Accessory corpus luteum induced by human chorionic gonadotropin on day 7 or days 7 and 13 of the estrous cycle affected follicular and luteal dynamics and luteolysis in lactating Holstein cows

Thiago O. Cunha,^{1,2} Leah R. Statz,¹ Rafael R. Domingues,² João Paulo N. Andrade,² Milo C. Wiltbank,² and João Paulo N. Martins^{1*}

¹Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison 53706

²Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Madison 53706

ABSTRACT

Our objective was to determine the effect of inducing an accessory corpus luteum (CL) with human chorionic gonadotropin (hCG; 3,300 IU) on d 7 (hCG7) or 2 accessory CL with hCG on d 7 and 13 (hCG7+13) of the estrous cycle in noninseminated lactating Holstein cows. Cows ($n = 86$) between 39 and 64 DIM were pretreated with an Ovsynch + CIDR protocol, and only synchronized cows were used ($n = 64$). The day of the last GnRH of Ovsynch was considered d 0 of the estrous cycle. Follicular and luteal dynamics of cows were evaluated daily during an entire estrous cycle by ovarian ultrasonography. Blood samples were collected daily to measure serum concentration of progesterone (P4). Cows were randomly assigned to CON ($n = 22$, no treatment), hCG7 ($n = 20$), or hCG7+13 ($n = 22$) treatments. Two cows from hCG7+13 failed to ovulate after hCG and were removed from the analyses post-hCG treatment. The first day of luteolysis was considered the day that P4 declined to more than 2 SD of the mean for the 4 consecutive P4 concentrations with the greatest mean in late diestrus for each individual cow. The P4 cut-off for complete luteolysis was <1.0 ng/mL. Mean P4 on d 7 (3.23 ± 0.16 ng/mL) did not differ among treatments. Cows treated with hCG had greater total luteal and original CL volume and serum P4 during diestrus than CON. Cows treated with hCG7+13 had greater serum P4 after d 13 of the cycle than hCG7. Cycles were classified as having atypical cycles if the dominant follicle or future dominant follicle at the time of luteolysis did not ovulate (delayed ovulation; CON, $n = 2$; hCG7, $n = 4$; hCG7+13, $n = 3$), had a short cycle (CON, $n = 1$), delayed (CON, $n = 2$) or incomplete luteolysis (CON, $n = 1$; hCG7, $n = 4$; hCG7+13, $n = 5$). The remainder of cycles with normal complete

luteolysis followed by ovulation were considered to be typical. Based on blood perfusion, the CON cow with incomplete luteolysis had 2 original CL remaining functional after first onset of luteolysis. The rest of the cows with incomplete luteolysis (9/10) had one or more CL regressing and at least one remaining functional after first onset of luteolysis. No specific pattern for CL side (ipsilateral vs. contralateral to a CL with complete regression) was observed for nonregressed CL. Cows with incomplete luteolysis had a second onset of luteolysis to undergo complete functional luteolysis. The proportion of cows with typical cycle was 73% (16/22) for CON, 60% (12/20) for hCG7, and 55% (11/20) for hCG7+13. Cows with typical cycles treated with hCG (hCG7 and hCG7+13) had a later onset of luteolysis, prolonged time to undergo complete luteolysis, and greater proportion of cows with 3 follicular waves than CON, resulting in a longer interovulatory interval for hCG7 and hCG7+13 than CON. In summary, accessory CL induced by hCG during diestrus not only altered follicular and luteal dynamics but also deferred and prolonged the luteolytic process.

Key words: hCG, corpus luteum, luteolysis, dairy cows

INTRODUCTION

Over the last 4 decades, a better understanding of reproductive physiology in the modern, high-producing dairy cow provided the basis for developing novel technologies and strategies that overcome challenges to reproductive performance on dairy farms (Thatcher, 2017; Cardoso Consentini et al., 2021). An example of these technologies widely adopted in US dairy farms is Ovsynch-based fertility programs for first service of lactating dairy cows, such as Double-Ovsynch (Wiltbank and Pursley, 2014; Carvalho et al., 2018; Martins and Pursley, 2016). Many studies have been performed to improve the original Ovsynch program and maximize fertility of lactating dairy cows receiving timed-AI

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*Corresponding author: jp.martins@wisc.edu

(Vasconcelos et al., 1999; Bisinotto et al., 2013; Wiltbank et al., 2015). One result that is consistent among studies is the positive association between circulating concentration of progesterone (**P4**) during the growth of the ovulatory follicle and pregnancy per AI (Fonseca et al., 1983; Martins et al., 2011; Bisinotto et al., 2015). Fertility programs generally use a GnRH-induced LH surge to control the onset of a new follicular wave and to cause ovulation and formation of an accessory corpus luteum (**CL**) to enhance circulating concentration of P4 during the growth of the pre-ovulatory follicle (Pursley and Martins, 2011; Wiltbank and Pursley, 2014; Carvalho et al., 2018). The effects of the formation of an accessory CL and increased circulating concentration of P4 in timed-AI programs have been reported (Bisinotto et al., 2015; Carvalho et al., 2018). However, the effects of inducing an accessory CL with hCG treatment during the normal estrous cycle on the dynamics of follicular and luteal development and the luteolytic process have not been well established. Understanding the physiologic changes that occur after induction of accessory CL during the estrous cycle may allow the development of new strategies to maximize the fertility of lactating dairy cows inseminated after estrus.

A common strategy to induce ovulation and formation of accessory CL in lactating dairy cows is by treatment with human chorionic gonadotropin (**hCG**) when a dominant follicle (**DF**) is present on the ovary. A recent study indicated that $\geq 2,000$ IU of hCG has greater ovulatory efficiency than GnRH (86 μ g of gonadorelin) when used on d 7 of the estrous cycle in lactating dairy cows (Cabrera et al., 2021). Cabrera et al. (2021) also determined that cows treated with 2,500 IU of hCG had a greater increase in serum P4 concentrations from d 7 to 14 of the estrous cycle and greater total luteal volume on d 14. These results may be due the formation of a new accessory CL combined with a luteotropic effect of hCG on the original CL because hCG binds to LH receptors in the CL and potentially increases P4 secretory capacity of luteal cells (De Rensis et al., 2010).

Most studies that used hCG to induce ovulation and formation of an accessory CL in cattle tested the effect of hCG post-AI or before embryo transfer (**ET**) on fertility (Nascimento et al., 2013; Besbaci et al., 2020). Their main objective was to use hCG to increase circulating concentration of P4 and improve early embryo development and survival (Lonergan, 2011). The results from those studies were inconsistent, with no differences (Schmitt et al., 1996b; Niles et al., 2019) or small improvements (Nascimento et al., 2013; Zolini et al., 2019) in pregnancy per AI or ET for cows and heifers treated with hCG. We only found one study by Sianangama and Rajamahendran (1996) that character-

ized follicular and luteal dynamics in nonbred lactating cows treated with hCG during the dominance period of the first follicular wave. That experiment had a small number of animals ($n = 6/\text{treatment}$), and the results suggested an effect of hCG on follicular dynamics, although not statistically significant, and no effect on luteal dynamics and estrous cycle length (Sianangama and Rajamahendran, 1996).

On the contrary, a recent study from our laboratory indicated that a single administration of 3,300 IU of hCG from d 5 to 7 of the estrous cycle increased the estrous cycle length in noninseminated lactating Holstein and Jersey cows (Cunha et al., 2021). Moreover, hCG decreased the proportion of multiparous cows returning to estrus (Cunha et al., 2021), indicating that accessory CL induced by hCG may affect luteal and luteolytic dynamics. The study did not collect ovarian and hormone data throughout the estrous cycle; therefore, it is still unclear how hCG prolonged the length of the estrous cycle. We speculated that this could be due to an increased incidence of cows with 3 follicular waves or a prolonged CL lifespan.

Hence, the objective of the present experiment was to characterize follicle and luteal dynamics and luteolysis in noninseminated lactating Holstein cows that form accessory CL due to treatment with hCG either on d 7 only or on both d 7 and 13 of the estrous cycle. Our first hypothesis was that hCG treatment on d 7 would increase the proportion of cows with 3 follicular waves compared with cows that did not receive any treatment (controls). Our second hypothesis was that cows with accessory CL induced by hCG would have a prolonged interovulatory interval (**IOI**). Our final hypothesis was that cows treated with hCG on d 7 and 13 would have follicles from the third wave growing in such synchrony that there would be a decrease in variation for the onset of functional luteolysis, synchronizing the time of luteolysis.

MATERIALS AND METHODS

All procedures involving animals were previously approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison (Protocol ID: A006164). No humane endpoints were observed during the study.

