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Inhibition of prostaglandin biosynthesis during postluteolysis and effects on CL regression, prolactin, and ovulation in heifers

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Abstract

The beginning of postluteolysis (progesterone, <1 ng mL^{-1}) in heifers was targeted by using 8 h after ultrasonic detection of a 25% decrease in CL area (cm^2) and was designated Hour 0. Flunixin meglumine (FM; n = 10) to inhibit $PGF_{2\alpha}$ secretion or vehicle (n = 9) were given intramuscularly at Hours 0, 4, 8, 16, 24, 32, and 40. The dose of FM was 2.5 mg/kg at each treatment. Blood sampling and measurement of the CL and dominant follicle were done every 8 h beginning 14 days postovulation in each group. Blood samples for detection of pulses of PRL and pulses of a metabolite of $PGF_{2\alpha}$ (PGFM) were obtained every hour for 24 h beginning at Hour 0. Pulse concentrations of both PGFM and PRL were lower in the FM group than in the vehicle group. Concentration of PRL was greatest at the peak of a PGFM pulse. Neither CL area (cm^2) nor progesterone concentration differed between groups during Hours 0 to 48 (postluteolysis). Ovulation occurred in nine of nine heifers in the vehicle group and in three of 10 heifers in the FM group. The anovulatory follicles in the FM group grew to 36.2 ± 2.9 mm, and the wall became thickened from apparent luteinization. The hypothesis that $PGF_{2\alpha}$ was involved in the continued P4 decrease and structural CL regression during postluteolysis was not supported. However, the hypotheses that pulses of PGFM and PRL were temporally related and that systemic FM treatment induced an anovulatory follicle were supported.

Keywords: Anovulation; Cattle; Luteolysis; $PGF_{2\alpha}$; Progesterone

1. Introduction

Prostaglandins are involved in many reproductive processes in cattle, such as luteolysis, ovulation, implantation, pregnancy, parturition, and postpartum physiology [1]. Secretion of $PGF_{2\alpha}$ by the endometrium on approximately Day 17 (Day 0 = ovulation) is well known for its fundamental role in luteolysis [2,3]. Assay of the concentration of a $PGF_{2\alpha}$ metabolite (PGFM) is often used to represent $PGF_{2\alpha}$ concentration in the systemic circulation [4]. The interval between PGFM pulse peaks is approximately 7 h during luteolysis in cattle [5,6]. Secretion of $PGF_{2\alpha}$ in pulses is a component of the luteolytic mechanism in cattle [7], as well as in sheep [8] and horses [9]. In cattle, the PGFM pulses are approximately 40% lower at the peak and the interpulse interval is 17% shorter during the postluteolytic period than during the luteolytic period [5,6].

For consistency among experiments, functional luteolysis in cattle is usually considered to end when

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progesterone (P4) decreases to 1.0 ng/mL, and postluteolysis has been defined as beginning at 0.9 ng/mL [10]. However, P4 concentrations gradually decline during postluteolysis. In one study with hourly sampling, P4 decreased from 0.9 to 0.1 ng/mL in 41 h in temporal association with a continuation of PGFM pulses [6]. It is unknown whether the continued secretion of $PGF_{2\alpha}$ is required during postluteolysis, either for the continued decrease in P4 concentration or continued morphologic regression of the CL.

After diminishing during postluteolysis, the secretion of prostaglandins increases late in the preovulatory period and plays a role in the ovulatory process [11,12]. Concentrations of $PGF_{2\alpha}$ and PGE_2 in follicular fluid increase between 24 and 30 h after the peak of the endogenous LH surge and at about the time of ovulation in cattle [13–15]. The prostaglandins apparently have an essential role in the ovulatory process in many species. Ovulation is blocked by the treatment with indomethacin, a prostaglandin inhibitor, in the doe rabbit [16], sow [17,18], ewe [19], woman [20], and mare [21], and by treatment with flunixin meglumine (FM) in the mare [22–24]. In the cow, ovulation is effectively blocked by intraovarian treatment with indomethacin [25] and intrafollicular treatment with a selective prostaglandin inhibitor, NS-398 [26]. However, the effect of systemic administration of a prostaglandin inhibitor during postluteolysis on follicle growth and anovulation and outcome of the induced anovulatory follicle in cattle has not been reported.

The synthesis of $PGF_{2\alpha}$ and PGE_2 involves release of arachidonic acid (AA) from membrane phospholipids, catalyzed by the hormone-responsive enzyme cytosolic phospholipase A_2 [27]. Flunixin meglumine, one of the commonly used nonsteroidal anti-inflammatory drugs (NSAIDs), inhibits prostaglandin synthesis by competing with AA for active binding sites on the cyclooxygenase (COX) enzyme [28,29]. The COX converts AA into PGH_2 that is further converted to other prostaglandins, such as $PGF_{2\alpha}$ and PGE_2 [29]. Treatment of heifers with FM (2.5 mg/kg body weight) every 8 h on Day 16 reduced the prominence of pulses of $PGF_{2\alpha}$ during preluteolysis and luteolysis, as represented by plasma PGFM concentration [30].

The PGFM pulses after the beginning of luteolysis are temporally associated with an increase in PRL concentrations and with an increase in PRL pulsatility in heifers [31] and mares [32]. Synchrony (pulse peaks at same hour) of the peaks of PGFM and PRL pulses in heifers is greatest during the 12 h before the end of luteolysis and the first 12 h of postluteolysis [31]. The concentration of PRL centralized to the peak of a PGFM pulse is greatest at the peak [31]. However, whether one hormone has a direct positive effect on the other or other factors are involved in the synchrony has not been determined. The role of PRL pulses during postluteolysis and in the growth of the dominant follicle is not known, but the postluteolytic period is temporally associated with continued regression of the structure of the CL and with final development of the dominant follicle of the ovulatory follicular wave.

