—Original Article—

Human chorionic gonadotropin (hCG)-induced ovulation occurs later but with equal occurrence in lactating dairy cows: comparing hCG and gonadotropin-releasing hormone protocols

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Abstract. This study assessed the effects of two hormones, human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone (GnRH), on ovulatory responses during different diestrous stages in lactating dairy cows. Estrous cycles of 21 cows were synchronized and were enrolled in stage 1 of the experiment. The cows were treated with a prostaglandin (PG) $F_{2\alpha}$ analog either 9 to 10 days [mid-diestrus (MD) group] or 5.5 to 6.5 days [early-diestrus (ED) group] after synchronized ovulation (day 0 = first $PGF_{2\alpha}$ administration). On day 2, the cows were administrated 250 μg GnRH or 3000 IU hCG. Ovulation was determined every 2 h from 24 to 36 h after GnRH or hCG administration, and then every 4 h up to 72 h until ovulation. Cows in stage 2 were administered these treatments in the reverse order. The results indicated that average ovulation times in cows treated with GnRH in the MD group (GnRH-MD group) and cows treated with GnRH in the ED group (GnRH-ED group) were 30.0 ± 1.0 h and 28.8 ± 0.4 h, respectively. However, ovulation times for cows treated with hCG in the MD group (hCG-MD group) and cows treated with hCG in the ED group (hCG-ED group) were 35.8 ± 4.6 h and 32.8 ± 2.2 h, respectively, and ovulation occurred significantly later in the hCG-treated groups than in the GnRH-treated groups. In summary, we found that hCG-induced ovulation occurred later than GnRH-induced ovulation regardless of different diestrous peroids; however, the two treatments did not differ in terms of percentage of ovulation.
Key words: Gonadotropin-releasing hormone (GnRH), Human chorionic gonadotropin (hCG), Ovulation time

Ovulation synchronization protocols, such as Ovsynch, comprise two doses of gonadotropin-releasing hormone (GnRH) and one dose of prostaglandin (PG) $F_{2\alpha}$ [1]. The use of GnRH induces a luteinizing hormone (LH) surge [2], resulting in ovulation at 26 to 32 h [1], with most cows ovulating between 28 and 30 h following GnRH administration [3, 4].

By binding directly to LH receptors [5, 6], human chorionic gonadotropin (hCG) can induce ovulation and can exert a luteotropic effect [7]. Additionally, unlike GnRH-induced ovulation by LH, which is affected by the negative feedback of systemic progesterone (P_4) concentration [3, 8, 9], ovulation is induced by hCG independent of the pituitary gland. Some researchers have tried to overcome restricted pregnancy rates by using hCG. In studies that have utilized synchronization protocols, substitution of the first GnRH dose of Pre-Ovsynch [5] and Ovsynch [10], and the second GnRH dose of Ovsynch with hCG [11, 12] has been reported. Generally, admin-

istration of hCG is associated with an increase in the percentage of ovulation when compared with GnRH administration; however, similar percentages of ovulation were found when P_4 concentration was lower than 1 ng/ml. Nevertheless, an improvement in synchronization and conception rates in cows was not observed following treatment with hCG. In contrast, researchers have speculated that increased P_4 concentration after artificial insemination could improve conception rates. In most cows receiving hCG after insemination, the development of a second corpus luteum (CL) was achieved, which was accompanied by elevated P_4 concentrations [13, 14]; however, improved conception rates in cows were observed in some [13, 15, 16] but not in all the trials [17, 18]. It has been reported that the interval between GnRH and artificial insemination influences pregnancy rate [19]; hence, we sought to determine the time of ovulation in cows after ovulation-inducing drug administration. To the best of our knowledge, earlier studies have focused on the percentage of ovulation after hCG treatment, disregarding the time interval between the hCG injection and occurrence of ovulation. Reducing the period between calving and the first insemination is important in the dairy industry. Our previous studies had focused on the practicability of the protocol (two low doses of $PGF_{2\alpha}$ with or without GnRH) during mid-diestrus [4, 20]; however, the benefit of using this protocol during early-diestrus is still unclear.

This study aimed to investigate the effects of hCG on ovulatory

responses during mid- and early-diestrus. We hypothesized that hCG-induced ovulation would occur earlier than GnRH-induced ovulation, but the percentage of ovulation would be similar regardless of the different diestrous periods.