Animals, Housing, and Feeding

From May to September 2019, a total of 86 lactating Holstein cows from 2 research farms in south central Wisconsin were enrolled in this study. Cows were housed in freestall barns and milked twice daily at the Emmons Blaine Dairy Cattle Center ($n = 23$; Arling-

Figure 1. Schematic diagram of the experimental design. The estrous cycle of lactating Holstein cows was synchronized with an Ovsynch + controlled internal drug-release insert (CIDR) protocol [GnRH 200 µg and CIDR insertion on d -9; CIDR removal and 25 mg of PGF_{2α} (dinoprost tromethamine) on d -2; PGF_{2α} on d -1; GnRH 200 µg on d 0]. After confirmation of ovulation by the last GnRH (d 0), cows were randomly assigned to each treatment. Cows in the control treatment did not receive any treatment. Cows in the hCG7 treatment received 3,300 IU of human chorionic gonadotropin (hCG) 7 d after the last GnRH (d 7 of the estrous cycle). Cows in the hCG7+13 treatment received 3,300 IU of hCG on d 7 and a second treatment with hCG on d 13 of the estrous cycle. Transrectal ultrasonography (US) and blood collection were performed on d -2 and daily from d 0 until spontaneous ovulation. After d 16 of the estrous cycle, Doppler US was performed in each corpus luteum (CL) until at least 2 consecutive days with blood flow $\leq 25\%$ in all CL of each cow.

ton, WI). At the US Dairy Forage Research Center (n = 63; Prairie du Sac, WI), cows were housed in tiestall barns and milked thrice daily. On both farms, cows had free access to water and fresh TMR delivered once daily, which consisted of corn and alfalfa silage as forage and corn-soybean meal-based concentrate. Individual milk yield from cows was measured daily, and BCS (scale from 1 = thin to 5 = fat; Edmonson et al., 1989) was evaluated on the day of enrollment. The daily average temperature-humidity index (THI; NRC, 1971) during the experimental period from the nearest meteorological station to both farms (Dane County Regional Airport, Madison, WI) was 63.5 ± 3.1 ($\pm$ SD), calculated as follows: $THI = (1.8 \times \text{temperature dry bulb} + 32) - [(0.55 - 0.0055 \times \text{relative humidity}) \times (1.8 \times \text{temperature dry bulb} - 26.8)]$.

Experimental Design and Selection of Animals

Cows between 39 and 64 DIM (mean $\pm$ SD = 45.2 ± 6.0) were submitted to a pretreatment for synchronization of ovulation, consisting of an Ovsynch + controlled internal drug-release insert (CIDR; Figure 1; Pursley et al., 1995). Briefly, cows received GnRH (200 µg i.m.; Factrel, Zoetis) and a P4 intravaginal insert (Eazi-Breed CIDR; Zoetis). Seven days later, the P4 insert was removed, and prostaglandin F_{2α} (PGF_{2α} 25 mg i.m.; Lutalyse HighCon, Zoetis) was administered, followed by another PGF_{2α} 1 d later and a second GnRH 1 d after the second PGF_{2α}. Only synchronized cows with complete CL regression 2 d after PGF_{2α} (serum P4 < 1.0 ng/mL) and ovulation by 2 d after the last GnRH

were used in the study (n = 64). The last GnRH of pretreatment was considered d 0 of the estrous cycle. After confirmation of synchronization, cows were randomly assigned to one of 3 treatments. Control cows (n = 22) did not receive any treatment except the synchronization protocol. Cows enrolled in the second treatment group (**hCG7**; n = 20) received an i.m. treatment with 3,300 IU of hCG (Chorulon, Merck Animal Health) 7 d after the last GnRH (d 7 of the cycle), and cows enrolled in the third treatment group (**hCG7+13**; n = 22) received 2 hCG treatments with 3,300 IU, one on d 7 and a second on d 13 after the last GnRH (d 7 and d 13 of the estrous cycle).

Ultrasonography

A technician blinded to treatments scanned and mapped ovaries using a transrectal ultrasonography fitted with a 5 to 10 MHz multifrequency linear array probe (SonoSite Edge, Fujifilm SonoSite Inc.) on the day of the first PGF_{2α} treatment of pretreatment (d -2) and daily from the day of GnRH (d 0) until the end of the cycle determined by spontaneous ovulation. Ovarian ultrasonographic videos of each cow were recorded and used for later measurement of follicular and luteal structures using a video analysis software for measurements (Kinovea version 0.9.1, <https://kinovea.org>). Measurements were performed by a technician blinded to treatments. The 10-mm distance between grid-scale on the ultrasound display was used to calibrate the measuring calipers of the software. The height and width of the largest cross-sectional size of

follicles with an antrum diameter ≥ 4 mm and any detectable CL were measured. Corpus luteum volume was calculated by the formula $V = 4/3 \times \pi \times R^3$ using a radius (R) calculated by the formula $R = (\text{Height}/2 + \text{Width}/2)/2$. Corpora lutea with fluid-filled central cavity also had the largest cross-sectional area of the cavity measured and subtracted from the total volume of the CL. Ovulation was characterized by the disappearance of the DF or co-dominant follicles, followed by the detection of a newly formed CL at the same location. Starting on d 16 of the estrous cycle, all cows had every CL present in the ovaries scanned daily using a color Doppler function to determine CL blood flow. Based on colored pixel intensity indicating blood flow, a subjective scale through vascularization scores (0 = no blood flow and 4 = maximum blood flow) based on Pugliesi et al. (2017) was used to evaluate the functionality of each CL. Doppler evaluations ended after at least 2 consecutive days with blood flow $\leq 25\%$ for all CL present in the ovaries.

Blood Sampling and P4 Assays

Blood samples were collected immediately before the first PGF_{2α} treatment and CIDR removal (d -2) and daily from d 0 until spontaneous ovulation. Samples were collected via coccygeal venipuncture into 10-mL evacuated serum collection tubes (Vacuette, Greiner Bio-One North America Inc.). After collection, blood samples were placed in a cooler with ice and transported to the laboratory. Samples were centrifuged (20 min at 2,000 $\times g$; 4°C) within 6 h, and serum aliquots were separated and frozen at -20°C until assayed. Progesterone concentrations were analyzed in serum by radioimmunoassay using a commercial kit (MP Biomedicals). Ten assays were performed and the sensitivity values of the assays were ≤ 0.05 ng/mL. All samples from the same cow were analyzed within the same assay, and cows from each week of enrollment and treatment were randomized for all assay rounds. Samples with P4 concentration = 2.0 ng/mL were incorporated in duplicates at the beginning, middle, and end of each assay for quality control and calculation of intra- and interassay coefficients of variation. Intra- and interassay coefficients of variation were 8.1 and 10.4%, respectively.

Definition of Luteolysis and Classification of Atypical Estrous Cycle

The first day of functional luteolysis was considered the day P4 declined by twice the SD of the mean for the 4 consecutive days serum P4 concentrations with the greatest mean in the late diestrus. The day of luteolysis onset was considered the day before the first day of

functional luteolysis. The circulating P4 concentration cut-off for complete functional luteolysis was <1.0 ng/mL.

Cows were categorized as having either a typical or an atypical estrous cycle. Cycles were classified as atypical with delayed ovulation if the DF or the future DF at the onset of luteolysis was not the ovulatory follicle (Sartori et al., 2004). Cows that had an early luteolysis or short cycle (≤ 16 d), delayed luteolysis onset ($> d 23$ of the cycle), or incomplete functional luteolysis (did not complete CL regression by 6 d after the onset of luteolysis) were also considered to have an atypical cycle. The remainder of cycles with onset of luteolysis ≤ 23 d of the cycle followed by complete luteolysis within 6 d after the onset and ovulation of the DF or future DF at the onset of luteolysis were considered typical. The effect of treatment on follicular and luteal dynamics after d 13 of the estrous cycle was analyzed separately for cows with typical and atypical cycles, aiming to eliminate misleading effects of atypical estrous cycle (delayed ovulation, and early, incomplete and delayed luteolysis) on the results. For cows classified with incomplete luteolysis, the day of second onset of luteolysis was based on the same criteria used for the first day of functional luteolysis.

Statistical Analysis

The total number of cows needed in this experiment was calculated using G*Power (Faul et al., 2007). A priori power analysis using the z test to calculate a difference between 2 independent proportions, 2-tailed, $\alpha = 0.05$, power $(1 - \beta) = 0.95$, indicated that 10 cows per treatment were required to detect a difference between 20 versus 90% in the proportion of cows with 3 follicular waves in the estrous cycle for control versus hCG7 or hCG7+13, respectively. We doubled the number of cows per treatment in the study to account for a large proportion of cows with atypical cycles in spontaneous estrous cycles (~50%) previously identified by Sartori et al. (2004).

This study used a complete randomized experimental design. Cows were randomly assigned to treatments after confirmation of ovulation by ultrasonography to the last GnRH of the pretreatment protocol. Cows that failed to ovulate to the d 7 or d 13 hCG (hCG7+13, $n = 2$) were removed from the statistical analysis for variables analyzed after each hCG treatment. All statistical analyses in this study were performed using SAS (version 9.4, SAS Institute Inc.).