The purpose of the current experiment during postluteolysis in cattle was to test the following hypotheses: (1) pulses of $PGF_{2\alpha}$ and PRL are temporally related; (2) $PGF_{2\alpha}$ is involved in the continued functional (P4 production) regression and structural regression of the CL during postluteolysis; and (3) systemic administration of FM interferes with ovulation. Consideration was also given to the effects of inhibition of prostaglandin synthesis by systemic FM treatment on the growth of the dominant follicle.

2. Materials and methods

2.1. Heifers

Dairy heifers (Holstein) aged 23 to 29 mo and weighing 560 ± 19 kg (range: 425–690 kg) were used during March and April in the northern temperate zone. Heifers were selected with docile temperament and no apparent abnormalities of the reproductive tract, as determined by ultrasound examination [33]. Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research. The heifers were kept under natural light in an open shelter and outdoor paddock and were maintained by *ad libitum* access to alfalfa and grass hay, water, and trace-mineralized salt. Heifers remained healthy and in good body condition throughout the experiment. The day of ovulation (Day 0) was determined by daily transrectal ultrasound examinations [33]. If more than one CL was present or the single CL was considered undersized (<3 cm^2) on Day 14, the heifer was not used.

2.2. Experimental design and treatments

The experimental protocol is illustrated (Fig. 1). Heifers were assigned randomly by replicate on Day 14 to a vehicle group and an FM-treated group (n = 10/group). Heifers were scanned by transrectal ultrasonography every 8 h starting at 7:00 AM on Day 14 to

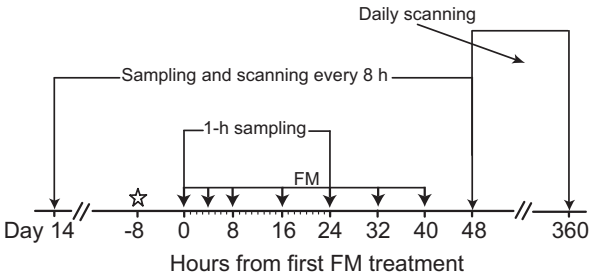

Fig. 1. Diagram of experimental design. The hour of detection of structural luteolysis is indicated by a star at Hour −8 and was designated as occurring at the 8-h scanning when maximum CL area (cm^2) had decreased to 25% of the area after the first examination 14 d postovulation. FM (2.5 mg/kilogram body weight) was given to heifers at each indicated hour.

determine the beginning of structural luteolysis, as indicated by a decrease in the size of the CL. The hour of first detection of structural luteolysis was defined as the scan at 8-h intervals when maximum CL area (cm^2) decreased by 25% of the area on Day 14. Within 2 h after the detection of beginning of structural luteolysis, heifers were sedated as described [34]. Sedation was done only for insertion of an indwelling catheter into a jugular vein, as described [35]. The first treatment was given 8 h after detection of structural luteolysis and was designated Hour 0. The 6 to 8-h delay between catheter insertion and the first treatment was used to minimize potential effects of sedation and catheterization on the experimental endpoints and to allow further CL regression. It was expected that the heifers would be in late luteolysis or early postluteolysis at Hour 0. The beginning of postluteolysis was defined as the 8-h sample when P4 first decreased to <1 ng/mL [3,36]. Heifers in the vehicle and FM groups were treated intramuscularly at Hours 0, 4, 8, 16, 24, 32, and 40. Heifers in the vehicle group were given 0.05 mL/kg of physiological saline, and heifers in the FM group were given FM at a dose of 2.5 mg/kg at each treatment. The FM dose and interval between treatments were based on the results of a previous study [30]. Decreases in P4 concentration and CL area (cm^2) were defined as functional and structural luteolysis, respectively.

A blood sample (10 mL) was taken through the jugular catheter each hour for a 24-h session from Hour 0 to Hour 24 to study concentration changes in PGFM and PRL and in the number, location, and prominence of PGFM and PRL pulses. Heifers had free access to water and hay between collections of hourly samples. In addition, blood samples for P4 assay were taken from the coccygeal vein every 8 h from 7:00 AM on Day 14 until Hour 48 to determine the end of functional luteolysis in each heifer. Data analyses for the hourly sampling were limited to the heifers that were late in luteolysis or at the beginning of postluteolysis at first treatment. It was judged that the heifers in late luteolysis or early postluteolysis at the hour of treatment would be most suited for testing the hypotheses; PRL pulses are most prominent, and the synchrony between PGFM and PRL pulses is greatest during the 12 h before and the 12 h after the end of luteolysis [31].

2.3. Identification of PGFM and PRL pulses

In each heifer during the 24-h session of hourly sampling, a transient hormone increase and decrease (fluctuation) encompassing at least four values in PGFM [3] or three values in PRL [31], including nadirs with a progressive increase on the ascending portion and progressive decrease on the descending portion, were evaluated. When the CV of the values composing a fluctuation was at least three times greater than the mean intra-assay CV, the fluctuation was defined as a pulse [3,5,37]. These definitions were based on reports that the pulsatile episodes of PGFM [4,38] and PRL [31] during luteolysis are detectable by sampling blood at 1-h intervals. Pulses of PGFM and PRL with a peak near the beginning or end of the 24-h sampling session were included in the CV analyses only if the peak was preceded or followed, respectively, by at least one lower value, but the lower value was not used to calculate nadir concentrations. Nadirs 1 and 2 were defined as the nadirs at the beginning and end of a pulse, respectively. When two identified PGFM peaks were separated by only 1 h, the two peaks were considered part of the same pulse, and the peak with the greatest concentration was used in the analyses; double peaks occurred in 13% of PGFM pulses.

Synchrony between PGFM and PRL pulses was defined as the occurrence of the peak of CV-identified pulses of both PGFM and PRL at the same hour. Pulses of PGFM were partitioned into nonprominent and prominent pulses based on pulses with peaks of <50 and ≥50 pg/mL, respectively, owing to the report that nonprominent pulses were ineffective [39]. The prominence of PRL pulses was also considered, using pulses with peaks of <10 and ≥10 ng/mL to represent nonprominent and prominent pulses, respectively, as reported [31]. Pulse prominence was considered in the temporal relationship between the concentrations of PRL during a PGFM pulse and concentrations of PGFM during a PRL pulse.