## Materials and Methods

Animals and management

This study, comprising about 60 Holstein cattle, was conducted on a dairy farm at the National Chung Hsing University (subtropical Taiwan) between August 2017 and April 2018. All the cows were housed in groups in free-stall barns with a slatted floor and equipped with free-stall bed mats, overhead fans, and a sprinkler system. Access to an outdoor shaded exercise yard was also available. Lactating dairy cows were fed with a total mixed ration (TMR) twice daily *ad libitum*. The diet (TMR) consisted of approximately 58% hay (Bermuda and alfalfa) and 42% concentrate (commercial milking cow concentrate, steam-flaked corn, and wheat bran), which was mixed with minerals (dicalcium phosphate and magnesium oxide) and vitamin E. Fresh water was provided *ad libitum*. All lactating dairy cows were milked twice daily, and the average daily milk yield was 25 kg per cow. During the experimental period, temperature and humidity were recorded at 0600 h, 1200 h, 1800 h, and 2400 h, and the thermal-humidity index (THI) was calculated. All the procedures were approved by the Animal Care and Use Committee for Biotechnology of the National Chung Hsing University (IACUC No. 106-058). The lactating dairy cows, between 50 and 70 days in milk (DIM) and with normal uterus involution and ovarian function was enrolled for this experiment. The parity of the cows ranged from 1 to 4, except for two cows that gave birth six and seven times, respectively [average parity: 2.9 ± 1.6 (mean $\pm$ SD)]. The body condition score (BCS) of the cows ranged from 2.5 to 3.25 based on a 5-point scale [21], and the average BCS was 3.03 ± 0.26.

Experimental design

A total of 21 lactating dairy cows were subjected to synchronization of the estrous cycle employing the modified Ovsynch-48 protocol, and the following experimental protocol was conducted on the animals after successful synchronization (Fig. 1). The modified Ovsynch-48 protocol involved administration of 250 µg GnRH (100 µg/ml Fertagyl/ gonadorelin; Intervet Deutschland, Unterschleißheim, Germany) intramuscularly (im) to cows having random estrous cycle; 7 days later, the cows received 375 µg of $PGF_{2\alpha}$ (250 µg/ml Estrumate/ cloprostenol sodium ; Intervet Deutschland), im (the time of first prostaglandin (PG) $F_{2\alpha}$ injection during synchronization; SynPG). A second dose of $PGF_{2\alpha}$ (250 µg) was administered after 24 h, and another dose of GnRH (250 µg) was administered 48 h after the first dose of $PGF_{2\alpha}$ (SynG2). If the cows were not synchronized, by presenting no signs of complete luteolysis/ovulation but were still between 50 and 70 DIM, the process of synchronization was repeated using a modified Ovsynch-48 protocol.

After successful synchronization, we first subdivided the cows in two groups: the mid-diestrus group (MD group; 9 to 10 days after ovulation) and the early-diestrus group (ED group; 5.5 to 6.5 days after ovulation). In the MD group, cows were enrolled in stage 1 (DIM: 76.6 ± 9.0) of the experimental protocol 9 to 10 days after ovulation of synchronization. The time of the first $PGF_{2\alpha}$ administration was defined as day 0 of stage 1. On days 0 and 1, cows were treated with 375 and 250 µg $PGF_{2\alpha}$, respectively. On day 2, the cows were administered im with 250 µg GnRH (GnRH-MD group) or 3000
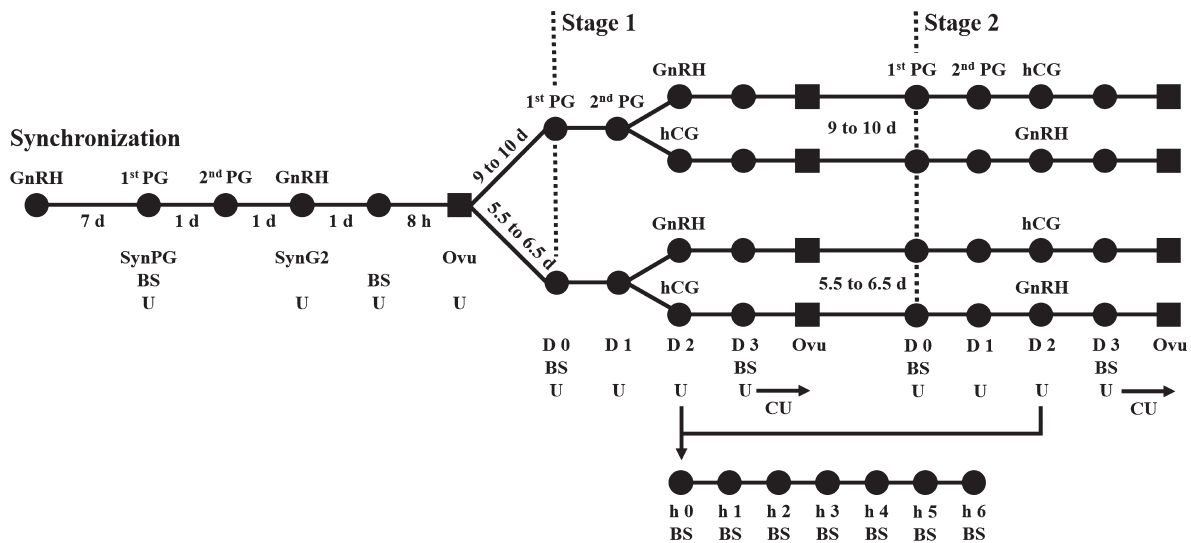
Fig. 1. Schematic representation of experimental procedures. Blood sampling (BS) for progesterone, luteinizing hormone, and human chorionic gonadotropin (hCG); consecutive ultrasound (CU) examination to determine ovulation every 2 h from 24 to 36 h after gonadotropin-releasing hormone (GnRH) or hCG treatments, then every 4 h up to 72 h; 1st and 2nd prostaglandin (PG), administration of 375 µg and 250 µg cloprostenol, respectively. GnRH, 250 µg of gonadorelin; hCG, 3000 IU of hCG; Ovu, observed ovulation time; SynG2, the time of GnRH administration; SynPG, the time of first $PGF_{2\alpha}$ injection during synchronization; U, ultrasonography for follicle and corpus luteum detection and measurements.

