Luteolysis, onset of estrus, and ovulation in Holstein heifers given prostaglandin $F_{2\alpha}$ concurrent with, or 24 hours prior to, removal of an intravaginal, progesterone-releasing device

M.A. Hittinger, J.D. Ambrose, J.P. Kastelic

Abstract

The objective was to determine the effects of giving prostaglandin $F_{2\alpha}$ (PGF) concurrent with, or 24 h before, removal of an intravaginal, progesterone-releasing (controlled internal drug release [CIDR]) device, on luteolysis, the synchrony of estrus and ovulation. Eighteen postpubertal Holstein heifers were given a CIDR and 100 μg gonadotropin releasing hormone (GnRH) and equally allocated to 3 groups. The PGF was given concurrently with CIDR removal after 7 or 8 d (groups D7/D7 and D8/D8, respectively) or given 1-d before removal of CIDR after 8 d (group D7/D8). There was no difference ($P > 0.75$) among groups in the intervals (h) from CIDR removal to onset of standing estrus and to ovulation (49.3 h ± 6.2 h and 77.5 h ± 9.0 h, respectively; least squares means ± standard error of means). We also determined if stage of the estrus cycle influenced the synchrony of estrus or ovulation. In heifers in metestrus at CIDR insertion (versus those at estrus or diestrus), intervals from CIDR removal to estrus and to ovulation were longer by 33.4 h ($P < 0.05$) and 38.5 h ($P = 0.01$), respectively. However, the interval from standing estrus to ovulation was not affected. Giving PGF concurrent with CIDR removal did not affect luteal regression, the synchrony of estrus, and ovulation; but heifers in metestrus at the initiation of treatment had longer intervals from CIDR removal to estrus and ovulation.

Résumé

L'objectif de cette étude était de déterminer les effets de l'administration de prostaglandine $F_{2\alpha}$ (PGF), simultanément, ou 24 h avant, le retrait d'un appareil intra-vaginal libérant de la progestérone (CIDR), sur la lutéolyse, le synchronisme de l'œstrus et l'ovulation. Un total de 18 taures pubères de race Holstein ont reçu un CIDR et 100 μg de gonadolibérine (GnRH) et réparties également dans 3 groupes. La PGF a été donnée simultanément avec le retrait du CIDR après 7 ou 8 j (respectivement, groupes D7/D7 et D8/D8), ou donnée 1 j avant le retrait du CIDR après 8 j (D7/D8). Aucune différence (P > 0,75) ne fut notée entre les groupes pour les intervalles (en h) entre le retrait du CIDR et, le début de l'œstrus et l'ovulation (respectivement de 49,3 ± 6,2 et 77,5 ± 9,0; moyenne des moindres carrés ± erreur-type de la moyenne). Nous avons également déterminé si le stade du cycle œstral influençait le synchronisme de l'œstrus ou de l'ovulation. Chez les taures au stade de metestrus lors de l'insertion du CIDR (versus celles au stade œstrus ou diestrus), les intervalles entre le retrait du CIDR et l'œstrus et de l'ovulation étaient plus longs, respectivement, de 33,4 h (P < 0,05) et 38,5 h (P = 0,01). Toutefois, l'intervalle entre l'œstrus et l'ovulation n'était pas modifié. L'administration simultanée de PGF et le retrait du CIDR n'a pas influencée la régression lutéale, le synchronisme de l'œstrus et l'ovulation; par contre, les taures au stade de metestrus au début du traitement avaient des intervalles plus longs entre le retrait du CIDR et l'œstrus et l'ovulation.

