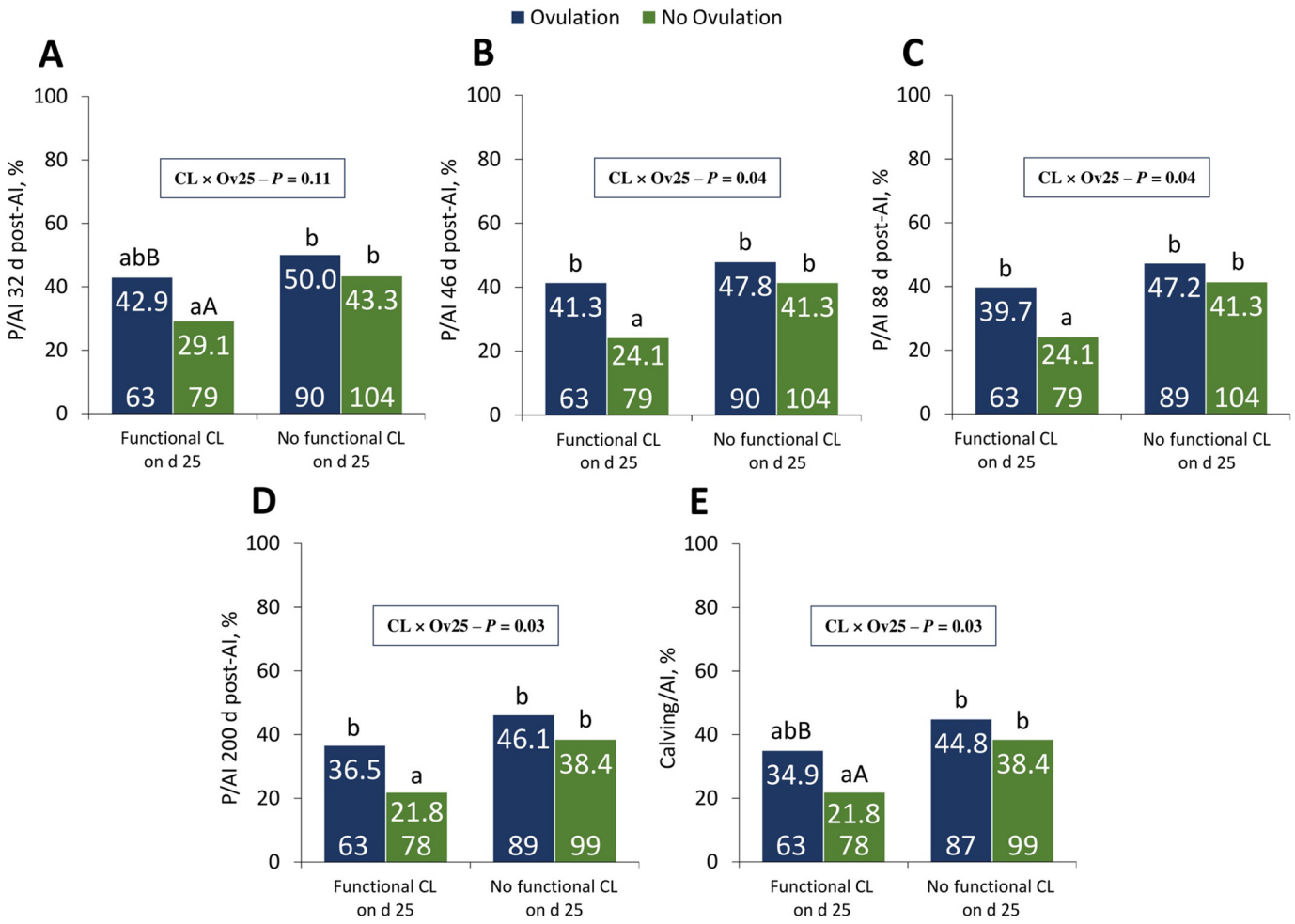

Figure 3. Association of the interaction between functional corpus luteum (CL) presence on d 25 after the previous AI and ovulatory response at the initiation of the Resynch-25 protocol with pregnancy per AI at (A) 32, (B) 46, (C) 88, and (D) 200 d post-AI, and (E) calving per AI. Different lowercase letters (a,b) denote differences ($P \leq 0.05$) in columns, obtained by pairwise comparisons of the means. Different uppercase letters (A,B) denote tendency to differences ($P > 0.05$ and ≤ 0.10) in columns, obtained by pairwise comparisons of the means. Numbers at the bottom of the columns indicate the service count for each group.