IU hCG (1,500 IU/ample Pregnyl/chorionic gonadotrophin; N.V. Organon, Netherlands) (hCG-MD group). If both ovulation within 72 h after GnRH or hCG administration as well as complete luteolysis occurred, the cows were further enrolled in stage 2 of the experimental protocol. Cows with partial luteolysis or absence of ovulation were not assigned to the next stage. During 9 to 10 days after ovulation of stage 1, the treatment protocol in stage 1 was reversed for cows in stage 2 (DIM: 91.0 ± 9.6) —i.e., cows treated with GnRH in stage 1 were administered hCG in stage 2 and vice versa.

In the ED group, all procedures were essentially the same as those used in the MD group except that the cows were treated with the first dose of $PGF_{2\alpha}$ between 5.5 and 6.5 days after ovulation of synchronization and stage 1. Cows treated with GnRH were allocated to the GnRH-ED group and the others to hCG-ED group. In stages 1 and 2, the average DIM in the ED group was 69.7 ± 4.2 and 80.1 ± 2.5, respectively.

Ultrasound examination for CL area, follicle diameter, and ovulation

Transrectal B-mode ultrasonography of the ovaries was performed using a portable scanner equipped with a 7.5-MHz linear-array transducer (SonoSite Ultrasound System; SonoSite, Bothell, WA, USA) at SynPG, 24 h after the second GnRH injection of the synchronization stage, and once a day from days 0 to 3 of stages 1 and 2. The method used to measure the longitudinal and transverse axes of the CL and to calculate the remaining CL area has been described previously [4].

The follicle diameter was determined by calculating the average of its longitudinal and transverse axes. The maximum follicle diameters at 24 h after the second GnRH dose of the synchronization stage and on day 3 of stages 1 and 2 were used to quantify the preovulatory follicle diameters. Ovulation was identified by the disappearance of preovulatory follicles followed by the appearance of a new CL at the same site in the ovary. During synchronization, ovulation was determined at 24 h and 32 h after the administration of second GnRH dose. During stages 1 and 2, ovulation was verified every 2 h from 24 to 36 h after GnRH or hCG injections, and then every 4 h up to 72 h until ovulation occurred. The time of disappearance of the preovulatory follicles was defined as the ovulation time.

Blood sampling and hormone analysis

Blood samples were collected from the coccygeal vessels and immediately refrigerated. The samples were then centrifuged (1,300 × g, 10 min), and the serum was harvested and stored at –20ºC until the assay. Blood sampling for analysis of P_4 concentrations was performed for all the cows at SynPG, at 24 h after the second dose of GnRH of the synchronization stage and on days 0 and 3 of stages 1 and 2. Serum P_4 concentrations were measured using an enzyme immunoassay kit (Progesterone ELISA, Demeditec Diagnostics GmbH, Kiel, Germany), with a sensitivity of 0.045 ng/ml. The average intra- and inter-assay coefficients of variation were 6.42% and 6.63%, respectively.

During stages 1 and 2, blood samples for analysis of LH and hCG concentrations were taken before and at every 1 h from 1 to 6 h after GnRH or hCG injections (Fig. 1). Serum LH concentrations were determined using an ELISA sandwich assay kit (LH DETECT for bovines with tetramethylbenzidine substrate; ReproPharm Vet, Nouzilly, France) in all the cows, with a sensitivity of 0.1 ng/ml. The intra- and inter-assay coefficients of variation for this assay were 2.5% and 6.0%, respectively. Serum hCG concentrations in 20 cows (GnRH-MD group = 4, hCG-MD group = 5, GnRH-ED group = 5, and hCG-ED group = 6) were estimated using an enzyme-linked fluorescent assay kit (VIDAS HCG, BioMérieux, Marcy-l'Étoile, France) on a MiniVidas automated analyzer (BioMérieux). The measurement range of this kit was 2 to 1500 mIU/ml, and the detection limit was 2 mIU/ml. The average intra- and inter-assay coefficients of variations were 5.2% and 5.6%, respectively.

Markers of complete luteolysis and successful synchronization

Complete luteolysis was identified by either a P_4 concentration < 1 ng/ml or a remaining CL area < 50% at 24 h after the second dose of GnRH of the synchronization stage and on day 3 of the stages 1 and 2 [4]. Successful synchronization was defined using the following three criteria: 1) P_4 concentration > 1 ng/ml with accompanying CL formation and at least one follicle with a diameter ≥ 8 mm at SynPG, 2) complete luteolysis at 24 h after the second dose of GnRH, and 3) ovulation of one or more follicles at 32 h after the second GnRH administration.

Milk production

Milk yield in each cow was recorded from days 0 to 3 of stages 1 and 2. Average milk production was calculated for each animal in stages 1 (29.3 ± 5.6 l/day) and 2 (28.4 ± 5.8 l/day).