Continuous data related to follicular dynamics, milk production, BCS were tested for differences by ANOVA, and differences among treatments and for typical versus atypical cycles were obtained by differences of least

squares means using the linear mixed model applying the MIXED procedure of SAS. The model considered the effect of parity and treatment as fixed effects. Parity was only maintained in the model when provided a lower value of the Akaike information criterion (AIC). Normality and homoscedasticity of residuals were assessed by visual inspection of studentized residual plots for each variable after fitting the model using the residual option of the MIXED procedure. When necessary, the assumption of normality was tested using the Shapiro-Wilk test within the UNIVARIATE procedure of SAS. Variables that did not fulfill assumptions were natural log-transformed for the analysis and actual means $\pm$ standard error of the mean of the data are presented for clarity. Means and SEM were obtained using the MEANS procedure.

Because the onset of functional luteolysis occurred on different days of the cycle among most cows and treatments, data at the end of the luteal phase relating to luteal dynamics were normalized for days relative to the onset of functional luteolysis (d -5 to 5).

Repeated measures variables such as the mean P4 concentration and CL volume over time (estrous cycle day or day relative to the onset of luteolysis) were analyzed using the MIXED procedure with the REPEATED statement with cow specified in the SUBJECT option of SAS. The model consisted of parity, treatment, time and the interaction treatment $\times$ time as fixed effects. Parity was only maintained in the model when provided a lower value of the AIC. For the MIXED procedure, fit statistic parameters for unstructured, compound symmetry, first-order autoregression, heterogeneous compound symmetry, and heterogeneous first-order autoregression variance structure were tested. The covariance structure with the lowest values for AIC was used for the analysis. The analysis was divided into 3 time periods based on the expected estrous cycle status in each phase, allowing comparisons between treatments with less variability of the residuals that could lead to distorted interpretations. The 3 phases for serum P4 and total luteal and CL volumes analysis are as follows: d -2 to 7 (pretreatment phase), d 7 to 14 (posttreatment phase before natural luteolysis), and d -5 to 5 relative to the onset of functional luteolysis.

Binary data such as double ovulation to d 0 GnRH and d 7 hCG, and the proportion of cows with typical estrous cycle, 3 follicular waves, and spontaneous double ovulation were analyzed by Fisher's exact test with the FREQ procedure. We used Fisher's exact test because the outcome with counts fewer than 5 was present in at least one of the treatment results from the variables listed.

Levene's test was used to assess if treatments had different variability for days at the onset of luteolysis. The GLM procedure with HOVTEST option in the MEANS statement was applied. If variances differed, the Welch's *t*-test within the GLM procedure was used for pairwise comparisons of 2 means.

All analyses used a 2-tailed test. Probability values were considered different when *P*-values were ≤ 0.05 , and trending toward significance when *P*-values were > 0.05 and ≤ 0.10 .

RESULTS

Average lactation number, BCS at enrollment, and milk production during the trial did not differ among treatments ($P = 0.34$; $P = 0.58$; $P = 0.91$; respectively). Overall mean lactation number and BCS were 2.3 ± 0.03 and 3.1 ± 0.04 , respectively. The overall mean daily milk production was 36.8 ± 0.9 kg for primiparous and 50.4 ± 1.0 kg for multiparous cows during the experiment. By the time of the first PGF_{2 α} of pre-treatment, all cows enrolled in the study had at least one CL present in one of the ovaries, and serum P4 was similar ($P = 0.26$) among treatments averaging 5.3 ± 0.4 ng/mL. Largest follicle diameter at the final GnRH of the pretreatment (d 0) averaged 15.0 ± 0.4 mm and did not differ among treatments (Table 1). No treatment differences were found for day of first follicular wave emergence (1.4 ± 0.1 d) and first-wave DF diameter at d 7 (13.1 ± 0.3 mm; Table 1).

Effect of Treatment on Follicular Dynamics

Treatment affected day of second follicular wave emergence (Table 1). As expected, the average day of second follicular wave emergence for cows that ovulated to the first hCG (hCG7 and hCG7+13) was earlier ($P < 0.001$) compared with the cows in the control group (8.8 ± 0.1 vs. d 12.1 ± 0.6 , respectively). Although a greater proportion of cows had double ovulation to hCG treatment on d 7 in hCG7+13 compared with hCG7, the DF diameter at d 13 did not differ for cows in hCG7 versus hCG7+13 (Table 1). Moreover, DF diameter at d 13 did not differ ($P = 0.18$) for cows with double ovulation and single ovulation to d 7 hCG (10.9 ± 0.4 vs. 10.1 ± 0.5 , respectively). Most cows with double ovulations to d 7 or 13 hCG were multiparous (14/17) and occurred by random chance. Interestingly, 3/4 of the cows with double ovulation to d 13 hCG also double ovulated to d 7 hCG.

Thirty-nine of 62 cows in this study were characterized as having typical cycles (Table 2). Supplemental Figure S1 (<http://digital.library.wisc.edu/1793/82420>;

Table 1. Effect of treatment (control, hCG7, and hCG7+13) on follicular dynamics of first and second follicular wave in lactating Holstein cows

Variable	Treatment ¹			P-value
	Control	hCG7	hCG7+13	
Cows treated, n	22	20	22	—
Primiparous, n	8	6	9	—
Ovulatory follicle size at d 0 ² , mm ± SEM	14.3 ± 0.6	14.9 ± 0.6	15.7 ± 0.6	0.23
Double ovulations after GnRH treatment at d 0, n/n	5/22	2/20	1/22	0.20
First follicular wave				
Day of emergence ³ ± SEM	1.3 ± 0.1	1.4 ± 0.2	1.5 ± 0.2	0.57
Dominant follicle diameter at d 7, mm ± SEM	13.1 ± 0.6	13.5 ± 0.4	12.8 ± 0.5	0.51
Failed to ovulate to d 7 hCG, n/n	—	0/20	1/22	—
Double ovulation to d 7 hCG, n/n	—	2/20 ^a	11/21 ^b	0.007
Primiparous	—	1/6	2/9	0.99
Multiparous	—	1/14	9/13	<0.01
Second follicular wave, n	22	20	21	—
Day of emergence ³ ± SEM	12.1 ± 0.6 ^a	8.6 ± 0.2 ^b	9.0 ± 0.2 ^b	<0.001
Dominant follicle diameter at d 13, ⁴ mm ± SEM	7.8 ± 0.5 ^a	11.0 ± 0.5 ^b	10.5 ± 0.4 ^b	<0.001
Failed to ovulate to d 13 hCG, n/n	—	—	1/21	—
Double ovulation to d 13 hCG, n/n	—	—	4/20	—
Primiparous	—	—	0/20	—
Multiparous	—	—	4/20	—

^{a,b}Mean values in the same row with different superscripts differ ($P \leq 0.05$).

¹Cows in the control treatment did not receive any treatment. Cows in the hCG7 treatment received 3,300 IU of human chorionic gonadotropin (hCG) 7 d after the last GnRH (d 7 of the estrous cycle). Cows in the hCG7+13 treatment received 3,300 IU of hCG on d 7 and a second treatment with hCG on d 13 of the estrous cycle.

²d 0 = day of last GnRH treatment.

³Day of emergence was the day that follicles with a diameter of ≥ 4 mm were detected by ultrasound.

⁴Only n = 13 cows were considered for the control at d 13 of the estrous cycle.

Martins, 2021) has examples of individual cows for serum concentration of P4, luteal volume, and CL Doppler scores for cows in each treatment classified with typical estrous cycles. Treatment did not affect the proportion of cows with typical cycles but increased the proportion of cows with 3 follicular waves compared with control cows (Table 2). Day of the emergence of the pre-ovulatory follicular wave was later for cows treated with hCG (hCG7 or hCG7+13) compared with controls (Table 2). Furthermore, pre-ovulatory follicle diameter at the onset of functional luteolysis was smaller for cows treated with hCG than controls (Table 2). Nevertheless, the age and maximum diameter of the pre-ovulatory follicle in the final wave did not differ among treatments (Table 2). Cows treated with hCG (hCG7 or hCG7+13) had a prolonged period between onset of functional luteolysis and ovulation and had a longer IOI (Table 2).