2.4. Ultrasound scanning

The ovaries were scanned by transrectal ultrasound every 8 h, beginning on day 14 until Hour 48 (Hour 0 = first treatment), and then daily until Hour 360 if ovulation did not occur. A duplex B-mode (gray-scale) and pulse-wave color Doppler ultrasound instrument (Aloka SSD 3500; Aloka America, Wallingford, CT, USA) equipped with a 7.5-MHz linear transducer was used for the scanning. The diameter of the four largest follicles and the maximum CL area (cm^2) were recorded. The four largest follicles were used so the growth of the one follicle among the four that became the preovulatory follicle could be assessed. The maximum CL area was determined using a B-mode still image and the tracing function. For CL with an anechoic fluid-filled cavity, the area of the cavity was subtracted from the total area [40,41]. Anovulation was defined as failure of ovulation of a dominant follicle that reached 15 to 17 mm and then maintained or increased in diameter for ≥5 days. Luteinization of an anovulatory follicle was assumed by thickening and increased echogenicity of the follicular wall by visual evaluation on the gray-scale image, as described [23]. The presence of blood flow in the luteinized wall of an anovulatory follicle was detected by color-Doppler signals, as described [42]. The gray-scale and color-Doppler observations were used only for preliminary information and were not analyzed critically.

2.5. End points

Comparisons between groups for the concentrations of PGFM and PRL were made during the 24-h session of hourly sampling. End points for the CV-identified PGFM and PRL pulses were the overall mean for all values in a pulse and area under curve of a pulse and concentrations at pulse events (peak, Nadir 1, Nadir 2, amplitude). Comparisons between groups were made of the intervals between Nadir 1 and Nadir 2 of a pulse, the interval from peak to peak and Nadir 2 to Nadir 1 of adjacent pulses, the number of PGFM and PRL pulses during the 24-h hourly sampling session, and the frequency of synchrony between PGFM and PRL pulses (synchrony = both pulses peak at same hour). The number of PGFM pulses/session was compared to the number of PRL pulses for each treatment group. The concentration profile of PRL during a PGFM pulse and concentration of PGFM during a PRL pulse were determined. Thus, the frequency of pulse synchrony and concentrations of one hormone relative to the other used either the PGFM pulse or the PRL pulse as the reference pulse.

In addition to the PGFM and PRL end points, P4 concentration, CL area (cm^2) at the 8-h scanning intervals for Hours 0 to 48, and the diameter of the dominant follicle from Hour 0 to ovulation or Hour 96 were compared between the two groups. Comparisons also were made between groups for the interval from first treatment to ovulation, the number of heifers in which the dominant follicle ovulated or did not ovulate, and the number of anovulatory follicles that apparently became luteinized based on increased thickening of the wall of the anovulatory follicle.

2.6. Hormone assays

Blood samples were immediately placed in ice water for 10 min before centrifuging (2000*g* for 10 min). The plasma was decanted and stored (−20°C) until assay. The plasma samples were assayed for PGFM by an ELISA that was developed and validated in our laboratory for use with bovine plasma [5]. Plasma concentrations of PRL were determined by an RIA that was described and validated for use in bovine plasma in our laboratory [43]. Plasma P4 concentrations were assayed with a solid-phase RIA kit containing antibody-coated tubes and I^{125}-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) as validated and described [3]. The intra- and interassay CV and sensitivity, respectively, were 10.1, 9.8%, and 13.7 pg/mL for PGFM, and were 10.0, 3.9%, and 0.39 ng/mL for PRL. The intraassay and sensitivity for P4, were 8.7%, and 0.02 ng/mL, respectively.

2.7. Statistical analyses

Data were examined for normality using the Shapiro-Wilk test. Data that were not normally distributed were transformed to natural logarithms, ranks, or square roots. Hourly concentrations of PGFM and PRL and the P4, CL, and follicle endpoints at 8-h intervals were analyzed for the main effects of group and hour and the interaction of group by hour. For analyses involving pulse prominence, a three-way factorial was used with group, hour, and prominence as factors. The SAS PROC MIXED procedure (Version 9.2; SAS Institute, Cary, NC, USA) was used for analysis, with a REPEATED statement to account for the autocorrelation between sequential measurements. The least significant difference (LSD) test was used for comparisons between hours within a group. Discrete or single-point data for characteristics and intervals for PGFM and PRL pulses were analyzed for differences between groups by Student's unpaired *t* test. The LSD test was used to locate differences among hours within each

group. Student's unpaired *t* test was used to locate differences between follicle diameters for each hour. Fisher's exact test and χ^2 distribution were used for comparisons of frequency data. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to $P \leq 0.1$ indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

3. Results

Probabilities for a group effect, hour effect, and a group-by-hour interaction for the factorial analyses are given in the figures and the probabilities for differences in discrete endpoints are given in the table or text. Concentrations of P4 at 8-h intervals indicated that one heifer in the vehicle group was in preluteolysis at the time of the first treatment and was not used in the analyses. In the remaining 19 heifers, a 25% reduction in CL area (cm^2) occurred on Day 18.0 ± 0.6 (Fig. 2). Eight h after the 25% reduction in CL area (hour of first treatment; Hour 0) the P4 concentration was <1 ng/mL in 15 heifers, indicating they were in postluteolysis. In the remaining four heifers, concentration was >1 ng/mL at Hour 0 (1.2–2.8 ng/mL). The mean P4 concentration for all heifers was 0.8 ± 0.1 ng/mL at Hour 0 and 0.2 ± 0.03 ng/mL at Hour 48. Therefore, all 19 heifers (9 and 10 in the vehicle and FM groups, respectively) were used in the analysis without subgrouping into heifers that were in luteolysis vs. postluteolysis at Hour 0. Both CL area and P4 concentration decreased progressively from Hours 0 to 48 (hour effect) with no significant difference between the vehicle and FM groups or an interaction in CL area (cm^2) or in P4 concentration during the 8-h intervals from Hours 0 to 48 (Fig. 2).