Statistical analysis

Statistical analysis of the data was performed using the SAS software - version 9.4 (SAS Institute, Raleigh, NC, USA). Statistical significance was set at P < 0.05. We considered $0.05 \leq P < 0.10$ as trends. Given the limited sample size and the non-normality of the distribution of the data, we performed group comparisons to test for differences in P_4 concentration, remaining CL area, follicular diameter on day 0, preovulatory follicle diameter, ovulation time, DIM, BCS, parity, average THI, and average milk production using the Kruskal-Wallis test (in GnRH-MD, hCG-MD, GnRH-ED, and hCG-ED groups) or the Wilcoxon rank-sum test (GnRH vs. hCG and MD vs. ED). In addition, a post-hoc test for the Kruskal-Wallis analysis was conducted as described in a previous study [22]. Differences in the percentages of complete luteolysis, overall percentages of ovulation, percentages of ovulation within 36 h of GnRH or hCG injection, and percentages of multiple ovulation (number of cows having more than one ovulated follicle/number of cows ovulating) were analyzed using the chi-square test and the Fisher's exact test for comparison among four groups and between two groups, respectively. Differences in LH and hCG concentrations measured at various time points (seven time points from 0 to 6 h; using a within-subject design) among the four groups (GnRH-MD, hCG-MD, GnRH-ED, and hCG-ED groups; using a between-subject design) were determined using a repeated measures ANOVA, and the Bonferroni's method was applied to perform multiple comparison.

Results

Synchronization

Synchronization was achieved for 24 estrous cycles from 21 cows. There were three cows that underwent the modified Ovsynch-48 protocol twice. The results of synchronization are shown in Table 1. Complete luteolysis could be identified in all the cows, and ovulation occurred in 20 synchronized cycles. The overall percentage of the synchronized cycles was 83.3% (20/24).

Stages 1 and 2

Before initiating the stage 1 experiment, one cow was excluded because of acute mastitis. In this study, because the sample size in the stages 1 or 2 of each treatment group was too small (n = 3 to 5), a sequence effect (i.e., at stages 1 and 2) on the percentages of complete luteolysis and ovulation was tested. Statistical analysis showed no sequence effect on these parameters (data not shown). Therefore, all the data from stages 1 and 2 were grouped together, and the cows were reassigned to two groups: 1) the GnRH and hCG groups, or 2) the ED and MD groups. Accordingly, four groups, i.e., GnRH-ED, GnRH-MD, hCG-ED, and hCG-MD, were taken into account for further statistical analysis. In total, there were nine cows in the GnRH-MD group, nine in hCG-MD group, eight in GnRH-ED group, and eight in the hCG-ED group. No significant differences were found among the four groups with regard to parity, BCS, DIM, average THI, and average milk production.

Except for five cows detected with more than one CL, all the remaining animals had only one CL. On day 0, the P_4 concentrations were higher in GnRH-MD than in the GnRH-ED and hCG-ED groups; however, no significant differences could be detected for P_4 concentration on day 3 (Table 2). On average, the values of the remaining CL area in the GnRH-MD group from day 1 to 3 were lower than those in the GnRH-ED group. Although there was each one cow with partial luteolysis in the GnRH-ED and hCG-ED groups,

Table 1. Results of the synchronization

Parameters	Values
Number of CL at SynPG	1.9 ± 1.1
P_4 at SynPG (ng/ml)	7.27 ± 2.74
P_4 at 24 h after second GnRH (ng/ml)	0.44 ± 0.11
Remaining CL area at 24 h after second GnRH (%)	37.0 ± 12.2
Complete luteolysis (%)	100.0 (24/24)
Preovulatory follicle diameter (mm)	14.3 ± 2.8
Ovulation (%)	83.3 (20/24)
Multiple ovulation (%)	15.0 (3/20)
Synchronization (%)	83.3 (20/24)

A total of 21 cows were synchronized for 24 estrous cycles. CL, corpus luteum; GnRH, gonadotropin-releasing hormone; P_4, progesterone; SynPG, the time point of first prostaglandin $F_{2\alpha}$ administration. Data are shown as mean ± SD.

the percentage of complete luteolysis was not statistically different among the four groups.

Mean LH profiles throughout the sampling period were affected by different groups, time periods, and a group by time interaction (all $P < 0.05$). During hours 1 to 3, higher LH concentrations were found in the GnRH-MD and GnRH-ED groups than those in the other two groups. Peak LH concentrations occurred at 2 h after GnRH administration in the GnRH-MD and GnRH-ED groups; the concentrations then decreased until they returned the baseline at 5 h after treatment. With the exception of one cow in the hCG-ED group that showed a spontaneous LH surge and had a similar profile to that observed for the GnRH-MD and GnRH-ED groups, the LH concentration of the cows in the hCG-MD and hCG-ED groups remained at baseline levels (Fig. 2).