(Traduit par Docteur Serge Messier)

Introduction

A protocol that allows timed artificial insemination (AI) in cattle through synchronization of ovulation, popularly referred to as "Ovsynch" (1), involves 2 treatments with gonadotropin releasing hormone (GnRH) given 9 d apart, and 1 prostaglandin $F_{2\alpha}$ (PGF) treatment 7 d after the 1st GnRH. The first GnRH treatment induces the release of luteinizing hormone (LH), resulting in ovulation of a responsive dominant follicle (if present), with emergence of a new follicular wave immediately after ovulation. The PGF is intended to induce luteolysis (GnRH-induced corpus luteum [CL], pre-existing CL, or both), and the 2nd GnRH is to synchronize ovulation of the new dominant follicle. Ovulation occurs 24 to 34 h after the 2nd GnRH injection (1,2), and cattle are typically timed-inseminated, either concurrent with the 2nd GnRH treatment or 12 to 16 h later.

The major advantage of the Ovsynch protocol, and similar protocols that synchronize ovulation, is that cattle can be bred without estrus detection. However, conception rates for heifers timed-

Dairy Research and Technology Centre (DRTC), Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta T6G 2P5 (Hittinger); DRTC — Alberta Agriculture, Food and Rural Development, 2nd Floor 6903 116 Street, Edmonton, Alberta T6H 5Z2 (Ambrose); Agriculture and Agri-Food Canada, Research Centre, 5403 1st Avenue, Box 3000, Lethbridge, Alberta T1J 4B1 (Kastelic).

Dr. Hittinger is currently enrolled in the DVM program at the Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4.

Address all correspondence and reprint requests to Dr. Divakar Ambrose; telephone: (780) 422-0807; fax: (780) 427-1439; e-mail: divakar.ambrose@gov.ab.ca

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inseminated following an Ovsynch protocol were poor (approximately 40%) in comparison to conception rates of heifers bred on detected estrus (3–5). Perhaps these poor conception rates are due to premature luteolysis and ovulation. In that regard, incorporation of an intravaginal, progesterone-releasing controlled internal drug release (CIDR) device (Bioniche Animal Health, Belleville, Ontario) into the Ovsynch protocol, could sustain progesterone concentrations high enough to prevent premature ovulation in cattle with premature luteolysis, thereby improving the odds of conception to timed-AI. However, few reports are available on the efficacy of CIDR-based protocols for the synchronization of ovulation in dairy heifers.

The label protocol for estrus synchronization using CIDR involves CIDR insertion on day 0 (without regard to the stage of the estrus cycle), followed by PGF treatment on day 6 and CIDR removal 1 d after PGF (day 7). A variation of this protocol, used successfully for synchronization of ovulation and timed-insemination (6,7), involves giving GnRH at CIDR insertion (day 0), PGF on day 7, and CIDR removal on day 8, based on the premise that the induced CL would be more responsive to PGF, given one more day to mature. Though $>$ 60% pregnancy rates were obtained (6), this protocol required animal handling on 5 occasions (including insemination). Increased handling may be stressful to the cattle and is time-consuming for producers.

The general objective of the present study was to determine if animal handling could be reduced without compromising luteal regression and synchrony of estrus and ovulation. Specific objectives were to determine, in Holstein heifers, the interval to luteal regression following PGF treatment, synchrony of estrus occurrence following CIDR removal, and the spontaneous ovulation response (in the absence of exogenous GnRH) when PGF was given concurrent with, or 24 h prior to, CIDR removal. We hypothesized that by administering PGF concurrent with CIDR removal, animal handling could be reduced without compromising luteolysis or the synchrony of estrus and ovulation.

Materials and methods

Animals

Postpubertal Holstein heifers (n = 18), approximately 15 mo of age, weighing between 380 and 420 kg were assigned randomly, but equally, to 1 of 3 treatment groups. Throughout the study, the heifers were housed indoors (in tie-stalls) with ad libitum access to feed and water, and were allowed access to an exercise pen for a minimum of 40 min daily. Prior to the initiation of the study, heifers were acclimatized to the indoor tie-stall environment over a 5 d period. The heifers were handled and cared for in accordance with the Canadian Council on Animal Care guidelines (8) and experimental procedures were approved by the Animal Policy and Welfare Committee, Department of Agricultural, Food and Nutritional Science, University of Alberta (Protocol 2000-26C).