considered. The effect of the greater GnRH dose on increasing ovulatory response at the initiation of the protocol potentially occurred by a partial recovery from the negative effect promoted by high serum P4 concentrations (Colazo et al., 2008). Despite the increase in the overall ovulatory response at the initiation of the protocol, the larger GnRH dose did not increase the overall P/AI 32, 46, 88 and 200 d after AI nor calving/AI. This is probably a consequence of the lack of improvement in overall synchronization by the larger GnRH dose. Parameters that indicate responsiveness to the treatments following the first GnRH of the protocol, such as proportion of cows with complete luteolysis and ovulatory response to G2, were not different among groups treated with the 2 different GnRH doses. A recent study from our laboratory using the greater dose of GnRH at the initiation of the Resynch-25 protocol also did not find an effect of dose on fertility of lactating dairy cows submitted to the Resynch-25 (Leão et al. 2024a). Different from the present study, in that study, we did not observe an effect of the larger dose of the first GnRH of the Resynch-25 on increasing ovulatory response at the initiation of the protocol in cows enrolled in a Resynch-25 program (Leão et al. 2024a).

Considering that estrous detection was not performed after AI in the present study, at the initiation of the Resynch-25 some cows are expected to be at the end or at the beginning of the estrous cycle (Ricci et al., 2017). Indeed, more than half (53.5%) of the nonpregnant cows did not have a functional CL (P4 <1.00 ng/mL) on d 25, indicating that they probably were in the estrus, proestrus, or early metestrus phase of the estrous cycle. In fact, only 31.5% of cows with P4 <1.00 ng/mL had a CL bigger than 10 mm in diameter visualized by ultrasonography. In this case, promoting ovulation with an exogenous

GnRH treatment may not be necessary, considering that these cows had just ovulated or were about to ovulate, regardless of the GnRH treatment, and would probably have their ovulatory wave onset initiate just before or after the GnRH treatment.

Indeed, nonpregnant cows starting the protocol without a functional CL on d 25 had better fertility compared with cows with a functional CL, even though no difference in ovulatory response at the initiation of the protocol was observed between these groups. Of the nonpregnant cows without CL at d 25, only 43.3% ovulated between d 25 and 29, but 90.9% of them had functional CL on d 32. The 43.3% of cows without a functional CL that ovulated in the beginning of the protocol were most likely in the proestrus or estrus phase of the estrous cycle and were about to ovulate regardless of GnRH treatment on d 25. On the other hand, the 56.7% of the cows without a functional CL at d 25 that did not ovulate between d 25 and 29 had likely ovulated just before d 25. The ovulation just before or after the initiation of the Resynch-25 protocol increases the chance of cows having a functional CL responsive to $PGF_{2\alpha}$ treatment on d 32 compared with cows that would ovulate just before NPD at d 32.

In addition to the benefit of controlling CL lifespan and responsiveness to $PGF_{2\alpha}$, ovulation just before or after the GnRH on d 25 ensures the initiation of a new follicular wave around d 25, increasing the chances of the presence of an ovulatory follicle within an optimal antral age at the time of the final GnRH of the resynchronization program (Cerri et al., 2009). These findings corroborate with a previous study from our laboratory that showed that P/AI 32 d post-AI of cows starting the Resynch-25 without a functional CL was 53.5% whereas P/AI 32 d post-AI of cows bearing a functional CL was only 39.7% (Leão et al., 2024a). These results contradict other findings that associated the absence of a CL at the initiation of the breeding-Ovsynch with a lower fertility (Denicol et al., 2012; Bisinotto at al., 2013, 2015). Nonetheless, these studies included first-service cows, and the absence of a CL at the initiation of the breeding-Ovsynch in these cows is an indicative of anovulatory condition (Bisinotto et al., 2010). Our studies only included cows receiving second and greater services when the chances of being anovular at the time of initiation of the Resynch-25 are not likely (Gümen et al., 2003), and the absence of a functional CL probably only indicates that cows are in the nonluteal phases of the estrous cycle. In addition, our study did not use reinsemination after detection of estrus and most of the cows had a functional CL on d 32, which reinforces the low chance of anovulatory condition.