Mean hCG profiles throughout the sampling period were affected by different groups, time periods, and a group by time interaction

Table 2. Luteolytic and ovulatory parameters in the four groups after data from stages 1 and 2 were pooled together

Parameters	GnRH-MD (n = 9)	hCG-MD (n = 9)	GnRH-ED (n = 8)	hCG-ED (n = 8)	P
P_4 on day 0 (ng/ml)	8.14 ± 0.72 [a]	7.96 ± 1.06 [ab]	4.69 ± 0.77 [b]	4.62 ± 0.62 [b]	0.0037
P_4 on day 3 (ng/ml)	0.43 ± 0.04	0.50 ± 0.05	0.87 ± 0.46	0.74 ± 0.27	0.5969
Remaining CL area on day 1 (%)	60.6 ± 2.7 [b]	69.8 ± 4.9 [ab]	82.4 ± 6.0 [a]	73.4 ± 5.4 [ab]	0.0222
Remaining CL area on day 2 (%)	44.6 ± 2.4 [b]	49.4 ± 2.4 [ab]	61.1 ± 5.0 [a]	54.7 ± 3.5 [ab]	0.0324
Remaining CL area on day 3 (%)	28.7 ± 1.1 [b]	35.0 ± 3.3 [ab]	43.4 ± 4.0 [a]	41.6 ± 4.7 [ab]	0.0127
Complete luteolysis (%)	100.0 (9/9)	100.0 (9/9)	87.5 (7/8)	87.5 (7/8)	0.4954
Follicle diameter on day 0 (mm)	16.4 ± 0.8 [a]	15.1 ± 1.2 [ab]	11.5 ± 1.2 [b]	14.3 ± 0.6 [ab]	0.0356
Preovulatory follicle diameter (mm)	17.1 ± 0.7	15.9 ± 1.1	15.0 ± 1.4	17.1 ± 1.0	0.3710
Ovulation time (h)	30.0 ± 1.0 [ab]	35.8 ± 4.6 [a]	28.8 ± 0.4 [b]	32.8 ± 2.2 [ab]	0.0285
Ovulation (%)	77.8 (7/9)	88.9 (8/9)	100.0 (8/8)	100.0 (8/8)	0.3061
Ovulation within 36 h (%)	77.8 (7/9)	77.8 (7/9)	100.0 (8/8)	87.5 (7/8)	0.5278
Multiple ovulation (%)	0.0 (0/7)	12.5 (1/8)	25.0 (2/8)	12.5 (1/8)	0.5558

CL, corpus luteum; P_4, progesterone; GnRH-MD, treated with gonadotropin-releasing hormone (GnRH) in the mid-diestrus group; GnRH-ED, treated with GnRH in the early-diestrus group; hCG-MD, treated with human chorionic gonadotropin (hCG) in the mid-diestrus group; hCG-ED, treated with hCG in the early-diestrus group. Data are shown as mean ± SEM. Different superscript letters indicate statistically significant difference between groups in the same row; Categorical and continuous data were tested by the chi-square test and the Kruskal-Wallis test, respectively.
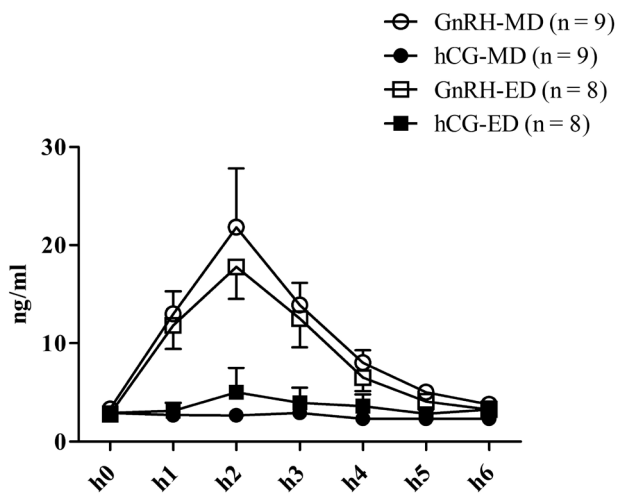
Fig. 2. Luteinizing hormone (LH) concentrations in lactating dairy cows receiving treatment in the GnRH-MD, hCG-MD, GnRH-ED, and hCG-ED groups. GnRH, gonadotropin-releasing hormone; GnRH-MD, treated with GnRH in the mid-diestrus group; GnRH-ED, treated with GnRH in the early-diestrus group; hCG-MD, treated with human chorionic gonadotropin (hCG) in the mid-diestrus group; hCG-ED, treated with hCG in the early-diestrus group. Data are shown as mean ± SEM. The P values of different groups, time periods, and a group by time interaction were < 0.05.
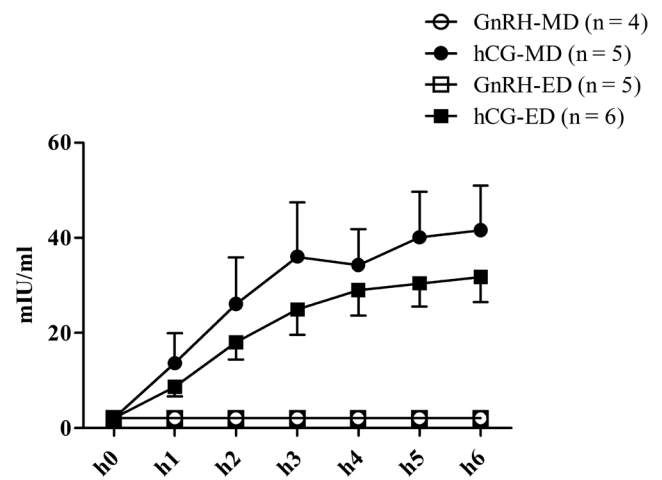
Fig. 3. Human chorionic gonadotropin (hCG) concentrations in lactating dairy cows receiving treatment in the GnRH-MD, hCG-MD, GnRH-ED, and hCG-ED groups. GnRH-MD, treated with gonadotropin-releasing hormone (GnRH) in the mid-diestrus group; GnRH-ED, treated with GnRH in the early-diestrus group; hCG-MD, treated with hCG in the mid-diestrus group; hCG-ED, treated with hCG in the early-diestrus group; Data are shown as mean ± SEM. The P values of different groups, time periods, and a group by time interaction were < 0.05.