Experimental protocols

On experimental day 0 (without regard to day of the estrus cycle), the vulva was cleaned with soapy water and a CIDR-device was gently placed intravaginally in each heifer. Approximately 3 cm of the plastic cord attached to the CIDR-device was trimmed (to minimize the length of the cord exterior to the vulvar lips). Immediately after CIDR insertion, heifers were given an injection of 100 μg gonadorelin acetate (Fertiline; Vetoquinol, Lavaltrie, Quebec), IM. There were 3 treatment groups, designated D7/D8, D8/D8, and D7/D7. Heifers in group D7/D8 received PGF (25 mg dinoprost tromethamine; Lutalyse; Pharmacia Animal Health, Orangeville, Ontario) on day 7 and the CIDR device was removed 24 h later (day 8). The CIDR device was removed concurrent with PGF treatment (on day 8 and day 7) in groups D8/D8 and D7/D7, respectively. The 2nd GnRH treatment, typically given 2 d after PGF in the standard Ovsynch program was omitted intentionally, to determine the approximate interval to spontaneous ovulation (after PGF treatment).

Blood sampling and progesterone assay

Blood samples were collected from the coccygeal vessels into heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) on days 0 and 17. In addition, samples were collected just prior to PGF treatment (day 7, 0 h) and then 4 more times at 12 h intervals (days 8 and 9). Samples were centrifuged for 20 min at 1500 $\times$ g and plasma was immediately harvested and stored at $-$20°C until analysis. Plasma progesterone concentrations were measured, in duplicate, by radioimmunoassay (Coat-A-Count Progesterone Invitro Diagnostic Test Kit; Diagnostic Products Corporation, Los Angeles, California, USA). The intra-assay coefficient of variation was $<$ 3%.

Ovarian ultrasonography

Ovaries of all heifers were examined on days 0, 2, 4, and 6 with a real-time ultrasound scanner (Aloka 500-V; Aloka Corporation, Tokyo, Japan) equipped with a 7.5 MHz linear-array transrectal transducer. Follicles were classified according to their diameter (class 1: $<$ 6 mm; class 2: $\geq$ 6 mm but $<$ 10 mm; class 3: $\geq$ 10 mm). Luteal status was monitored by ultrasound at each examination, based on size (diameter) and echodensity of the CL. When a newly emerged class 2 follicle became at least 2 mm larger (in diameter) than the next growing follicle, it was considered the dominant follicle. Ovaries were examined by ultrasound just prior to PGF treatment, then once daily for the next 48 h, and every 8 h thereafter (starting 56 h after PGF) to confirm ovulation, or to 144 h after PGF treatment if ovulation was not detected.

Estrus detection

To aid in estrus detection, Kamar heat detection devices (Kamar, Steamboat Springs, Colorado, USA) were placed on the tail head of all heifers on day 6. Starting on day 8, heifers were allowed access to an exercise pen for 30 to 40 min, thrice daily (05:00, 13:00, and 21:00 h), and observed for estrus behavior.

Stage of the estrus cycle

Though not a preplanned objective, there was an opportunity to determine the stage of the estrus cycle when the synchronization protocols were initiated. This was possible retroactively, on the basis of external signs (including behavior), ultrasonographic assessment of ovarian structures, and plasma progesterone concentrations. Heifers with overt behavioral signs of estrus, at least 1 class 3 follicle (in the absence of a distinct luteal structure), and plasma progesterone concentrations $<$ 1.0 ng/mL, were considered in estrus. Heifers with a sanguineous vaginal discharge, the absence of both a large follicle and distinct luteal tissue, the presence of a new CL or corpus

hemorrhagicum, and/or plasma progesterone concentrations < 1.0 ng/mL were considered in metestrus. Heifers with no overt signs of estrus, a distinct CL detected by ultrasonography, and plasma progesterone concentrations > 1.0 ng/mL were considered in diestrus.