Lopes et al. (2013) also observed a higher P/AI in cows with high serum P4 concentrations at the start of the resynchronization program, either on d 32 or 39 after the previous AI, compared with cows with low serum P4. This different result may be because of the interval between previous AI and GnRH administration and differences in the experimental design. Unlike d 25, the absence of a CL on d 32 or 39 after previous AI may indicate atypical estrous cycles or an asynchrony to the previous synchronization program, potentially leading to a reduced P/AI. Additionally, in contrast with the present study, cows in that study were monitored daily for estrous expression using tail painting, which led to the selection of a different population of cows to be enrolled in the resynchronization protocol. They also evaluated the effect of a pre-synchronization with GnRH administered 7 d before the start resynchronization (Lopes et al., 2013). About half of the cows in that study received this pre-synchronization treatment, and the absence of a CL at G1 probably reflects a lack of ovulatory response to the strategy, potentially resulting in a lower synchrony and reduced fertility.

The association between ovulation at the beginning of the protocol and better fertility found corroborates with other studies that highlight the importance of ovulation at the initiation of Ovsynch-like protocols for first (Giordano et al., 2013; Valdez-Arciniega et al., 2023) and subsequent services (Bisinotto et al., 2015). However, this enhanced fertility promoted by ovulation at the initiation of the protocol was only observed in cows that had a functional CL on d 25. The lack of association between ovulation at the initiation of the protocol and better fertility in cows without a functional CL on d 25 is likely correlated with cows in early metestrus at the initiation of the protocol. Cows in metestrus represented part of the nonpregnant cows that did not have a functional CL at d 25 and did not ovulate after GnRH treatment. However, these cows had started a new follicular wave just before the initiation of the protocol, and their synchrony and fertility are probably similar to the ones that ovulate at the initiation of the protocol (Vasconcelos et al., 1999).

In addition, cows in proestrus and estrus on d 25 also did not have a CL and most likely ovulated after the initiation of the protocol. However, these cows were already about to have an ovulation induced by an endogenous GnRH-induced LH surge. Therefore, strategies that improve ovulatory response probably did not affect these cows. This suggests that only cows with a functional CL at the initiation of the Resynch-25 are likely to benefit from increased ovulation to the first GnRH of the Resynch-25 program. The accurate identification of cows with a functional CL by the utilization of technologies such as cow-side blood P4 test and color Doppler ultrasonography might help to assess the efficiency of these strategies that aim to increase ovulatory response at the initiation of the Resynch-25, helping to develop their application.

An unexpected finding of the present study was the greater proportion of cows treated with a second GnRH

on d 27 post-AI undergoing complete luteolysis on d 34. Ovulation occurs around 28 h after a GnRH treatment (Pursley et al., 1995), and nonpregnant cows without a CL on d 25 that ovulated after the GnRH on d 27 post-AI would have only one CL approximately 4- to 5-d old at the time of the first $PGF_{2\alpha}$ on d 32. This CL would be too young and unlikely to regress upon a single exogenous $PGF_{2\alpha}$ treatment (Nascimento et al., 2014). In these cows, the additional $PGF_{2\alpha}$ treatment on d 33 was probably crucial for the complete CL regression of this newly formed CL (Wiltbank et al., 2015). Furthermore, cows that did not have or had a small CL on d 32 were enrolled in an Ovsynch + CIDR protocol. This strategy was already adopted by the farm based on studies that have shown that these cows have reduced fertility if they continue the Resynch-25 protocol and starting an Ovsynch + CIDR increases their chance of becoming pregnant (Sterry et al., 2006; Giordano et al., 2016; Wijma et al., 2017, 2018; Pérez et al., 2020). However, none of the cows that ovulated between d 27 and d 29 were assigned to the Ovsynch + CIDR protocol, so were not excluded from the complete luteolysis analysis. Thus, a potential explanation for the improved luteolysis may be that the extra GnRH treatment on d 27 post-AI led to the formation of an accessory CL in cows that already had a CL on d 25 (Souza et al., 2009). These cows would then have 2 CLs on d 32, an old and a newly formed CL. The induction of luteolysis in the older CL can also improve the regression of the newer CL (Baez et al., 2017). This difference in proportion of cows with complete luteolysis by d 34 between the 2 GnRH Day treatments could also be a consequence of a statistical type I error promoted by random chance.