Table 3. Time of ovulation after data from stages 1 and 2 were pooled together

Group	Ovulation time (h) after GnRH or hCG injection						
	≤ 28	≤ 30	≤ 32	≤ 34	≤ 36	> 36	no ovulation
GnRH-MD (n = 9)	4	0	2	1	0	0	2
hCG-MD (n = 9)	0	4	2	1	0	1	1
GnRH-ED (n = 8)	5	3	0	0	0	0	0
hCG-ED (n = 8)	1	3	3	0	0	1	0

hCG, human chorionic gonadotropin; GnRH, gonadotropin-releasing hormone; GnRH-MD, treated with GnRH in the mid-diestrus group; GnRH-ED, treated with GnRH in the early-diestrus group; hCG-MD, treated with hCG in the mid-diestrus group; hCG-ED, treated with hCG in the early-diestrus group; > 36: ovulation occurred between 40 h and 72 h.

(all P < 0.05). Except for 0 and 1 h, the hCG concentrations were higher in the hCG-MD and hCG-ED groups than in the other two groups. The hCG concentrations between 0 and 6 h in the GnRH-MD and GnRH-ED groups were 2 mIU/ml below the detection limit. We observed an increase of the hCG concentration during the first three hours in the hCG-MD and hCG-ED groups, followed by a slight deceleration in the rate of increase (Fig. 3).

As shown in Table 3, all the cows ovulated between 28 and 32 h after GnRH administration (with the exception of one cow that ovulated at 34 h and two cows that did not ovulate). One cow, which was initially identified with partial luteolysis, ovulated at 28 h. However, ovulation occurred between 30 and 32 h in the majority of cows treated with hCG. Two cows were observed to ovulate at 34 h and 68 h, and one cow that did not ovulate in the hCG-MD group. In the hCG-ED group, one cow with a spontaneous LH

surge ovulated at 28 h, and one cow ovulated at 48 h. In the cow showing partial luteolysis, ovulation occurred at 32 h. Overall, the occurrence of ovulation in the hCG-MD group was later than that in the GnRH-ED group (Table 2). Although we excluded data from the cows ovulating later than 36 h after GnRH or hCG administration, the ovulation time still tended to be different between these two groups (P = 0.06). The average follicle diameter on day 0 was smaller in the GnRH-ED group than in the GnRH-MD group; however, the mean values for preovulatory follicle diameters varied between 15 and 17 mm and were not different among the four groups. Furthermore, the overall percentage of ovulation and the percentage of ovulation within 36 h of treatment were not different among the groups. There was one cow in the hCG-MD group, two in GnRH-ED group, and one in hCG-ED group that showed multiple ovulation; however, the percentage of multiple ovulation was not different among groups.

Comparisons between the GnRH and hCG groups

With respect to luteolysis, there was no difference in P_4 concentration or in the remaining CL area between the GnRH and hCG groups (Table 4). The percentage of complete luteolysis was 94.1% for both the groups. Regarding ovulatory responses, excluding ovulation time, there were no significant differences between the GnRH and hCG groups in terms of follicle diameter on day 0, preovulatory follicle diameter, the percentage of overall ovulation, the percentage of ovulation within 36 h, and the percentage of multiple ovulation. Collectively, ovulation in the GnRH group occurred earlier than in the hCG group. After excluding two cows that ovulated later than 36 h post-treatment, we observed that the period between GnRH injection and ovulation in the GnRH group was shorter than that in the hCG group (29.3 ± 0.5 h *vs*. 30.9 ± 0.4 h, P = 0.0240; mean ± SEM).

Comparisons between the MD and ED groups

We did not observe any significant differences in ovulatory responses between the MD and ED groups, except for differences in follicle diameters on day 0, which were significantly smaller in the ED group than in the MD group (Table 5). With respect to the CL, the P_4 concentration in the MD group was higher on day 0 than in the ED group. Although there were no significant differences between the two groups in terms of P_4 concentrations on day 3, the remaining CL area between days 1 and 3 were lower in the MD group than in the ED group. The percentages of complete luteolysis were 100% and 87.5% in the MD and ED groups, respectively; however, this difference was not statistically significant.