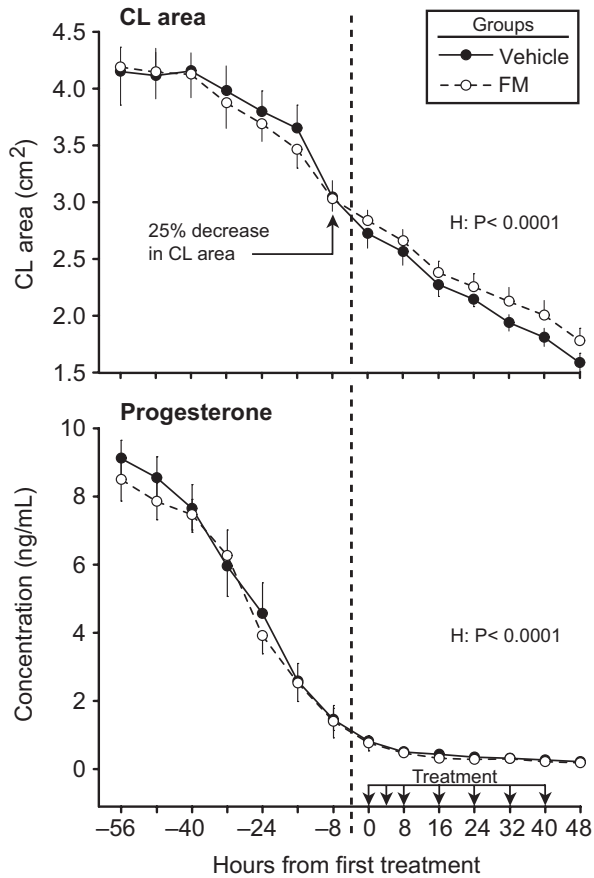

Fig. 2. Mean ± SEM area (cm^2) of the CL and concentrations of progesterone in a vehicle group (n = 9) and a FM group (n = 10) treated at Hours 0, 4, 8, 16, 24, 32, and 40 (Hour 0 = 8 h after detection of structural luteolysis as indicated). The factorial analysis was done from Hours 0 to 48. Main effect of Hour (H) is shown. There was not a main effect of group or a group by hour interaction for either endpoint.

Concentrations of PGFM during the 24 h of hourly sampling had significant group and hour effects and an interaction that approached significance (Fig. 3). The group effect represented a lower mean PGFM concentration in the FM group (23.2 ± 3.1 pg/mL) than in the vehicle group (40.7 ± 4.4 pg/mL). The tendency for an interaction was attributable primarily to significantly reduced concentrations in the FM group during Hours 6 to 14, 17, and 22 to 24. There were fewer CV-identified pulses of PGFM/heifer during a 24-h session in the FM group than in the vehicle group (Table 1). The PGFM pulses had a group effect from lower overall concentrations in the FM group than in the vehicle group (Fig. 3, Table 1). The hour effect was significant as expected. An interaction represented in part lower concentrations in the FM group at −1, 1, and 2 h (0 h = peak). Most discrete or single-point characteristics of PGFM pulses were similar between groups, but the concentration at Nadir 2 was less and the area under the curve tended to be less in the FM group (Table 1). If only three values for a PGFM pulse had been accepted for pulse identification, additional pulses of PGFM would have been detected by the CV method in only 1 of 9 heifers in the vehicle group compared to 7 of 10 heifers in the FM group ($P < 0.02$).

The hourly PRL concentrations had only an hour effect, reflecting transient increases ($P \leq 0.05$) over Hours 5 to 13 and Hours 15 to 21 (Fig. 3). There were fewer CV-identified PRL pulses/heifer in the FM group than in the vehicle group (Table 1). Although the factorial analysis of pulses showed only the expected hour

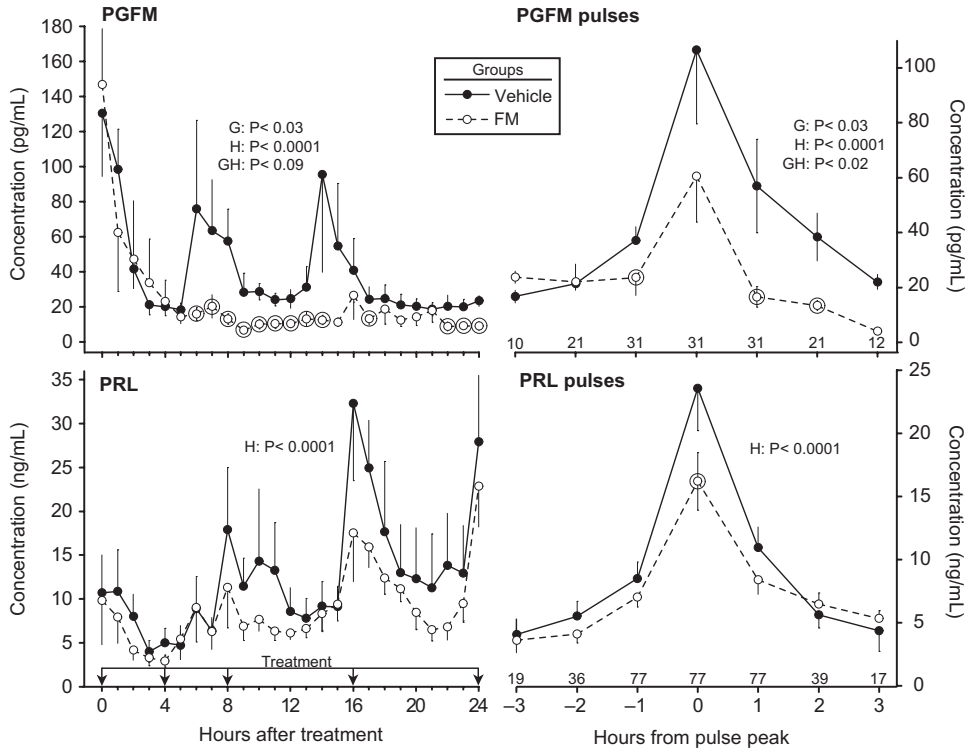

Fig. 3. Mean ± SEM hourly and pulse concentrations for PGFM and PRL in a vehicle group (n = 9) and a FM-treated group (n = 10). The combined number of observations for pulses are shown above the hour scale for each hour of a pulse. Main effects of group (G) and hour (H) and the interaction (GH) are shown. A circle around an FM mean indicates a difference ($P < 0.05$) from the vehicle mean at that hour.