Despite this observed increase in luteolysis rate, the second GnRH on d 27 post-AI did not improve the fertility parameters evaluated. It surprisingly tended to decrease the overall P/AI on d 32 post-AI compared with treatment with GnRH only on d 25. This tendency was not likely occasioned by differences in ovulatory response at the initiation of the protocol or to G2, ovulatory follicle size, or double ovulations as these parameters were similar between cows treated with GnRH only on d 25 or on d 25 and 27. Interestingly, a similar reduction in fertility was observed by Cabrera et al. (2021) when replacing the first GnRH of the breeding-Ovsynch by human chorionic gonadotropin (**hCG**). One potential explanation for the decreased fertility may be the greater stimulation of the follicles' LH receptors, promoted by the long half-life of hCG, leading to a premature oocyte maturation (Revah and Butler, 1996). In the present study, cows treated with the second GnRH on d 27 most likely had an extra LH surge release, increasing the exposure of the dominant follicle to LH, which may negatively affected oocyte quality and P/AI at d 32 post-AI. We did not observe any differences in P/AI on d 46, 88, and 200, nor in calving/AI. This suggests that the negative effect of the additional GnRH treatment 27 d after the previous AI impaired the early establishment of pregnancy, corroborating with the hypothesis that its negative effect may occur on the oocyte.

Conversely, in a recent study from our laboratory, the extra treatment with GnRH on d 27 did not affect P/AI of cows enrolled in the Resynch-25 protocol (Leão et al., 2024b). We actually observed a reduced total pregnancy loss that led to a tendency of a greater calving/AI for multiparous cows treated with the extra GnRH compared with the ones receiving a single GnRH treatment at the initiation of the Resynch-25. Different from the present study, Leão et al. (2024b) administered the 2 GnRH treatments 48 h apart and G2 48 h after the first $PGF_{2\alpha}$, instead of 56 h. In addition, nonpregnant cows were also resinseminated after detection of estrus in that study, so a different population of cows was used. These differences may have led to different ovulatory responses at the initiation and end of the Resynch-25, and multiparous cows not reinseminated and treated with the extra GnRH on d 27 may have benefited from it.

Finally, the hypothesis that the combination of the larger dose with the GnRH treatment on d 25 and 27 would promote an additive effect was not confirmed. The absence of an individual effect of each strategy alone led to no differences in the parameters evaluated when both strategies were applied together, and no benefit of their concomitant use was observed.

CONCLUSIONS

The 200 µg GnRH (gonadorelin hydrochloride) dose successfully increased the ovulatory response at the initiation of the Resynch-25 protocol but did not improve P/AI when compared with the 100 µg GnRH dose. The additional GnRH treatment on d 27 after the previous AI did not increase ovulatory response at the initiation of the protocol and tended to decrease P/AI 32 d after AI but did not affect P/AI 46, 88 and 200 d post-AI nor calving/AI. Furthermore, the ovulation at the initiation of the Resynch-25 protocol was associated with a better fertility only in cows that had a functional CL on d 25. Data generated in this study suggests that the use of technologies that allow the accurate identification of CL functionality on d 25 after previous AI may help to define strategies that lead to increased fertility after resynchronization and an optimized usage of reproductive hormones in dairy farms.

NOTES

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Nonstandard abbreviations used: CIDR = controlled internal drug release; CL = corpus luteum; ET = embryo transfer; G1 = first SnRH of the Ovsynch protocol; G2 = last GnRH of the Ovsynch protocol; hCG = human chorionic gonadotropin; IBI = interval between inseminations; NPD = nonpregnant diagnosis; P/AI = pregnancy per AI; P4 = progesterone; RIA = radioimmunoassay; ROC = receiver operating characteristic; TAI = timed AI.

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ORCIDS

Iago M. R. Leão, https://orcid.org/0000-0001-7017-3642
Carlos E. C. Consentini, https://orcid.org/0000-0002-4781-2466
Marcelo S. El Azzi, https://orcid.org/0000-0002-2589-0965
Everaldo Anta-Galván, https://orcid.org/0000-0001-5528-5582
Teresita Valdés-Arciniega, https://orcid.org/0000-0001-5187-5905
Lucas Oliveira e Silva, https://orcid.org/0000-0002-8394-2583
Roberto Sartori, https://orcid.org/0000-0001-6917-0057
João Paulo N. Martins https://orcid.org/0000-0003-0452-3207