Table 4. Luteolytic and ovulatory parameters in the GnRH and hCG groups

Parameters	GnRH (n = 17)	hCG (n = 17)	P
P_4 on day 0 (ng/ml)	6.52 ± 0.67	6.39 ± 0.74	0.7977
P_4 on day 3 (ng/ml)	0.63 ± 0.21	0.61 ± 0.13	0.2431
Remaining CL area on day 1 (%)	70.8 ± 4.1	71.5 ± 3.5	0.6088
Remaining CL area on day 2 (%)	52.4 ± 3.3	51.9 ± 2.1	0.7846
Remaining CL area on day 3 (%)	35.6 ± 2.6	38.1 ± 2.9	0.4540
Complete luteolysis (%)	94.1 (16/17)	94.1 (16/17)	1.0000
Follicle diameter on day 0 (mm)	13.8 ± 0.9	14.7 ± 0.7	0.6729
Preovulatory follicle diameter (mm)	16.0 ± 0.8	16.5 ± 0.7	0.6296
Ovulation time (h)	29.3 ± 0.5 [b]	34.3 ± 2.5 [a]	0.0098
Ovulation (%)	88.2 (15/17)	94.1 (16/17)	1.0000
Ovulation within 36 h (%)	88.2 (15/17)	82.4 (14/17)	1.0000
Multiple ovulation (%)	13.3 (2/15)	12.5 (2/16)	1.0000

CL, corpus luteum; P_4, progesterone; GnRH, cows treated with gonadotropin-releasing hormone; hCG, cows treated with human chorionic gonadotropin. Data are shown as mean ± SEM. Different superscript letters indicate statistically significant difference between groups in the same row; Categorical and continuous data were tested by the Fisher's exact test and the Wilcoxon rank-sum test, respectively.

Table 5. Luteolytic and ovulatory parameters in the MD and ED groups

Parameters	MD (n = 18)	ED (n = 16)	P
P_4 on day 0 (ng/ml)	8.05 ± 0.62 [a]	4.66 ± 0.48 [b]	0.0009
P_4 on day 3 (ng/ml)	0.46 ± 0.03	0.81 ± 0.26	0.5500
Remaining CL area on day 1 (%)	65.2 ± 2.9 [b]	77.9 ± 4.1 [a]	0.0126
Remaining CL area on day 2 (%)	47.0 ± 1.8 [b]	57.9 ± 3.1 [a]	0.0126
Remaining CL area on day 3 (%)	31.8 ± 1.9 [b]	42.5 ± 3.0 [a]	0.0082
Complete luteolysis (%)	100 (18/18)	87.5 (14/16)	0.2139
Follicle diameter on day 0 (mm)	15.8 ± 0.7 [a]	12.8 ± 0.8 [b]	0.0208
Preovulatory follicle diameter (mm)	16.5 ± 0.6	16.0 ± 0.9	0.8927
Ovulation (%)	83.3 (15/18)	100.0 (16/16)	0.2299
Ovulation within 36 h (%)	77.8 (14/18)	93.8 (15/16)	0.3402
Multiple ovulation (%)	6.7 (1/15)	18.8 (3/16)	0.5996

CL, corpus luteum; P_4, progesterone; MD, all cows treated with gonadotropin-releasing hormone (GnRH) or human chorionic gonadotropin (hCG) in the mid-diestrus; ED, all cows treated with GnRH or hCG in the early-diestrus. Data are shown as mean ± SEM. Different superscript letters indicate statistically significant difference between groups in the same row; Categorical and continuous data were tested by the Fisher's exact test and the Wilcoxon rank-sum test, respectively.

Discussion

In this study, we aimed to investigate the effects of hCG induction during mid- and early-diestrus on ovulatory responses in lactating dairy cows. Human chorionic gonadotropin acts directly on LH receptors and thus induces ovulation independent of the pituitary [6]; therefore, we hypothesized that ovulation after hCG administration may occur earlier than ovulation after GnRH administration. Contrary to our initial predictions, our results suggest that the hCG-induced ovulation time was 2 to 4 h later than GnRH-induced ovulation. Previous studies have reported an LH surge occurs 1 or 2 h after a GnRH pulse [23–25], and our findings were in line with these studies. Additionally, the ovulation time determined in our study had a number of similarities with that reported by Giordano *et al.* (2012) [3] and replicated our earlier data [4]. Other researchers have demonstrated that the serum concentration of hCG was above the baseline even at 66 h in cows treated with 3000 IU hCG (1000 IU iv and 2000 IU im) [26]. Another study reported that hCG concentration reaches a maximum at 4 h after intramuscular hCG administration (3300 IU), remains at plateau between 4 and 12 h, and decreases to baseline values at 72 h post-treatment [13]. In our study, we found that hCG concentrations were still increasing between 4 and 6 h after hCG injection, indicating that more time would be needed for the hCG to reach its peak concentration as compared to the time that GnRH-induced LH would take to reach its peak concentration to induce ovulation. Despite the fact that higher hCG concentrations may be achieved in a short period of time after intravenous administration, effects of different routes of administration (intravenous *vs.* intramuscular injection) on ovulation time should be explored.