effect (Fig. 3), the discrete data indicated that area under the curve and concentration at the pulse peak and at Nadir 2 were significantly less in the FM group than in the vehicle group (Table 1). Nadir 1 and amplitude of PRL pulses approached lower concentrations in the FM group. The intervals between all PRL pulse events were greater or approached being greater in the FM group than in the vehicle group. In each of the vehicle and FM groups, there were more ($P < 0.0001$) PRL pulses than PGFM pulses (Table 1).

During a PGFM pulse with a peak (0 h) of <50 vs. ≥50 pg/mL representing nonprominent vs. prominent pulses, respectively, the associated concentrations of PRL showed only an interaction of prominence (<50 vs. ≥50 pg/mL) and hour (Fig. 4). The interaction represented an increase in PRL concentrations in each of the vehicle and FM groups between −1 and 0 h and a decrease between 0 and 1 h for the prominent PGFM pulses. There were no changes in PRL during nonprominent PGFM pulses. The effect of treatment group and all interactions involving group were not significant. The PRL overall concentration during prominent PGFM pulses combined for vehicle and FM groups (24.2 ± 6.2 ng/mL) was greater ($P < 0.02$) than during the combined nonprominent PGFM pulses (9.1 ± 2.0 ng/mL). In addition, the area under the PRL curve (ng/mL h) was greater ($P < 0.05$) in each group when associated with a prominent PGFM pulse (vehicle, 19.6 ± 6.1; FM, 21.8 ± 18.4) than when associated with a nonprominent pulse (vehicle, 6.4 ± 1.5; FM, 5.8 ± 2.2). Combined for both groups, the peak of a prominent PGFM pulse was synchronized (same hour) with the peak of a prominent PRL pulse (≥10 ng/mL) in 8/17 (47%) pulses. In contrast, the peak of a prominent PRL pulse was synchronized with the peak of a prominent PGFM pulse in 8/50 (16%) prominent PRL pulses (8 of 17 vs. 8 of 50, $P < 0.01$).

The concentrations of PGFM centralized to the peak of a nonprominent PRL pulse (<10 ng/mL) and to the peak of a prominent pulse (≥10 ng/mL) are shown (Fig. 4). At −1 to 1 h of a PRL pulse, the associated concentrations of PGFM showed a group effect (vehicle, 48.3 ± 7.7 pg/mL; FM, 16.4 ± 2.1 pg/mL) and an hour effect (−1 h, 28.2 ± 5.3 pg/mL^{a}; 0 h, 45.0 ± 10.4 pg/mL^{b}; 1 h, 28.2 ± 6.3 pg/mL^{a}); means with different letters are different ($P < 0.05$). The prominence effect

Table 1
Mean ± SEM for discrete end points during PGFM and PRL pulses in heifers treated with vehicle (n = 9) or FM (n = 10).

	PGFM			PRL		
Pulse characteristic	Vehicle	FM	Probability	Vehicle	FM	Probability
No. pulses	24	7	—	44	33	—
No. pulses/24h	2.7 ± 0.4	0.7 ± 0.2	P < 0.0001	4.9 ± 0.3	3.3 ± 0.3	P < 0.0002
PGFM (pg/mL); PRL (ng/mL)						
Overall[a]	48.5 ± 6.8	26.5 ± 4.9	P < 0.03	11.8 ± 1.1	8.6 ± 0.7	NS
At Nadir 1[b]	19.6 ± 3.1	13.1 ± 5.4	NS	6.5 ± 1.1	4.5 ± 0.7	P < 0.07
At peak	105.1 ± 26.9	59.1 ± 16.7	NS	23.7 ± 2.4	16.3 ± 2.3	P < 0.04
At Nadir 2[b]	14.8 ± 1.8	7.3 ± 1.5	P < 0.02	7.8 ± 1.5	5.1 ± 0.6	P < 0.05
Amplitude[c]	82.0 ± 28.1	46.0 ± 17.0	NS	18.1 ± 3.2	12.3 ± 2.3	P < 0.08
Area under curve[d]	100.5 ± 33.4	50.9 ± 19.1	P < 0.1	19.8 ± 3.2	11.9 ± 2.6	P < 0.04
Intervals (h)						
Nadir 1 to Nadir 2[b]	4.8 ± 0.4	4.0 ± 0.7	NS	3.5 ± 0.2	4.6 ± 0.4	P < 0.01
Nadir 1 to peak[b]	2.2 ± 0.2	2.3 ± 0.5	NS	1.7 ± 0.2	2.4 ± 0.3	P < 0.01
Peak to Nadir 2[b]	2.4 ± 0.3	1.7 ± 0.3	P < 0.1	1.8 ± 0.2	2.3 ± 0.3	P < 0.07
Peak to peak	6.1 ± 0.7	—	—	3.7 ± 0.3	5.0 ± 0.6	P < 0.02
Nadir 2 to Nadir 1[b]	1.0 ± 0.4	—	—	0.4 ± 0.2	1.0 ± 0.5	P < 0.1

NS, not significant.
[a] Overall refers to the average of all values in the pulse.
[b] Nadir 1 and Nadir 2 are at the beginning and end of a pulse, respectively. The interval from Nadir 2 to Nadir 1 refers to adjacent pulses.
[c] Amplitude is the concentration at the peak minus the concentration at Nadir 1.
[d] Area under the curve (ng/mL h) of a pulse was assessed for −1, 0, and 1 h.

was not significant, and the only significant interaction was for prominence by hour. Significant changes in PGFM concentrations among hours were found only in the vehicle group with a prominent PRL pulse (≥10 ng/mL) as shown (Fig. 4).