Effect of Treatment on Luteal Dynamics and Luteolysis

Circulating P4 concentration and luteal volume did not differ among treatments until d 7 of the estrous cycle (Figure 2). On average, cows in hCG7 or hCG7+13 had a greater number of CL (CL number on day of luteolysis: Control = 1.2 ± 0.1 ; hCG7 = 2.3 ± 0.1 ; hCG7+13 = 3.5 ± 0.2 $P < 0.001$), circulating concentrations of

P4 (Figure 2A), and total luteal volume (Figure 2B) during diestrus (d 9 to 13) than control cows (Table 3). In contrast, no differences in serum P4 and luteal volume were observed between hCG treatments (hCG7 vs. hCG7+13) within the same period (Figure 2), even with a greater proportion of cows with double ovulation following hCG administration on d 7 in the hCG7+13 treatment compared with hCG7 treatment (Table 1). Human chorionic gonadotropin treatment on d 7 of the estrous cycle enhanced growth of the original CL from d 7 to 9 compared with control cows, resulting in greater ($P < 0.01$) original CL volume for hCG7 and hCG7+13 cows between d 10 and 13 than controls (Figure 2C). Control cows maintained ($P > 0.10$) similar original CL volume between d 8 and 14 of the estrous cycle and between d -5 and 0 relative to the onset of functional luteolysis (Figure 2). In contrast, the original CL volume in hCG7 and hCG7+13 decreased on some days ($P < 0.05$) between d -5 and 0 relative to luteolysis (Figure 2C). However, the second hCG on d 13 of the estrous cycle in the hCG7+13 maintained a larger original CL volume for an extended period during the estrous cycle than hCG7 (Figure 2C). From d -5 to d 0 relative to the onset of luteolysis, circulating concentrations of P4 and luteal volume were different among treatments, except at d -5 when luteal volume only had a tendency ($P = 0.07$) to be different between hCG7 and hCG7+13 (Figure 2B).

Table 2. Effect of treatment (control, hCG7, and hCG7+13) on the proportion of cows with typical estrous cycle, 2 or 3 follicular waves, follicular dynamics of the pre-ovulatory follicle, day of spontaneous ovulation and interovulatory interval in lactating Holstein cows

Variable	Treatment ¹			P-value
	Control	hCG7	hCG7+13	
Cows with typical cycle, % (n/n)	72 (16/22)	60 (12/20)	55 (11/20)	0.30
Primiparous	88 (7/8)	66 (4/6)	78 (7/9)	0.82
Multiparous	64 (9/14)	58 (8/14)	36 (4/11)	0.43
Proportion of typical cows with 2 waves, n/n	12/16	1/12	0/11	<0.001
Proportion of typical cows with 3 waves, n/n	4/16	11/12	11/11	<0.001
Pre-ovulatory follicular wave				
Day of emergence, ² mm ± SEM	13.2 ± 0.8 ^a	17.2 ± 1.0 ^b	17.6 ± 0.7 ^b	<0.001
Pre-ovulatory follicle diameter at onset of functional luteolysis, mm ± SEM	12.5 ± 0.9 ^a	9.0 ± 0.9 ^b	8.2 ± 0.8 ^b	0.009
Pre-ovulatory follicle diameter before ovulation, mm ± SEM	15.9 ± 0.5	16.4 ± 0.3	16.8 ± 0.5	0.41
Age of pre-ovulatory follicle at ovulation, ³ d ± SEM	10.6 ± 0.4	9.5 ± 0.6	9.8 ± 0.4	0.29
Interval between onset of functional luteolysis and spontaneous ovulation, d ± SEM	4.8 ± 0.4 ^a	6.2 ± 0.3 ^{b*}	7.5 ± 0.7 ^{b*}	0.002
Day of the cycle at spontaneous ovulation ± SEM	23.8 ± 0.5 ^a	26.7 ± 0.4 ^b	27.5 ± 0.8 ^b	<0.001
Cows with spontaneous double ovulation, n/n	3/16	2/12	0/11	0.41
Interovulatory interval, d ± SEM	22.0 ± 0.5 ^a	24.9 ± 0.4 ^b	25.8 ± 0.8 ^b	<0.001
2 waves	21.6 ± 0.4	22.0	—	—
3 waves	24.5 ± 0.6	25.2 ± 0.3	25.8 ± 0.8	0.48

^{a,b}Mean values in the same row with different superscripts differ ($P \leq 0.05$).

¹Cows in the control treatment did not receive any treatment. Cows in the hCG7 treatment received 3,300 IU of human chorionic gonadotropin (hCG) 7 d after the last GnRH (d 7 of the estrous cycle). Cows in the hCG7+13 treatment received 3,300 IU of hCG on d 7 and a second treatment with hCG on d 13 of the estrous cycle.

²Day of emergence was the day that follicles with a diameter of ≥ 4 mm were detected by ultrasound.

³Interval between the day of emergence and the day before ovulation.

*Mean values in the same row with asterisks tended to differ ($0.05 < P \leq 0.10$).

Cows treated with hCG had later onset of functional luteolysis during the estrous cycle (Table 4). However, the day of the cycle at the onset of functional luteolysis did not differ between control cows with 3 follicular waves and cows receiving hCG treatments that also had 3 follicular waves (Table 4). Cows with accessory CL also had a prolonged period between the onset of functional luteolysis and time to complete luteolysis (serum P4 < 1 ng/mL; Figure 3A; Table 4). Interaction treatment by day relative to functional luteolysis was observed on accessory CL volume during luteolysis, indicating that the rate of accessory CL volume reduction after the onset of luteolysis differed among treatments (Figure 3C). Treatment with hCG and/or accessory CL presence had an effect not only on the timing of functional luteolysis but also on structural luteolysis timing. Although the period between the onset of functional luteolysis and P4 < 1 ng/mL was shorter for cows in control with 3 follicular waves than hCG treatments, the interval between onset of functional luteolysis and ovulation was similar for cows with 3 follicular waves between treatments (Table 4). Furthermore, the IOI did not differ between treatments in cows with 3 follicular waves (Table 1).

When cows with typical and atypical estrous cycle were combined within each treatment, hCG treatments reduced the variation in timing of the onset of luteolysis among cows in hCG7 or hCG7+13 compared with

CON (Figure 4A). Variation of day of complete luteolysis and IOI did not differ ($P > 0.10$) between treatments, as indicated in Figure 4 (B and C, respectively).

Atypical Versus Typical Estrous Cycles

Parity ($P = 0.15$), BCS ($P = 0.32$), and milk production ($P = 0.15$) did not differ between cows with typical and atypical cycles. A total of 23/62 (37.1%) cows had an atypical estrous cycle. One cow in control had an early luteolysis onset and short cycle with only one follicular wave, 13 d at the onset of luteolysis and an IOI of 16 d (Supplemental Figure S2; <http://digital.library.wisc.edu/1793/82420>; Martins, 2021). Ten cows had an atypical cycle with delayed ovulation of the DF or future DF at the time of luteolysis not subsequently ovulating (control, $n = 1$; hCG7, $n = 4$; hCG7+13, $n = 4$; Supplemental Figure S3; <http://digital.library.wisc.edu/1793/82420>; Martins, 2021). Regardless of treatment or parity, day of onset of luteolysis did not differ ($P = 0.64$) for cows with delayed ovulation and typical estrous cycles (Table 5; Figure 5). These cows with delayed ovulation had one ($n = 2/10$) or 2 ($n = 8/10$) additional follicular waves after the onset of luteolysis and a total of 3 to 5 waves during the estrous cycle. Due to the additional follicular wave(s), the period between the onset of luteolysis and ovulation was longer ($P < 0.001$) for cows with delayed ovulation than for cows

Figure 2. Effect of treatment (control, hCG7, and hCG7+13) on mean ($\pm$ SEM) serum progesterone (P4) concentrations (A), total luteal volume (B), original corpus luteum (CL; C), and accessories CL (D) volume during an entire estrous cycle in lactating dairy cows with typical cycle. (A; serum P4): From d -2 to 7 of the cycle ($n = 64$): estrous cycle day (Day): $P < 0.001$; treatment (TRT): $P = 0.73$; and Day $\times$ TRT: $P = 0.22$. From d 7 to 14 of the cycle ($n = 62$): Day: $P < 0.001$; TRT: $P < 0.001$; and Day $\times$ TRT: $P < 0.001$. From d -5 until d 5 relative to the onset of functional luteolysis ($n = 39$, only cows with typical estrous cycles): Day: $P < 0.001$; TRT: $P < 0.001$; and Day $\times$ TRT: $P < 0.001$. (B; total luteal volume): From d 2 to 7 of the cycle ($n = 64$): Day: $P < 0.001$; TRT: $P = 0.28$; and Day $\times$ TRT: $P = 0.23$. From d 7 to 14 of the cycle ($n = 62$): Day: $P < 0.001$; TRT: $P < 0.001$; and Day $\times$ TRT: $P < 0.001$. From d -5 until d 5 relative to the onset of functional luteolysis ($n = 39$, only cows with typical estrous cycle): Day: $P < 0.001$; TRT: $P < 0.001$; and Day $\times$ TRT: $P < 0.001$. (C; original CL volume): Only cows with typical estrous cycle and single ovulation at d 0 (Control, $n = 13$; hCG7, $n = 11$; hCG7+13, $n = 10$) were used in these analyses. From d 2 to 7: Day: $P < 0.001$; TRT: $P = 0.55$; and Day $\times$ TRT: $P = 0.17$. From d 7 to 14 of the cycle ($n = 62$): Day: $P < 0.001$; TRT: $P < 0.001$; and Day $\times$ TRT: $P = 0.54$. From d -5 until d 5 relative to the onset of functional luteolysis: Day: $P < 0.01$; TRT: $P < 0.01$; and Day $\times$ TRT: $P = 0.01$. (D; accessories CL volume): Only cows with typical estrous cycle and single ovulation at d 0 (Control, $n = 13$; hCG7, $n = 11$; hCG7+13, $n = 10$) were used in these analyses. From d 10 to 14: Day: $P < 0.001$; TRT: $P = 0.31$; and Day $\times$ TRT: $P = 0.06$. From d -5 until d 5 relative to the onset of functional luteolysis: Day: $P < 0.001$; TRT: $P < 0.001$; and Day $\times$ TRT: $P < 0.001$. Different letters (a-c) within a day represent differences between LSM ($P \leq 0.05$), and letters with asterisks within a day represent a tendency ($0.05 < P \leq 0.10$) for a difference. hCG = human chorionic gonadotropin.