Statistical analysis

Intervals from CIDR removal to standing estrus and to ovulation, interval from standing estrus to ovulation, and diameter of the ovulatory follicle on the last examination prior to ovulation were analyzed using the general linear model (PROC GLM; SAS, Cary, North Carolina, USA) analysis of variance (ANOVA) (9). For plasma progesterone concentrations, the effects of treatment, time of sampling, and their interaction, as well as the effects of stage of cycle, time of sampling and their interaction were analyzed using the mixed model procedure (PROC MIXED; SAS). Based on fit statistics for AIC, AICC, and BIC criteria, the covariance structure of the repeated measures was modeled using 'ante-dependence' with random effect for heifer and means separated by the probability of difference (PDIFF) function of SAS. For all analyses, differences were considered significant when $P < 0.05$.

Results

For plasma progesterone concentrations, there were significant effects of treatment ($P < 0.04$), time ($P < 0.01$), and a treatment by time interaction ($P < 0.01$). Mean (± standard error of mean [$s_{\bar{x}}$]) progesterone concentrations (ng/mL) at the time of GnRH treatment (day 0) did not differ significantly ($P > 0.12$) among groups D7/D7 (6.5 ± 1.4), D8/D8 (2.8 ± 1.4), and D7/D8 (2.7 ± 1.4). Likewise, there were no significant differences among treatment groups for progesterone concentrations at PGF treatment (0 h), or at 36 and 48 h after PGF treatment; however, there were differences among groups at 12 and 24 h after PGF (Figure 1). Progesterone concentrations declined to approximately 1.0 ng/mL within 12 h after PGF injection in the D7/D7 and D8/D8 groups (CIDR removed concurrent with PGF). However, in heifers in the D7/D8 group (CIDR removed 24 h after PGF), progesterone declined to about 2.0 ng/mL by 24 h after PGF and subsequently declined to < 1.0 ng/mL 12 h after CIDR removal (Figure 1). Progesterone concentrations 7 d after estrus (day 17) in the heifers that ovulated were 4.5 ± 0.5, 3.4 ± 0.5, and 3.2 ± 0.5 ng/mL ($P > 0.19$) for D7/D7, D8/D8, and D7/D8, respectively.

Based on ultrasonographic examinations and progesterone concentrations, spontaneous CL regression occurred prior to PGF treatment in 5 of the 18 heifers. However, none of these heifers was found to be in estrus prior to CIDR removal. Mean plasma progesterone concentration in these heifers at the time of PGF treatment (day 7) was 2.3 ng/mL (versus 6.5 ng/mL in the other heifers). The CIDR devices were retained in all heifers until removal occurred as scheduled.

The interval from CIDR removal to standing estrus did not differ significantly ($P = 0.80$) among groups (mean, 49.3 ± 6.2; range, 46.2 to 51.5 h). Similarly, the mean intervals (h) from CIDR removal to ovulation ($P = 0.95$) and from standing estrus to ovulation ($P = 0.14$) were not significantly different among groups (77.5 ± 9.0, range, 76.0 to 79.8; 31.0 ± 2.5, range, 27.6 to 35.5, respectively). The average day the dominant follicle was first identified as a class 2