At least one PGFM pulse was detected in each heifer in the vehicle group. In the FM group, PGFM pulses were detected in six heifers, and no pulses were detected in four heifers (0 of 9 vs. 4 of 10; P < 0.05). Analysis of these two FM subgroups for the hourly concentration of PRL during the 24-h session showed subgroup and hour effects (Fig. 5). The subgroup effect represented greater overall PRL concentration in the heifers with PGFM pulses (10.8 ± 0.8 ng/mL) than in the heifers with no PGFM pulses (7.3 ± 0.8 ng/mL). Although the interaction was not significant, PRL concentrations were lower in the heifers with no detected PGFM pulses at Hours 5 to 8 and 13.

The diameter of the dominant follicle for Hours 0 to 96 showed an hour effect and an interaction of group by hour (Fig. 6). The interaction represented greater diameter of the dominant follicle in the FM group at Hour 72 (approached significance; P ≤ 0.1) and at Hour 96 (P < 0.05). The number of heifers that ovulated was greater (P < 0.0004) in the vehicle group (9 of 9) than in the FM group (3 of 10). For the heifers that ovulated, the interval from Hour 40 (last treatment) to ovulation was not different between the vehicle group (61.3 ± 12.5 h) and the FM group (48.0 ± 8.0 h). The increasing diameter of the seven anovulatory follicles in the FM group is shown (Fig. 6). The anovulatory follicles reached a maximum diameter of 36.2 ± 2.9 mm. Two of seven anovulatory follicles collapsed during scanning at Hours 144 and 148. The remaining anovulatory follicles (n = 5) collapsed or began to regress 10 days after the first treatment. It was estimated that gray-scale indicators of luteinization of the follicle wall (increased thickness and echogenicity) of the anovulatory follicles began at Hour 92 ± 13. Color-Doppler signals of blood flow were detected in all luteinized follicles.

4. Discussion

The technique of using an ultrasonically detected 25% reduction in CL area (cm^2) and beginning the treatments 8 h later for targeting late luteolysis and postluteolysis for beginning the treatment challenges was successful in 95% of heifers. Most heifers (79%) were in postluteolysis (P4 <1 ng/mL) at the first treatment (Hour 0). The remaining 21% were in the later portion of functional luteolysis (P4 mean, 1.7 ng/mL). Beginning treatment at the 8-h interval with 25% in CL reduction, rather than 8-h later, would have resulted in 50% of heifers being in luteolysis.

The PGFM concentrations and pulsatility during postluteolysis in the dairy heifers in the vehicle group

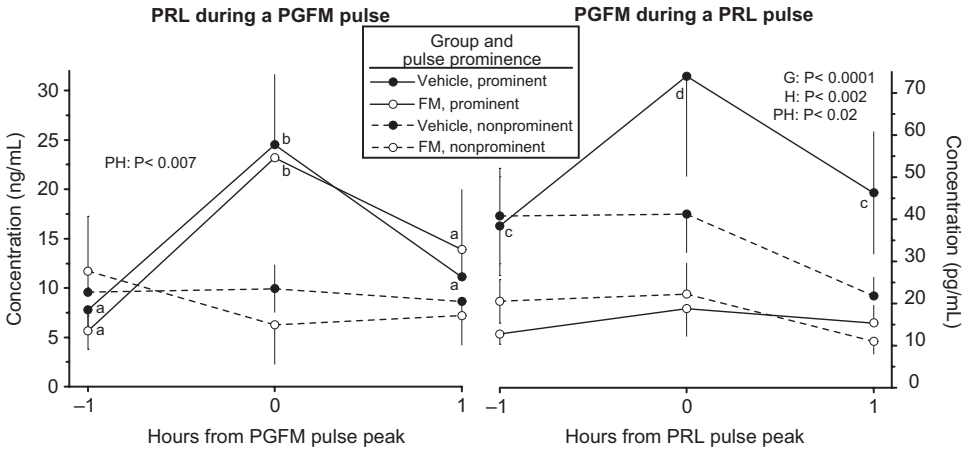

Fig. 4. Mean ± SEM for concentrations of PRL within the hours of a PGFM pulse centralized to the PGFM peak (left panel) and concentrations of PGFM within the hours of a PRL pulse centralized to the PRL peak (right panel) in the vehicle group and the FM-treated group. Each group is partitioned into prominent pulses and nonprominent pulses, as indicated by peak concentrations (PGFM ≥50 vs. <50 pg/mL; PRL ≥10 vs. <10 ng/mL). In the three-way factorial analysis (group, hour, prominence) for PRL concentrations during a PGFM pulse, only the interaction of PH was significant. For PGFM concentrations during a PRL pulse, the main effect of group (G) and hour (H) are shown.

ab = means within the vehicle group with prominent pulses and within the FM group with prominent pulses with different letters are different among hours. cd = means within the vehicle group with prominent pulses with different letters are different ($P < 0.05$) among hours. The only significant interaction was PH.

seemed similar to those of previous reports [5,44]. Treatment with FM during postluteolysis in the current study effectively reduced the concentrations and pulses of PGFM. This was indicated in the FM group by the approximately 2-fold reduction in each of the mean hourly concentrations, overall pulse concentrations, and area under the curve of PGFM pulses and a 4-fold reduction in number of pulses. These results indicated