Table 3. Effect of treatment (control, hCG7, and hCG7+13) on total luteal volume, original and accessory corpus luteum (CL) volume and circulating progesterone (P4) concentrations on d 7, d 13 and d of onset of functional luteolysis in lactating dairy cows classified with typical estrous cycle

Variable	Treatment			P-value
	Control (n = 16)	hCG7 (n = 12)	hCG7+13 (n = 11)	
d 7 of the estrous cycle				
Total luteal volume, mm ³ ± SEM	6,374 ± 758	7,022 ± 1,182	7,009 ± 669	0.83
Serum P4, ng/mL ± SEM	3.2 ± 0.3	3.3 ± 0.5	3.6 ± 0.3	0.46
d 13 of the estrous cycle				
Total luteal volume, mm ³ ± SEM	7,792 ± 731 ^a	16,789 ± 2,109 ^b	18,584 ± 1,352 ^b	<0.001
Original CL volume, mm ³ ± SEM	7,726 ± 759 ^a	11,834 ± 1,949 ^b	12,099 ± 1,601 ^b	0.001
Original CL volume only in cows with single ovulation to d 0 GnRH, ² mm ³ ± SEM	6,646 ± 596 ^a	10,809 ± 1,814 ^b	11,208 ± 1,471 ^b	0.001
Total accessory CL volume, mm ³ ± SEM	—	4,951 ± 399	6,370 ± 622	0.20
Serum P4, ng/mL ± SEM	7.0 ± 0.5 ^a	9.6 ± 0.7 ^b	13.8 ± 2.1 ^b	<0.001
Day of onset of functional luteolysis				
Number of CL ± SEM	1.2 ± 0.1 ^a	2.3 ± 0.1 ^b	3.5 ± 0.2 ^c	<0.001
Total luteal volume, mm ³ ± SEM	7,798 ± 717 ^a	14,681 ± 1,333 ^b	24,519 ± 1,459 ^c	<0.001
Original CL volume, mm ³ ± SEM	7,798 ± 717 ^a	7,053 ± 1,222 ^a	11,379 ± 1,445 ^b	<0.001
Original CL volume only in cows with single ovulation to d 0 GnRH, ² mm ³ ± SEM	6,929 ± 673 ^a	6,251 ± 1,011 ^a	10,985 ± 1,188 ^b	<0.01
Total luteal volume for d 7 accessory CL, mm ³ ± SEM	—	7,628 ± 723	11,187 ± 1,352	0.02
Total luteal volume for d 13 accessory CL, mm ³ ± SEM	—	—	2,148 ± 286	—
Serum P4, ng/mL ± SEM	6.6 ± 0.5 ^a	10.0 ± 0.9 ^b	21.7 ± 3.2 ^c	<0.001

^{a-c}Mean values in the same row with different superscripts differ ($P \leq 0.05$).

¹Cows in the control treatment did not receive any treatment. Cows in the hCG7 treatment received 3,300 IU of human chorionic gonadotropin (hCG) 7 d after the last GnRH (d 7 of the estrous cycle). Cows in the hCG7+13 treatment received 3,300 IU of hCG on d 7 and a second treatment with hCG on d 13 of the estrous cycle.

²Only cows with single ovulation at d 0 (control = 13; hCG7 = 11; hCG7+13 = 10).

Table 4. Effect of treatment (control, hCG7, and hCG7+13) on luteal and luteolysis dynamics in lactating dairy cows classified with typical estrous cycle with 2 and 3 follicular waves

Variable	Treatment ¹			P-value
	Control (n = 16)	hCG7 (n = 12)	hCG7+13 (n = 11)	
Day of the cycle at onset of functional luteolysis ± SEM	18.0 ± 0.5 ^{a,A}	19.5 ± 0.3 ^b	19.0 ± 0.3 ^{ab,B}	0.03
2 Follicular waves	17.6 ± 0.5	18.0	—	—
3 Follicular waves	19.0 ± 1.1	19.6 ± 0.2	19.0 ± 0.3	0.21
Day of the cycle at P4 ² <1.0 ng/mL ± SEM	19.6 ± 0.5 ^a	22.0 ± 0.4 ^{b,A}	23.2 ± 0.4 ^{b,B}	<0.001
2 Follicular waves	19.2 ± 0.5	19.0	—	—
3 Follicular waves	20.8 ± 0.9 ^{a,A}	22.3 ± 0.4 ^{ab,B}	23.2 ± 0.4 ^b	0.02
Interval between onset of functional luteolysis and P4 <1.00 ng/mL, d ± SEM	1.6 ± 0.2 ^a	2.5 ± 0.3 ^b	4.2 ± 0.4 ^c	0.001
2 Follicular waves	1.5 ± 0.2	1.0	—	—
3 Follicular waves	1.8 ± 0.3 ^a	2.6 ± 0.2 ^b	4.2 ± 0.4 ^c	0.001
Interval between onset of functional luteolysis and ovulation, d ± SEM	5.8 ± 0.4 ^a	7.2 ± 0.3 ^b	8.5 ± 0.7 ^b	<0.001
2 Follicular waves	5.2 ± 0.2 ^x	5.0	—	—
3 Follicular waves	7.5 ± 1.3 ^y	7.4 ± 0.3	8.5 ± 0.7	0.38
Interval between P4 <1.00 ng/mL and ovulation, d ± SEM	4.2 ± 0.4	4.7 ± 0.4	4.3 ± 0.5	0.64
2 Follicular waves	3.6 ± 0.1 ^x	4.0	—	—
3 Follicular waves	5.8 ± 1.3 ^y	4.7 ± 0.4	4.3 ± 0.5	0.37
Interval between P4 ≤0.50 ng/mL and ovulation, d ± SEM	4.1 ± 0.4 ^a	4.5 ± 0.4	3.5 ± 0.5	0.11
2 Follicular waves	3.6 ± 0.1 ^x	4.0	—	—
3 Follicular waves	5.5 ± 1.3 ^{y,A}	4.5 ± 0.4 ^A	3.5 ± 0.5 ^B	0.08

^{a-c}Mean values in the same row with different superscripts differ ($P \leq 0.05$).

^{x,y}Mean values for 2 versus 3 follicular waves in the same variable with different superscripts differ ($P \leq 0.05$).

^{A,B}Mean values with different uppercase letters have a tendency ($0.05 < P \leq 0.10$) to differ.

¹Cows in the control treatment did not receive any treatment. Cows in the hCG7 treatment received 3,300 IU of human chorionic gonadotropin (hCG) 7 d after the last GnRH (d 7 of the estrous cycle). Cows in the hCG7+13 treatment received 3,300 IU of hCG on d 7 and a second treatment with hCG on d 13 of the estrous cycle.

²P4 = progesterone.

Figure 3. (A) Effect of treatment on the mean ($\pm$ SEM) percentage of circulating progesterone (P4) concentration; (B) total luteal volume percentage after the onset of functional luteolysis; (C) accessories corpus luteum (CL) volume percentage after the onset of functional luteolysis; (D) comparisons among percentage of original CL volume in the control treatment versus original CL of human chorionic gonadotropin (hCG)-treated cows versus d 7 induced CL of hCG-treated cows. The analysis only considers cows with typical estrous cycle ($n = 39$). The circulating P4 concentration and volume of CL on d 0 (onset of luteolysis) was designated as 100% for each cow. Main effects and their interaction are shown on each panel. Different lowercase letters (a–c) within a day represent differences between least squares means ($P \leq 0.05$), and * or † represent a tendency ($0.05 < P \leq 0.10$) for a difference.

with typical cycle, resulting in a prolonged ($P < 0.001$) IOI for cows with delayed ovulation compared with cows with typical cycles (Table 5).