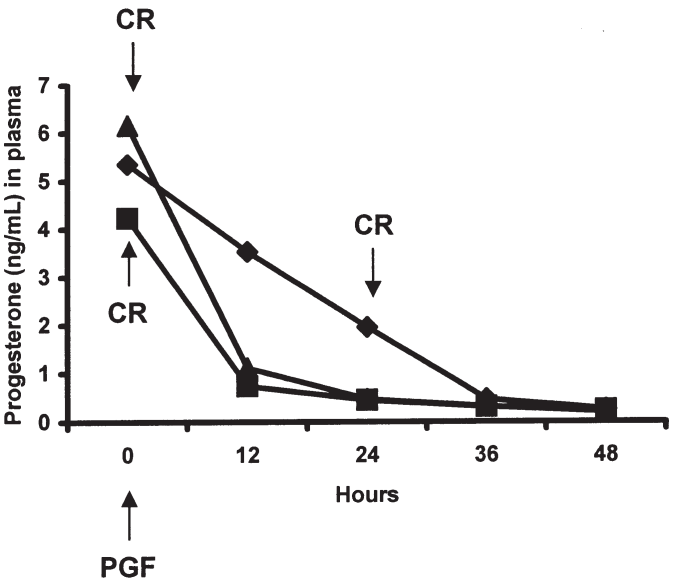

Figure 1. Plasma progesterone concentrations during the 48 h period following prostaglandin $F_{2\alpha}$ (PGF) injection (0 h). The controlled internal drug releasing (CIDR) device was removed concurrent with PGF injection in treatment groups D7/D7 (▲) and D8/D8 (■), but was removed 24 h later in the D7/D8 (◆) group. There were significant effects of treatment ($P < 0.04$), time ($P < 0.01$), and a treatment by time interaction ($P < 0.01$); progesterone concentrations were higher ($P < 0.05$) in the D7/D8 group than in the other 2 groups 12 and 24 h after PGF treatment.

follicle ranged from 5.0 to 5.8 d after GnRH treatment for all 3 groups ($P > 0.25$). When individual heifers were considered, the range was from 2 to 8 d. There was no difference ($P = 0.58$) among treatment groups in the diameter (mm) of the preovulatory follicle (13.5; range, 13.1 to 14.0). Only 5 of 18 heifers (28%) ovulated in response to the first GnRH, but 94% (16/17) ovulated spontaneously following PGF-induced luteolysis. In one heifer of group D7/D8, adhesions involving the reproductive tract were detected in the first 2 ultrasound examinations. Therefore, ovarian changes and spontaneous ovulation were not consistently determined for this heifer.

The number of heifers in estrus, metestrus, and diestrus, respectively, was 1, 0, and 5 for group D7/D7; 3, 1 and 2 for group D8/D8; and 1, 2, and 3 for group D7/D8. Ovulation in response to GnRH treatment, in heifers at estrus, metestrus, and diestrus was 5/5, 0/3, and 0/10, respectively. For plasma progesterone concentrations (Table I), there were effects of stage of estrus cycle ($P < 0.02$), time ($P < 0.01$), and an interaction of stage of cycle and time ($P < 0.01$). Progesterone concentrations were highest at the time of the GnRH injection (day 0) in diestrus stage heifers, but were highest in metestrus heifers 24, 36, and 48 h after PGF treatment. There was no significant effect due to stage of the cycle on plasma progesterone concentrations at the remaining sampling times. The interval (h) from CIDR removal to standing estrus was longer ($P < 0.05$) in heifers in metestrus (78.5 ± 5.3) than those in estrus (45.8 ± 3.3) or diestrus (44.5 ± 2.5). Similarly, the interval (h) from CIDR removal to ovulation was longer ($P = 0.01$) in metestrus stage heifers (112.0 ± 10.5) than those in estrus (76.0 ± 7.5) or diestrus (71.0 ± 4.7). The stage of the estrus cycle had no effect on either the interval from standing estrus to ovulation (mean, 31.4 h; $P > 0.75$) or on the diameter of the preovulatory follicle (mean, 13.2 mm; $P = 0.15$). However, preovulatory follicles were numerically smaller in metestrus stage

Table I. Plasma progesterone concentrations (least squares means ± standard error of mean [$s_{\bar{x}}$]) in heifers according to stage of the estrus cycle at the start of treatment. These heifers were given gonadotropin releasing hormone (GnRH) and a controlled internal drug release (CIDR) device (day 0), with CIDR removal after 7 or 8 d and prostaglandin $F_{2\alpha}$ (PGF) given concurrent with, or 24 h before CIDR removal. There were significant effects of stage of estrus cycle ($P < 0.02$), time ($P < 0.01$), and an interaction of stage of cycle and time ($P < 0.01$)