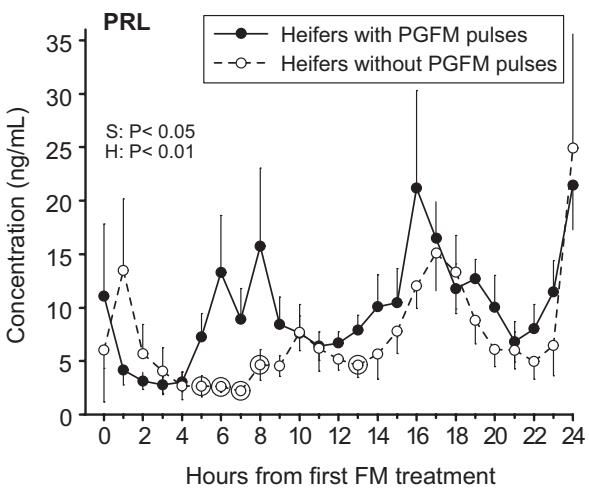

Fig. 5. Mean ± SEM for concentrations during hourly blood sampling of PRL in heifers of the FM group with PGFM pulses (n = 6) and without PGFM pulses (n = 4). Main effects of subgroup (S) and hour (H) are shown. A circle around a subgroup mean for heifers without PGFM pulses indicates a difference ($P < 0.05$) from the mean for the other subgroup.

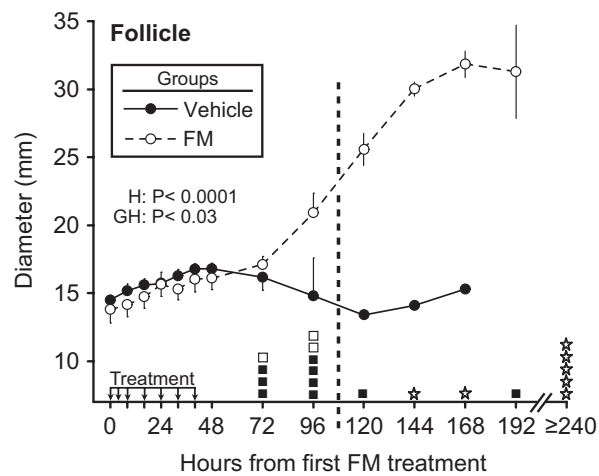

Fig. 6. Mean ± SEM diameter of the dominant follicle in a vehicle group (n = 9) and a FM group (n = 10) treated at Hours 0, 4, 8, 16, 24, 32, and 40 (Hour 0 = 8 h after detection of structural luteolysis). The factorial analysis was done from Hours 0 to 96. Main effect of hour (H) and interaction of GH are shown. The squares above the hour scale represent the hours of ovulation in individuals of the vehicle group (solid squares) and FM group (open squares). The stars represent the hour of detection of collapse or regression of the luteinized anovulatory follicle in individuals in the FM group.

that the effect of FM treatment on reduction in PGFM concentrations and pulses in heifers during postluteolysis was similar to the reported effects during preluteolysis and luteolysis with the same FM dose [30]. The PGFM concentrations in the current study in the FM group were reduced to below baseline levels, as indicated by hourly concentrations for most hours and by the nadirs of PGFM pulses.

Hypothesis 1: That pulses of $PGF_{2\alpha}$ and PRL are temporally related during postluteolysis— was supported. This was indicated by (1) the reduction in PGFM concentrations and pulses in the FM group was associated with a reduction in number of pulses of PRL, reduced area under the curve of PRL pulses, and the significant or approaching significant decrease in PRL concentrations at the components of the PRL pulses; (2) increased concentration of PRL at the peak and area under the curve when centralized to the peak of a prominent PGFM pulse (≥50 pg/mL) in the vehicle and FM groups; (3) reduced PRL concentrations in the FM group in heifers that did not have PGFM pulses; and (4) considerable synchrony (47%; peaks at same hour) of a prominent PGFM pulse (≥50 pg/mL) with a prominent PRL pulse (≥10 ng/mL). The partitioning of PGFM pulses according to nonprominence and prominence was justified by the increase in PRL centralized to a PGFM pulse peak only when the PGFM pulses were prominent.

The reduction in number of PRL pulses in the FM group was consistent with the increased peak-to-peak interval and the wider base (Nadir 1 to Nadir 2) of a PRL pulse. The increase in PGFM concentrations during the hours of a prominent PRL pulse in the vehicle group but not in the FM group may have been related to inhibition of $PGF_{2\alpha}$ biosynthesis in the FM group. More PRL pulses than PGFM pulses in the vehicle group cannot be attributed to the CV-identification requirement that four values were used to detect PGFM pulses, whereas three values were used to detect PRL pulses. Only 1 of 9 heifers in the vehicle group had additional PGFM pulses that would have been identified if only three values were required. The lower frequency of synchrony between PGFM and PRL pulses when a PRL pulse was the reference pulse was a reflection of the greater number of PRL than PGFM pulses. That is, more PRL pulses than PGFM pulses necessitated that many PRL pulses would not be synchronized with a PGFM pulse.

It is unknown whether the temporal relationships between PGFM and PRL pulses represented a positive effect of one on the other or whether other factors are involved. In this regard, an increase in estradiol occurs during luteolysis [45] and within the hours of a PGFM pulse [44]. The prominence of estradiol-induced PGFM pulses increases with increasing estradiol doses [46]. Furthermore, PRL concentrations increase before estrus in sheep and rats and is thought to be associated with the increase in estradiol, as indicated by the increase in concentration of PRL after estradiol administration [47]. Secretion of PRL is also stimulated by estradiol in bovine pituitary-cell cultures [48]. Further study will be required to determine if $PGF_{2\alpha}$ affects PRL or PRL affects $PGF_{2\alpha}$ and to clarify the role of other factors in $PGF_{2\alpha}$ and PRL pulse synchrony.