As expected, the percentage of ovulation (including percentage of ovulation within 36 h) was not different among the groups receiving different treatments in this study. One report has indicated that the percentage of ovulation after hCG injection was significantly higher than that after GnRH administration in a high P_4 environment; however, no difference in the percentage of ovulation was observed when the P_4 concentration was below 1 ng/ml [5]. In this study, almost all the cows showed complete luteolysis that yielded similar percentages of ovulation when groups administered with different treatments were compared. In contrast, although no significant differences in the percentage of ovulation could be detected regarding the different diestrous periods, the percentage of ovulation was higher in the ED group than in the MD group. In this study, atretic follicles were observed in two non-ovulating cows in the GnRH-MD and hCG-MD groups, and in one cow that ovulated at 68 h, a preovulatory follicle developed into a cyst, followed by the formation of a new follicle on day 2. These results were in accordance with our previous findings showing that a few follicles in the mid-estrous cycle result in atresia or form cysts rather than ovulate after ovulation induction [4]. Moreover, fewer LH receptors in the granulosa cells have been shown in atretic follicles [27] or cysts [28] than in dominant follicles. Thus, we can speculate that non-ovulation of follicles in the mid-estrous cycle, which results in atresia or cyst-formation, may be due to an underlying loss of LH receptors.

No significant differences were detected in preovulatory follicle diameters among the four groups. Diameter of a preovulatory follicle is described to be in a range of 10–22 mm, and follicle size during the highest peak of ovulation is between 15–17 mm [29, 30]. In the current study, most of the preovulatory follicle diameters were higher than 10 mm (11–21 mm), with the exception of three follicles that were lower than 10 mm. The occurrence of three preovulatory follicles below 10 mm are likely to be related to two distinctive situations: 1) double ovulation occurred, with preovulatory follicles having smaller diameters (8.3 and 9.6 mm), 2) the expected preovulatory follicle turned into a cyst, and a new follicle formed on day 2 ovulated at 68 h after hCG administration. Although there was no effect of diestrous period on the diameters of preovulatory follicles, the size of dominant follicles in the MD group on day 0 was higher than that in the ED group in this study. Other studies have relied on only a single measurement to determine the preovulatory follicle size at the time of $PGF_{2\alpha}$ treatment, a second dose of GnRH, or artificial insemination using the Ovsynch protocol [31–33]. However, we measured the size of follicles several times, i.e., once a day from day 0 to day 3. Given that the groups did not exhibit differences in their preovulatory follicle diameters, the dominant follicle diameter on day 0 in the MD group was bigger than that in the ED group. Thus, we speculate that the dominant follicle had a more potential to grow in the ED group rather than in the MD group.

Interestingly, we observed that one follicle in the GnRH-MD group with 17.6 × 14.0 mm diameter on day 3 was detected at 28 h after GnRH injection and subsequently disappeared at 30 h. However, since development of a CL was not detected 5 days later, it was identified as a case of anovulation. In addition to endothelial cells, fibroblasts, and immune cells, the CL is composed of large and small luteal cells [34], which are transformed from granulosa and theca cells, respectively [35]. Apoptosis of granulosa cells has been demonstrated when the follicles undergo atresia [36, 37]. From our observations, we can speculate that the follicle described above underwent a process of gradual atresia with apoptosis of the granulosa cells instead of being transformed into large luteal cells.

The percentages of complete luteolysis were 100% in both the GnRH-MD and hCG-MD groups, which is consistent with our previous findings [4, 20] and provides evidence once again towards the advantage of using two low doses of $PGF_{2\alpha}$ for the complete regression of mature CL. In this study, we found two cows with partial luteolysis as indicated by parameters, such as P_4 concentration above 1 ng/ml and remaining CL area more than 50% in the ED group during stage 1. Valldecabres-Torres and co-workers (2012) showed that a single standard dose or double dose of $PGF_{2\alpha}$ for CL aged 5.5 days resulted in 80% and 100% of complete luteolysis, respectively [38]. Considering a period of seven days between the first GnRH and $PGF_{2\alpha}$ in the Ovsynch protocol [1] and 28 to 30 h ovulation time after GnRH administration [3], we expected to achieve a similar effect using two low doses of $PGF_{2\alpha}$. Although we achieved a high proportion (87.5%) of complete luteolysis in the ED group, partial luteolysis occurred in some animals, which were consequently excluded from the next stage of the experiment. To prevent partial luteolysis and non-ovulation, we suggest that the optimal interval from ovulation to the first $PGF_{2\alpha}$ treatment should be designed to fall on any day between 6.5 and 9 days in further experiments having a similar design.

In summary, we provide evidence that hCG-induced ovulation occurred later than GnRH-induced ovulation, but the percentages of

ovulation did not differ between hCG and GnRH treatment during mid- and early-diestrus. Even with high percentages of ovulation and complete luteolysis, risk of non-ovulation and partial luteolysis may exist when an ovulation protocol is started in the early and middle phases of diestrus, respectively.

Acknowledgments

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