	Plasma progesterone concentration (ng/mL)			
Day or time	Estrus	Metestrus	Diestrus	Probability
D0 (at GnRH)	0.4 ± 0.9[a]	0.4 ± 1.1[a]	6.8 ± 0.6[b]	< 0.01
D7 or D8 (at PGF)	4.2 ± 1.3	7.5 ± 1.7	5.1 ± 0.9	= 0.30
Interval after PGF (h)				
12	1.4 ± 0.6	3.5 ± 0.8	1.5 ± 0.5	= 0.06
24	0.5 ± 0.3[a]	2.1 ± 0.4[b]	0.8 ± 0.2[a]	< 0.02
36	0.3 ± 0.1[a]	0.8 ± 0.1[b]	0.3 ± 0.1[a]	< 0.01
48	0.3 ± 0.0[a]	0.4 ± 0.1[b]	0.2 ± 0.0[a]	< 0.01
D17 (7 d after estrus)	4.0 ± 0.6	2.3 ± 0.8	4.0 ± 0.4	= 0.08

[a,b] Within a row, means a significant difference between numbers with those superscripts

heifers (11.8 mm) compared to heifers of estrus (14.1 mm) or diestrus (13.6 mm).

Discussion

The hypothesis that giving PGF concurrent with CIDR removal (thereby decreasing animal handling) would not compromise CL regression or the synchrony of estrus and ovulation was supported. Plasma progesterone concentrations in all heifers from groups D7/D7 and D8/D8 (PGF given concurrent with CIDR removal) declined precipitously to about 1.0 ng/mL by 12 h after PGF treatment/CIDR removal, indicating that PGF-induced luteolysis occurred synchronously. In group D7/D8, progesterone concentrations declined to approximately 2.0 ng/mL by 24 h after PGF treatment (due to the presence of the CIDR device), consistent with progesterone concentrations (2.8 ng/mL) in ovariectomized cattle given a CIDR (10). In heifers of the D7/D8 group, progesterone concentrations subsequently declined below 1.0 ng/mL only after CIDR removal. Therefore, although the presence of the CIDR for 24 h after PGF in the D7/D8 group prevented a direct comparison of luteolysis among the 3 groups, there is clear, compelling evidence that luteolysis was similar among the groups. Regardless of whether PGF was given concurrent with CIDR removal or 24 h earlier, functional regression of the CL was comparable among treatments.

One concern with the utilization of the Ovsynch protocol for timed-AI in heifers is spontaneous luteolysis, accompanied by premature estrus and ovulation, resulting in post-ovulation fixed-time AI and very low fertility (4). However, in the 5 heifers in the present study that underwent spontaneous luteolysis, the CIDR device kept progesterone concentrations elevated (> 2 ng/mL) and prevented the occurrence of premature estrus and ovulation. A modification of the protocol used in group D7/D8 has been successfully used in Holstein heifers (6,7); ovulation was synchronized by a 2nd GnRH treatment and pregnancy rates to timed-AI exceeded 60%. In the present study, plasma progesterone concentrations were maintained by the CIDR and decreased below 1.0 ng/mL by 12 h after CIDR removal in heifers from all 3 groups. There was no difference among groups in the interval from CIDR removal to estrus, indicating that synchrony of estrus was unaffected by the protocol. In a previous study utilizing a 7 d CIDR protocol (11), the interval from CIDR removal to onset of estrus was influenced by PGF treatment (44.6 versus 40.0 h, when PGF was given at CIDR removal versus 24 h earlier; $P < 0.05$) but not the rate of synchronous estrus. Since none of the heifers in the present study was detected to be in estrus prior to CIDR removal (including the 5 that underwent spontaneous luteolysis), the presence of a CIDR device apparently prevented the premature onset of estrus.

With the exception of 1, all heifers ovulated spontaneously following CIDR removal, with no difference among groups in the intervals from CIDR removal to ovulation and from standing estrus to ovulation. Therefore, the timing of PGF treatment did not affect the synchrony of ovulation. Furthermore, all heifers that ovulated had elevated (> 3.0 ng/mL) plasma progesterone concentrations 7 d after estrus, consistent with the presence of a functional CL.