Hypothesis 2: That $PGF_{2\alpha}$ is utilized in the continued functional and structural regression of the CL during postluteolysis— was not supported. The decrease in P4 concentration and in CL area (cm^2) did not differ between groups during the 48 h of postluteolysis; both end points decreased progressively during the 48 h and therefore the hypothesis was adequately considered. During Hours 0 to 48, mean P4 concentration decreased from 0.8 to 0.2 ng/mL. Nine heifers reached 0.1 ng/mL by Hour 48. A previous preliminary conclusion indicated that P4 decreases from 0.9 to 0.1 ng/mL in 41 h [6] which seems close to the current results. The reported time required for the completion of luteolysis to 1 ng/mL is at least partly dependent on the prominence of $PGF_{2\alpha}$ secretion as represented by PGFM concentrations [30,39].

The reduction in both PGFM and PRL by FM treatment precluded distinguishing between the effects of $PGF_{2\alpha}$ and PRL. However, in the current study during postluteolysis, the reduction in both PGFM and PRL in the FM group indicated that neither circulatory $PGF_{2\alpha}$ nor PRL were involved in the continued functional and structural CL regression after P4 decreased to <1 ng/mL. A reservation is that residual PGFM or PRL after the FM treatment may have had an effect. A literature search did not disclose compelling indications that PRL has a luteal role during or after luteolysis in cattle. However, the findings that the PRL gene is expressed in luteal cells and PRL protein is present in the smooth muscle of the intraluteal arterioles and endothelial cells are at least compatible with a role of PRL in luteolysis in cattle [49]. Reduction of PGFM with FM in heifers beginning during preluteolysis delayed the first decrease in P4; however, PRL concentrations were not studied [30]. The negative results in the current study did not clarify whether the continued functional and structural CL regression after P4 reaches <1 ng/mL is a carry-over effect of the previously active luteolytic

mechanism or whether an active mechanism continues to be involved. In this regard, the apoptosis process in the steroidogenic and accessory cells of the CL is mediated by local factors, such as endothelin-1, cytokines, vasoactive peptides, and nitric oxide [50–52].

The reduction in $PGF_{2\alpha}$ and PRL during Hours 0–48 did not alter the growth of the dominant follicle. The follicle increased in the combined treated and vehicle groups from a mean of 13.5 mm at Hour 0 to 16.5 mm at Hour 48 (8 h after last treatment). This finding represents the first reported indication that PRL is not a requirement for growth of the preovulatory follicle during postluteolysis. Ovulation in the 12 heifers that ovulated occurred between Hours 48 and 72 in four heifers and between Hours 72 and 96 in six heifers. Therefore, it is assumed that anovulation in the FM group began primarily between Hours 48 and 96 or within 2 days after the last FM treatment. The precise temporal relationship between the last treatment of FM and anovulation and the associated concentrations of PGFM and PRL were not determined. However, the half-life of FM after the last treatment increases substantially in cattle when repeated treatments are given; FM half-life in cattle has been reported as 4 h after a single treatment of 2.2 mg/kg and 26 h after four daily treatments [53]. Most likely, therefore, the continued effects of FM approached the expected time of ovulation. However, specific study with frequent sampling of blood and frequent scanning for ovulation is indicated.

Hypothesis 3: That systemic administration of FM interferes with ovulation— was supported. Repeated intramuscular FM treatment of 2.5 mg/kg for 2 days after the end of luteolysis efficiently blocked ovulation, as indicated by fewer heifers ovulating in the FM group (3 of 10) than in the vehicle group (9 of 9). The ovulation blockage by FM likely involved inhibition of the COX activity and consequently prostaglandin biosynthesis. In this regard, the local increase in concentration of prostaglandins induces vasodilatation in the follicle blood vessels, augments vascular permeability, and stimulates the degradation of extracellular matrix that are involved in the follicle rupture [19,54]. In mares, repeated systemic FM treatment also effectively blocks ovulation, leading to the development of the syndrome of hemorrhagic anovulatory follicle [22–24]. In cattle, the inhibition of prostaglandins after the LH surge by intraovarian treatment with indomethacin [25] and intrafollicular treatment with a selective COX-2 inhibitor (NS-398 [26]) effectively blocks ovulation. However, the intramuscular or intrauterine treatment with indomethacin apparently did not sufficiently inhibit local prostaglandin secretion [25]. The efficacy of the intramuscular FM treatment in the current study used a less invasive and more practical experimental approach for blocking ovulation in cattle, compared to the intraovarian or intrafollicular treatment previously reported [25,26].

The diameter of the dominant anovulatory follicle in the FM group increased after the expected time of ovulation (Hours 72 and 96) and continued to increase to an average of 36 mm and was >40 mm in three heifers. The initiation of luteinization of the anovulatory follicle wall seemed to be near the expected time of ovulation, indicating that the inhibition of PGs blocked ovulation but did not affect LH-induced luteinization of the follicular wall. The presence of an anovulatory follicle was observed for ≥10 days in five out of seven heifers. The color-Doppler signals of blood flow in the luteinized wall indicated that this area was functional and most likely secreting P4, but the P4 concentration was not determined. In this regard, reported circulating P4 concentrations in heifers with induced luteinized anovulatory follicles were similar to concentrations in heifers with a spontaneous CL [26].

The ultrasonography of FM-induced luteinized anovulatory follicles resembled those reported for spontaneous luteinized cystic follicles in cattle [55–57]. Cystic anovulatory follicles cause economic loss in the diary industry [55,56,58]. The endocrine and cellular changes after the formation of cysts have been characterized (reviewed in [56]), but the cellular and molecular events that take place before the development of cysts remain poorly understood [59]. The similar characteristics of anovulatory follicles in the FM-treated heifers in the current study and in spontaneous cystic follicles in reported studies [55–57] indicate that systemic FM treatment may be useful for study of the etiology, pathology, and treatment of anovulatory cysts.

In conclusion, repeated treatment of heifers with FM beginning near the mean onset of postluteolysis (mean, P4 <0.8 ng/mL) reduced the concentrations and number of pulses of PGFM and PRL. The reduced $PGF_{2\alpha}$ and PRL did not alter the concentrations of P4 or CL area (cm^2) or growth of the dominant follicle during postluteolysis (48 h after the first FM treatment). The FM treatment blocked ovulation in 70% of heifers.

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