Two cows in control had delayed luteolysis with the onset of luteolysis on d 32 and 34 of the estrous cycle, 4 and 3 follicular waves during the estrous cycle, respectively, and 35 and 38 d of IOI, respectively (Supplemental Figure S4; <http://digital.library.wisc.edu/1793/82420>; Martins, 2021).

Mean day of first onset of luteolysis for cows with incomplete luteolysis (control, $n = 1$; hCG7, $n = 4$; hCG7+13, $n = 5$; Figure 5; Supplemental Figure S5; <http://digital.library.wisc.edu/1793/82420>; Martins, 2021) did not differ ($P = 0.14$) compared with cows

with typical cycles (Table 5). However, cows with incomplete luteolysis had complete functional luteolysis ($P4 < 1$ ng/mL) later ($P < 0.001$) in the cycle compared with cows with a typical cycle (Table 5). All cows with incomplete luteolysis had a second onset of luteolysis to undergo complete functional luteolysis (Figure 5; Table 5). The mean $\pm$ SEM for the interval between the first and second onset of luteolysis was 17.4 ± 2.1 d (range: 9–29 d). The cow with incomplete luteolysis in control had double ovulation to d 0 GnRH and 2 original ipsilateral CL during the estrous cycle (Table 6). Based on Doppler from the first onset of luteolysis to ovulation, both CL had score >1 and did not undergo complete regression. In addition, this cow had serum concentra-

Figure 4. Box-and-whisker plot and scatter plot representing the distribution of cows within treatment for the day of the estrous cycle for the first onset of functional luteolysis (A), completion of luteolysis (B), and interovulatory interval (C). Open dots (o) represent cows with atypical estrous cycle, and solid dots (●) represent cows with typical estrous cycle. In the box-and-whisker plot, diamond markers (◆) indicate the mean within treatments. The bottom of the box indicates the 25th percentile, the middle is the median, and the top represents the 75th percentile. The bottom and top whiskers denote the 10th and 90th percentiles, respectively. The probability of different variance among treatments for day to the first onset of functional luteolysis is shown in A. Cows in the control did not receive any treatment. Cows in the hCG7 treatment received 3,300 IU of human chorionic gonadotropin (hCG) 7 d after the last GnRH (d 7 of the estrous cycle). Cows in the hCG7+13 treatment received 3,300 IU of hCG on d 7 and a second treatment with hCG on d 13 of the estrous cycle.

tions of $P4 > 1 \text{ ng/mL}$ between the first and second onset of luteolysis (Supplemental Figure S5; cow #5498). The other 9/10 cows with incomplete luteolysis had one or more CL regressing (Doppler score ≤ 1) and at least one remaining functional between the first and second onset of luteolysis (Doppler score > 1 ; Table 6; Figures 6, 7, and 8; and Supplemental Figure S5). Some cows ($n = 4/10$) had one or more functional CL regression in one ovary, but all CL remained functional in the contralateral ovary between the first and second onset of luteolysis (Table 6; Figures 6 and 7). Surprisingly, $n = 6/10$ cows had ipsilateral CL, in which one or more CL underwent complete regression, and at least one CL remained functional on the same ovary until the second onset of luteolysis (Table 6; Figures 8 and 9). Figure 10 shows the luteal dynamics of a cow with complete luteolysis classified with a typical cycle.

Although most CL that did not undergo regression were accessory CL, a few original CL also did not undergo complete regression (Table 6). Of the cows with accessory CL (hCG treatments) and incomplete luteolysis ($n = 9$), one cow had accessory CL regression after the first onset of luteolysis, but the original CL did not regress (Table 6, cow #6601). In $n = 6/9$ cows, the opposite occurred; the original CL regressed after the first onset of luteolysis, but at least one accessory CL did not regress (Table 6; Figure 6; Supplemental Figure S5). In $n = 2/9$ cows, some accessory CL regressed, but the original CL and one accessory CL did not regress (Table 6; Figure 7).

After the first onset of luteolysis, all cows with incomplete luteolysis had an additional follicular wave and a total of 4 follicular waves, except for one cow in the hCG7+13 that had 2 follicular waves after the first onset of luteolysis and a total of 5 follicular waves during the estrous cycle. Cows with incomplete luteolysis had a prolonged ($P < 0.001$) IOI compared with cows with a typical cycle (Table 5). The interval from complete luteolysis to ovulation did not differ ($P = 0.41$) among cows with incomplete luteolysis and typical cycle (Table 5).

DISCUSSION

This study aimed to determine the effect of hCG administration either on d 7 only or on both d 7 and 13 on follicular and CL development dynamics and luteolysis during the estrous cycle in noninseminated high-producing lactating Holstein cows. Even though the use of hCG in cattle has been studied for more than 5 decades, to our knowledge, less than a handful of studies with small sample sizes have evaluated the effects of hCG administered during diestrus on ovarian dynamics in noninseminated lactating dairy cows (Helmer and

Table 5. Differences in luteolysis and estrous cycle characteristics among lactating dairy cows classified with typical estrous cycle, delayed ovulation and incomplete luteolysis

Variable	Typical cycle (n = 39)	Atypical cycle		P-value
		Delayed ovulation (n = 10)	Incomplete luteolysis (n = 10)	
Day of the cycle at onset of functional luteolysis $\pm$ SEM	18.7 $\pm$ 0.2	19.1 $\pm$ 1.2	19.9 $\pm$ 0.7	0.33
Day of the cycle at the second onset of functional luteolysis $\pm$ SEM	—	—	37.3 $\pm$ 0.3	—
Day of the cycle at P4 ¹ <1.0 ng/mL $\pm$ SEM (range)	21.3 $\pm$ 0.4 ^a (16–25)	21.8 $\pm$ 1.3 ^a (16–29)	38.6 $\pm$ 2.5 ^b (28–50)	<0.001
Interval between the first onset of functional luteolysis and P4 <1.00 ng/mL, d $\pm$ SEM (range)	2.6 $\pm$ 0.2 ^a (1–6)	2.8 $\pm$ 0.4 ^a (1–5)	17.9 $\pm$ 2.5 ^b (10–30)	<0.001
Interval between first onset of functional luteolysis and ovulation, d $\pm$ SEM (range)	6.9 $\pm$ 0.3 ^a (4–13)	21.6 $\pm$ 2.8 ^b (9–33)	20.2 $\pm$ 1.8 ^b (14–26)	<0.001
Interval between P4 <1.00 ng/mL and ovulation, d $\pm$ SEM	4.4 $\pm$ 0.2 ^a	18.8 $\pm$ 2.9 ^b	3.9 $\pm$ 0.5 ^a	<0.001
Interval between P4 $\leq$ 0.50 ng/mL and ovulation, d $\pm$ SEM	4.0 $\pm$ 0.2 ^a	18.4 $\pm$ 2.9 ^b	3.4 $\pm$ 0.4 ^a	<0.001
Interovulatory interval, d $\pm$ SEM (range)	24.0 $\pm$ 0.4 ^a (19–31)	38.7 $\pm$ 2.8 ^b (28–49)	38.9 $\pm$ 2.4 ^b (30–44)	<0.001

^{a,b}Mean values in the same row with different superscripts differ ($P \leq 0.05$).

¹P4 = progesterone.

Britt, 1986; Sianangama and Rajamahendran, 1996). Most studies used hCG to induce ovulation of a DF, formation of an accessory CL and increase circulating P4 concentration in inseminated cows and heifers (Schmitt et al., 1996b; Santos et al., 2001; Nascimento et al., 2013) or before embryo transfer (Vasconcelos et al., 2011; Niles et al., 2019). These studies aimed to test the effect of increased circulating P4 concentrations induced by hCG on early embryo development (Rizos et al., 2012) and fertility in cattle. However, the effect of

the formation of an accessory CL after hCG treatment on estrous cycle characteristics in nonpregnant inseminated cows is unclear due to the possible presence of an embryo and the effects of interferon tau on the timing of the luteolysis process. Even with the confounding effect of AI and ET, Niles et al. (2019) indicated that nonpregnant heifers after AI and ET that were treated with hCG on d 7 had a delay in luteolysis compared with nonpregnant untreated heifers. In agreement, a recent study from our laboratory using noninseminated

Figure 5. Mean ($\pm$ SEM) serum progesterone concentrations relative to the first onset of luteolysis in lactating dairy cows with typical estrous cycle (n = 39) and relative to first and second onset of luteolysis in lactating dairy cows with atypical estrous cycle with incomplete luteolysis (n = 8) or delayed ovulation (n = 9). Atypical estrous cycle with incomplete luteolysis: cows with incomplete functional luteolysis that did not complete corpus luteum regression by 6 d after the first onset of luteolysis. Atypical estrous cycle with delayed ovulation: cows with the dominant follicle or future dominant follicle at the time of luteolysis that did not ovulate.