Less than one-third of the heifers ovulated in response to the first GnRH injection in the present study. In a previous study (2), the ovulation response to the first GnRH treatment was lower in randomly cycling Holstein heifers (54%) than in cows (90%). In other studies, 50% to 58% of randomly cycling Holstein heifers ovulated (7,12) in response to the 1st GnRH injection, and 33% to 50% ovulation rates were reported (13) for crossbred dairy heifers. The reasons for the reduced rate of ovulation (to the 1st GnRH treatment) in dairy heifers are not well understood. However, it has been suggested that a more rapid turnover of follicular waves in heifers than in lactating cows increases the odds of administering the 1st GnRH injection in the absence of an active dominant follicle (14).

The average day the dominant follicle was first identified as a class 2 follicle, ranged from 5.0 to 5.8 d for all 3 groups. Even though this data was not based on daily ovarian ultrasonography, it is in agreement with that of Ambrose et al (6) who reported a mean of 5.2 d when using a protocol comparable to that used in group D7/D8 of the present study. Thus, although a large proportion of heifers failed to ovulate in response to the GnRH treatment, development of a new dominant follicle occurred following GnRH treatment in all heifers.

The stage of the estrus cycle when GnRH treatment is initiated is known to influence the ovulation response (12). For example,

following the 1st GnRH treatment in an Ovsynch protocol in heifers, ovulation rates were 0%, 64%, and 100% when the protocol was initiated in metestrus, diestrus, and proestrus, respectively (12). In the present study, 5/18 heifers were in estrus at the time of the GnRH treatment, and all 5 ovulated. However, none of the other heifers ovulated. Although the absence of ovulation in metestrus heifers was expected (because of the absence of a class 3 follicle), the complete lack of ovulatory response in diestrus stage heifers was rather unexpected. The GnRH product (Fertiline) used in this study has been tested and found effective to induce LH release in dairy heifers (7). Therefore, there was no reason to believe that GnRH-induced LH release did not occur. It seems likely that the heifers in diestrus were all at a stage of the estrus cycle (days 9 to 11 of the cycle) when a follicle responsive to GnRH-induced LH surge was absent (12).

Heifers in diestrus had the highest concentration of progesterone on day 0 (at the time of the GnRH treatment). Although progesterone concentrations 24, 36, and 48 h after PGF were highest in heifers that were in metestrus, they remained $<$ 1.0 ng/mL, 36 and 48 h after PGF treatment, indicating functional regression of the CL. That progesterone concentrations were highest in metestrus heifers 24 to 48 h after PGF was consistent with this group having the (numerically) highest progesterone concentration at the time of PGF treatment. However, plasma progesterone concentrations in these heifers were $<$ 1.0 ng/mL by 36 h after PGF treatment, indicating complete luteolysis. Consistent with the longer interval for progesterone concentrations to reach a baseline, onset of estrus and ovulation following CIDR removal, were delayed in metestrus heifers. They also tended to ovulate smaller follicles. If a 2nd injection of GnRH had been given, ovulation in these heifers would likely have occurred sooner. It has been previously reported (12) that GnRH treatment failed to synchronize the emergence of a new follicular wave in heifers when the treatment was initiated in metestrus (day 2 of the cycle); the resulting ovulation of an aged follicle may affect subsequent functional competence of the oocyte and CL. Moreira et al (12) found that the day of the cycle at initiation of the Ovsynch/timed-AI protocol affected synchrony, and suggested early diestrus (between days 5 and 10 of the cycle) as the optimal time for initiation of GnRH treatment in heifers.

In conclusion, giving PGF concurrent with CIDR removal did not affect luteal regression or the synchrony of estrus and ovulation in dairy heifers. Therefore, giving GnRH at CIDR insertion and PGF at CIDR removal (7 or 8 d later) should effectively synchronize estrus and ovulation in dairy heifers. To enable fixed-time AI, a 2nd injection of GnRH should be given approximately 48 h after PGF, with timed-AI performed either concurrent with GnRH or 12 to 16 h later. Large-scale field trials using the tested protocols and modified protocols (including a 2nd GnRH to synchronize ovulation) are essential to determine the pregnancy outcome in dairy heifers following AI at detected estrus and timed-AI, respectively.

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