Figure 6. Patterns of luteal development (luteal volume) and serum progesterone (P4) concentration, and ovarian sonographic B-Mode and color Doppler images of corpora lutea (CL) of a lactating Holstein cow (#6,499) treated with human chorionic gonadotropin (hCG) on d 7 of the estrous cycle (hCG7) and classified with an atypical estrous cycle due to incomplete luteolysis. The original CLd0 on the left ovary regressed after the first onset of luteolysis, but the contralateral CLd7 did not regress. CLd0 = original CL; CLd7 = accessory CL formed after hCG treatment on d 7.

Table 6. Circulating concentrations of progesterone (P4), corpora lutea (CL) number and side (LO = left ovary; RO = right ovary) of each specific CL location on day of first onset of functional luteolysis and 9 d later for each cow with incomplete luteolysis¹

Cow	Treatment ²	Day of first onset of luteolysis				9 d after first onset of luteolysis			
		CL before the first onset of luteolysis				Remaining CL (Doppler score >1) after the first onset of luteolysis			
		P4 (ng/mL)	CL number	LO	RO	P4 (ng/mL)	CL number	LO	RO
5,498	Control	8.6	2	—	—	5.2	2	—	2x(CLd0)
5,820	hCG7	11.1	2	CLd0 and CLd7	2x(CLd0)	2.7	1	CLd7	—
6,499	hCG7	7.7	2	CLd7	—	2.4	1	CLd7	—
6,601	hCG7	10.5	2	CLd0 and CLd7	CLd0	5.3	1	CLd0	—
8,856	hCG7	10.3	2	CLd0 and CLd7	—	4.0	1	CLd7	—
6,006	hCG7+13	14.0	4	2x(CLd7)	CLd0 and CLd13	2.1	2	—	CLd0 and CLd13
6,081	hCG7+13	12.7	5	2x(CLd7) and CLd13	CLd0 and CLd13	1.2	2	—	CLd0 and CLd13
6,467	hCG7+13	11.8	4	CLd0, 2x(CLd7), and CLd13	—	2.0	1	CLd7	—
8,457	hCG7+13	22.6	5	CLd0 and CLd13	2x(CLd7) and CLd13	4.8	3	—	2x(CLd7) and CLd13
8,938	hCG7+13	28.4	3	CLd0, CLd7, and CLd13	—	2.9	1	CLd13	—

¹CLd0 = original CL; CLd7 = accessory CL formed after human chorionic gonadotropin (hCG) treatment on d 7; CLd13 = accessory CL formed after hCG treatment on d 13.

²Cows in the control treatment did not receive any treatment. Cows in the hCG7 treatment received 3,300 IU of hCG 7 d after the last GnRH (d 7 of the estrous cycle). Cows in the hCG7+13 treatment received 3,300 IU of hCG on d 7 and a second treatment with hCG on d 13 of the estrous cycle.

cows determined that a single administration of 3,300 IU of hCG from d 5 to 7 of the estrous cycle reduced the proportion of multiparous cows returning to estrous and prolonged the estrous cycle (Cunha et al., 2021). In farms that use detection of estrus to rebreed cows and heifers post-AI, the use of hCG post-AI could affect their reproductive performance. Thus, to continue using this tool to enhance fertility of lactating dairy cows, it is necessary to understand the physiological effects of the use of hCG to induce ovulation and accessory CL formation during diestrus.

In the present study, we used 3,300 IU hCG to induce ovulation of a DF and the formation of accessory CL in noninseminated lactating Holstein cows, which resulted in a high ovulation rate; only 2 of 42 (4.8%) cows did not ovulate and 3/64 (4.6%) hCG treatments failed to induce ovulation. We also successfully increased serum

P4 concentrations during diestrus due to increased total luteal volume by growth of the original CL and formation of new accessory CL, consistent with other studies (Sianangama and Rajamahendran, 1992, Fricke et al., 1993, Santos et al., 2001). Our results indicated that hCG administered on d 7 caused an increase in the original CL volume that was maintained during most of the diestrus period. The second administration of hCG in hCG7+13 cows allowed maintenance of the increased volume of the original CL after d 13 and extension of the greater volume before the onset of functional luteolysis compared with cows receiving hCG7. Schmitt et al. (1996a) determined that the original CL of cows treated with hCG on d 5 had an increase in the size of small and large luteal cells at d 17 of the cycle compared with control cows (saline), but they did not find an increase in the number of small and large luteal

Figure 7. Patterns of luteal development (luteal volume) and serum progesterone (P4) concentration, and ovarian sonographic B-Mode and color Doppler images of corpora lutea (CL) of a lactating Holstein cow (#6,006) treated with human chorionic gonadotropin (hCG) on d 7 and 13 of the estrous cycle (hCG7+13) and classified with an atypical estrous cycle due to incomplete luteolysis. Two accessories CLd7 in the left ovary regressed after the first onset of luteolysis, but the contralateral original CLd0 and accessory CLd13 did not regress. CLd0 = original CL; CLd7 = accessory CL formed after hCG treatment on d 7; CLd13 = accessory CL formed after hCG treatment on d 13.

cells. In the present study, the increase in the volume of original CL for cows treated with hCG may be explained by a hypertrophy effect of hCG on luteal cells. Although original CL volume in hCG7 and hCG7+13 cows decreased before the onset of functional luteolysis, serum P4 concentration was maintained during the same period, most likely due to the development and increase in the size of the accessory CL in the same period.

A greater proportion of cows treated with hCG on d 7 had estrous cycles with 3 follicular waves than control cows, confirming our first hypothesis. Inducing ovulation of the first-wave DF using hCG on d 7 of the estrous cycle led to an earlier emergence of the second wave on d 8 to 9, subsequent regression of the second-wave DF, and emergence of the third wave on d 16 to 17. The days of follicular wave emergence were similar to cows with noninduced 3 follicular waves estrous cycles previously reported, with emergence of the second wave on d 8 or 9 of the estrous cycle and the third wave on d 16 or 17 (Ginther et al., 1989; Sartori et al., 2004). The second follicular wave emergence in control cows with 2 waves was later in the cycle (d 11.7 ± 0.6). The earlier emergence of the second follicular wave and the increased circulating P4 concentration in hCG-treated cows most likely led to early atresia of the second-wave DF, before luteolysis, and emergence of a third follicular wave in the hCG7 cows. In hCG7+13 cows, the third-wave emergence was already expected

to occur after induced ovulation of the second-wave DF with hCG on d 13 of the cycle.

Our second hypothesis that cows treated with hCG would have a prolonged IOI than control cows was also confirmed. The longer IOI for cows treated with hCG seemed to relate primarily to the increase in the proportion of cows with 3 follicular waves. Previous studies reported that cows and heifers with 3-follicular wave cycles have longer IOI than 2-wave cycles (Ginther et al., 1989; Diaz et al., 1998; Bleach et al., 2004). The longer IOI has also been related to an earlier emergence of the second wave and later onset of luteolysis in nontreated cows and heifers (Savio et al., 1990; Jaiswal et al., 2009), as occurred in hCG7 3-waves cows in the present study. After the onset of luteolysis, these cows took longer to ovulate than control cows because the third-wave DF had to grow large enough to produce sufficient E2 to induce a pre-ovulatory GnRH surge. The occurrence of 3 follicular waves and later onset of luteolysis were crucial factors in prolonging the IOI in our study.

The initiation of luteolysis in the bovine is characterized by endometrial pulse release of $\text{PGF}_{2\alpha}$ in response to hypothalamic oxytocin (reviewed by McCracken et al., 1999). The regulation of $\text{PGF}_{2\alpha}$ secretion is a result of an interaction among circulating concentrations of estradiol- 17β (E2), P4, and oxytocin (OXT) and the changes in their respective receptors in the endometrium (Flint et al., 1994; Robinson et al., 2001). It seems

Figure 8. Patterns of luteal development (luteal volume) and serum progesterone (P4) concentration, and ovarian color Doppler images of corpora lutea (CL) of a lactating Holstein cow (#8,856) treated with human chorionic gonadotropin (hCG) on d 7 of the estrous cycle (hCG7) and classified with an atypical estrous cycle due to incomplete luteolysis. The original CLd0 in the left ovary regressed after the first onset of luteolysis, but the ipsilateral accessory CLd7 did not regress. This cow did not have any CL on the right ovary. CLd0 = original CL; CLd7 = accessory CL formed after hCG treatment on d 7.

Figure 9. Patterns of luteal development (luteal volume) and serum progesterone (P4) concentration, and ovarian sonographic and color Doppler images of corpora lutea (CL) of a lactating Holstein cow (#5,820) treated with human chorionic gonadotropin (hCG) on d 7 of the estrous cycle (hCG7) and classified with an atypical estrous cycle due to incomplete luteolysis. The original CLd0 in the left ovary regressed after the first onset of luteolysis, but the ipsilateral accessory CLd7 did not regress. This cow did not have any CL on the right ovary. CLd0 = original CL; CLd7 = accessory CL formed after hCG treatment on d 7.

that both circulating concentrations of E2 produced by the DF and the endometrial E2 responsiveness are the main factors that may initiate upregulation of OXT receptors (OXTR) and timing of luteolysis (Domingues et al., 2020). Araujo et al. (2009) successfully prevented the rise of follicular E2 and prolonged the luteal phase in dairy heifers after daily follicle ablation between d 9 and 15 of the estrous cycle. The prolonged luteal phase caused by successive follicular aspirations was reversed with exogenous E2 administration on d 13 and 15 of the estrous cycle. In a recent study, Domingues et al. (2020) temporally associated a later upregulation of endometrial OXT mRNA with later onset of luteolysis in heifers with 3 waves during the cycle. In that study, the

Figure 10. Patterns of luteal development (luteal volume) and serum progesterone (P4) concentration, and ovarian sonographic color Doppler images of the corpora lutea (CL) of a lactating Holstein cow (#6,450) treated with human chorionic gonadotropin (hCG) on d 7 and 13 of the estrous cycle (hCG7+13) and classified with a typical estrous cycle. CLd0 = original CL; CLd7 = accessory CL formed after hCG treatment on d 7; CLd13 = accessory CL formed after hCG treatment on d 13.

author suggested that in heifers with 2 or 3 waves cycle, the downregulation of P4 and estradiol- β receptors removes their inhibitory effects on estradiol- α receptors at a similar time during the cycle. Their hypothesis was that in cycles with 3 follicular waves, the second-wave DF lost dominance and E2 secretory capacity before estradiol- α receptors could be activated. In addition, upregulation of OXTR was delayed until adequate circulating E2 concentrations from either the second or third follicular wave activate estradiol- α receptors. The difference in day for onset of luteolysis and the decreased variation in the onset of functional luteolysis observed in our study for cows treated with hCG7 and hCG7+13 compared with control support the role of follicles in the timing of luteolysis onset in cattle. It also confirmed our final hypothesis that cows treated with hCG on d 7 and 13 would have follicles from the third wave growing in such synchrony that there would be a reduction in variation for the onset of luteolysis. Decreasing this variance could benefit rebreeding strategies, especially resynchronization of ovulation for timed-AI protocols before pregnancy diagnosis that aim to control the follicular wave of the preovulatory follicle with a GnRH injection (Giordano et al., 2012, Baruselli et al., 2018, Palhão et al., 2020).

One surprising finding in this study was the protracted interval for complete functional luteolysis in hCG-treated cows. Previous reports using lactating Holstein cows and heifers indicated that natural complete functional luteolysis takes between 1 and 2 d (Sartori et al., 2004; Ginther et al., 2007), which is consistent with our findings in control cows. For cows in the treatment hCG7+13, our results suggest that the youngest accessory CL induced by hCG on d13 of the cycle and ~6 d old at the onset of luteolysis might have prolonged the luteolysis process. A single administration of GnRH agonist (5 μ g of buserelin) from d 12 to 16 of the estrous cycle also prolonged the average length of the estrous cycle, indicating that cows with accessory CL formed later in diestrus might have induced a later luteolysis onset (Macmillan et al., 1985). Moreover, studies have indicated that CL 7 d old or younger may not be completely responsive to the administration of exogenous PGF_{2 α} (Tsai and Wiltbank, 1998; Nascimento et al., 2014). In some cows in the hCG treatments, more endometrial pulses of PGF_{2 α} , which occur in intervals of 7 to 12 h (Ginther et al., 2007, 2010), may have been required to achieve complete luteolysis. Yet, this would only partially explain the prolonged luteolysis interval since hCG7 cows with older accessory CL (~12 d old) at the onset of luteolysis also had prolonged luteolysis.

Another indication of tardy complete luteolysis was the slower decrease in the volume of the original CL

in both hCG7 and hCG7+13 compared with controls. These results suggested a longer luteolysis of older CL in cows treated with hCG. Furthermore, hCG treatment on d 5 of the estrous cycle has been reported to increase the proportion of small and large luteal cells of the original CL in earlier stages of development at d 17 compared with untreated cows (Schmitt et al., 1996a). They also suggested that hCG may have delayed the progression of luteal cells through the stages of development that lead to involution and regression (Schmitt et al., 1996a). Perhaps this delay in small and large luteal cell development has altered the ability of the CL to undergo complete regression after endometrial PGF_{2 α} pulses. Other factors that may have contributed to the prolonged functional luteolysis in hCG treatment were the greater CL number and total luteal volume for cows at the onset of luteolysis. The ~50% and ~330% greater mean serum P4 concentrations at onset of luteolysis for hCG7 and hCG7+13, respectively, than control cows, does not seem to explain the prolonged luteolysis process in cows treated with hCG (1.6 ± 0.2 d vs. 2.5 ± 0.2 d vs. 4.2 ± 0.2 d for control, hCG7 and hCG7+13, respectively) because the clearance rate of circulating P4 occurs relatively fast in lactating dairy cows (Sangsrivong et al., 2002). The average half-life of P4 in dairy cows is approximately 35 min (Miller et al., 1963).

All these potential factors that extended the luteolytic process in hCG-treated cows could also have impacted the capacity of cows to complete CL regression and undergo complete functional luteolysis. A previous study identified delayed or incomplete luteolysis induced by exogenous PGF_{2 α} analogs in lactating dairy cows (Martins et al., 2011). Also, Greco et al. (2018) identified approximately 18% of lactating Holstein cows with no spontaneous luteolysis (P4 > 1.00 ng/mL) by d 23 of the estrous cycle. However, those cows did not have accessory CL and were not followed after d 23 of the estrous cycle. It appears that cows with no luteolysis in that study may not have had luteolytic PGFM pulses and a significant decrease in circulating P4 concentrations by d 23 of the estrous cycle (Greco et al., 2018). In the present study, we collected data from all cows until subsequent spontaneous ovulation. All cows in our study had an onset of luteolysis with a significant decrease in circulating P4 concentrations during the estrous cycle, early or late, that most likely characterize luteolytic PGF_{2 α} pulses. Ten cows (n = 10/62) did not achieve P4 < 1.00 ng/mL after the onset of luteolysis and had incomplete luteolysis. Most of them were in hCG treatments (n = 9/10), suggesting that induction of accessory CL using hCG may disrupt some physiological processes essential for completion of

functional luteolysis. The majority of cows with incomplete luteolysis ($n = 9/10$) also had one or more CL with complete structural regression based on Doppler evaluations and a significant decrease in circulating concentrations of P4 approximately d 21 of the estrous cycle. Differently, cows with delayed luteolysis did not significantly decrease serum P4 concentration until approximately d 33 of the estrous cycle. These results indicated that cows with incomplete luteolysis had luteolytic endometrial pulses of PGF_{2α}, but at least one CL did not respond to the PGF_{2α} pulses either because the pulses were inadequate to induce complete luteolysis or the CL was refractory to PGF_{2α} action. All cows with incomplete luteolysis needed a second onset of luteolysis to undergo complete functional luteolysis. In addition, it appears that another luteal phase with similar duration to a normal estrous cycle was necessary to prepare the endometrium for the second onset of luteolysis.

Besides cows with incomplete or delayed luteolysis, we also identified cows with delayed ovulation and a short estrous cycle. These classifications of atypical estrous cycle have been reported in previous studies (Savio et al., 1990; Sartori et al., 2004). In the present experiment, most cows with atypical estrous cycles had delayed ovulation (10/23) or incomplete luteolysis (10/23). Moreover, the majority of them were in hCG treatments (17/20), suggesting that induction of accessory CL with hCG during diestrus may disrupt luteolysis and ovulation. These results also agree with a recent study from our laboratory that determined that a single hCG treatment from d 5 to 7 of the estrous cycle decreased the proportion of lactating multiparous Holstein cows returning in estrus until d 32 of the cycle (Cunha et al., 2021). In addition, cows returning to estrus after treatment had a prolonged estrous cycle (Cunha et al., 2021). Thus, the effects of hCG on the estrous cycle dynamics must be considered in farms that use detection of estrus as a rebreeding strategy and use hCG post-AI or before ET to improve fertility (Nascimento et al., 2013; Niles et al., 2019).

In conclusion, hCG administration on d 7 and d 7 and 13 of the estrous cycle augmented the original CL volume, increased serum concentration of P4 during diestrus and the proportion of cows with 3 follicular waves, and decreased the variability in the onset of luteolysis. Nevertheless, hCG treatments also prolonged luteolysis and IOI, and increased the incidence of cows with impaired luteolysis and delayed ovulation. These data indicate that forming accessory CL with the use of hCG disrupted natural luteolysis and ovulation, which are critical physiological events that regulate estrous cycle length.

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