Original Article

Predictable ovulation time and increased serum estradiol concentration achieved by GnRH administration at 56-hour proestrus in lactating dairy cows

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Abstract

The feasibility of modified ShortSynch protocols was evaluated to determine the effects of prolonged proestrus on elevation of circulating estradiol (E_2) levels and synchronous ovulation in lactating dairy cows. Cows (n = 15) ranging between 50 and 70 days postpartum were pre-synchronized by a modified ShortSynch protocol (375 mg PGF-1 day-250 mg of PGF-1 day-GnRH) to induce ovulation. After the initial ovulation, a switch-back design (stages 1 and 2, respectively) was used to compare the effects of GnRH administration at 48 versus 56 h after PGF (length of proestrus) in the same cow with modified ShortSynch protocols. Seven to eight days after ovulation, the first PGF treatment (defined as Hour 0) was administered to the cows, with a second PGF given at Hour 24. Cows were randomly allocated to receive GnRH either at Hour 48 (G48 group) or Hour 56 (G56 group) in stage 1 and vice versa in stage 2. All cows showed complete luteal regression and 93.3% (28/30) ovulated between 28 and 32 hours after GnRH administration. Extending the interval of proestrus by eight hours had an effect ($P < 0.05$) on preovulatory E_2 levels, with the E_2 concentration for G56 higher than that for G48 at GnRH administration (41.0 ± 3.3 vs. 33.3 ± 3.9 pg/mL; $P = 0.02$). The present study suggests that modified ShortSynch protocols could help achieve high synchronization rates with a predictable time of ovulation after GnRH administration, in addition to promoting higher circulating E_2 levels with the extension of proestrus from 48 to 56 hours.

Keywords: ovulation time, proestrus, estradiol, GnRH, dairy cattle

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Introduction

Synchronization protocols are common methods of herd management that enable timed AI and reduce estrus detection in cattle (Wiltbank and Pursley 2014); nevertheless, cows that did not exhibit estrus were reported to have lower preovulatory circulating estradiol (E_2) levels and lower pregnancy rates (Bó and Cedeño 2018; Perry, *et al*. 2014), indicating that insufficient preovulatory E_2 would compromise fertility. Therefore, an increasing number of studies have started to recognize the importance of preovulatory E_2 levels, modifying protocols either by adding exogenous E_2 (Jinks, *et al*. 2013; Martins, *et al*. 2017) or by extending the interval between PGF and GnRH administrations and thereby prolonging proestrus (Bridges, *et al*. 2010). Although extension of proestrus can benefit pregnancy establishment by optimizing uterine gene expression (Bridges, *et al*. 2012; de la Mata, *et al*. 2018) and inducing a subsequent CL with higher steroidogenic capacity (Nunez-Olivera, *et al*. 2020), a spontaneous LH surge will result in ovulation before GnRH administration (Bridges, *et al*. 2014) and lead to asynchrony between timing of AI and timing of ovulation. For instance, the pregnancy outcomes of Cosynch protocols, which have a longer proestrus than Ovsynch, were often substantially lower (Borchardt, *et al*. 2018; DeJarnette and Marshall 2003) or significantly decreased compared to those of Ovsynch (Brusveen, *et al*. 2008; Geary, *et al*. 1998). This poor performance could be due to the fact that Cosynch involved timed AI at 28 h before the expected GnRH-induced ovulation, instead of 8 to 16 hours prior to ovulation, which would maximize fertility (Borchardt, *et al*. 2018; Stevenson 2016), whereas the second GnRH would be redundant if ovulation were triggered by a natural LH surge. Therefore, a predictable time of ovulation in protocols with various intervals of proestrus should be evaluated in order to optimize the suitable timing for fixed AI protocols as well as to assist pregnancy establishment by optimizing the E_2 level.

Modified protocols for synchronization of the estrous cycle (e.g., G6G, Double Ovsynch) are often used to pre-synchronize cattle, enabling initiation of Ovsynch at an optimal time and thereby increasing pregnancy rates to timed AI (Bello, *et al*. 2006; Souza, *et al*. 2008) but these protocols involve numerous hormone treatments. ShortSynch, a novel ovulation induction protocol reported in beef cattle, uses ultrasonographic imaging to select cattle with a CL ≥ 18 mm in diameter for PGF treatment, in lieu of the "blanket" first GnRH treatment in the Ovsynch protocol (Funakura, *et al*. 2018). Although treated cattle showed a synchronization rate of 89.2% along with a reasonable pregnancy rate of 60.4%, the synchronization outcome may still be further improved by adding additional means. For instance, the application of a second PGF dosage, both two-standard dose (Atanasov, *et al*. 2021) and two-lower doses (Liu, *et al*. 2017), has been proved to improve the rate of complete luteolysis and favored pregnancy establishment (Kasimanickam, *et al*. 2009). In addition, the optimal interval from ovulation to the first PGF was suggested to be set between 6.5 and 9 days in order to prevent chances of partial luteolysis and non-ovulation during synchronization (Liu, *et al*. 2019). Considering the long-term goals of optimizing fertility and minimizing the use of exogenous hormones, modification of ShortSynch protocols with additional PGF and date of induction may provide alternative strategies for reproductive management.

Our objective was to evaluate the effects of modified ShortSynch protocols with 48 or 56 hours of proestrus on synchronous ovulation in dairy cattle. We tested the hypotheses that extending the duration of proestrus in a ShortSynch protocol by eight hours 1) increases preovulatory E_2 concentrations; 2) results in synchronous ovulation.

Materials and Methods

Cows and management: Lactating Holstein dairy cows from the National Chung Hsing University livestock farm, Taichung, Taiwan, were used in this experiment, which was conducted from November 2018 to April 2019. All cows were kept in semi-outdoor, free-stall facilities with good ventilation and a water spraying system (to promote cooling). Cows were fed a total mixed ration containing grass hay and concentrate twice daily, with *ad libitum* access to water. Temperature and humidity were recorded daily at 0600, 1200, 1800 and 2400 for calculation of the thermal humidity index (THI) (Liu, *et al*. 2018). All procedures were approved by the Animal Care and Use Committee for the Biotechnology Center of the National Chung Hsing University (Protocol No. 107-115).

Fifteen lactating Holstein cows, parity 1 to 3, with no apparent reproductive abnormalities based on their reproductive clinical history and transrectal examination, were used. After enrollment, the cows underwent evaluation for body condition score (BCS) using a five-point scale (from 1 = thin to 5 = fat) (Edmonson, *et al*. 1989), and all cows had BCSs between 2.50 to 3.25. Milk yield was recorded twice daily, and all cows had daily milk production of 30.5 ± 5.3 kg (mean ± SD). The cows were observed thrice daily for the detection of estrous behaviors, which include mounting, being mounted or showing vaginal mucus discharge. Whether a cow expressed estrous behavior will be used as a binary variable for further analysis.

Experimental design: In order to synchronize ovulation for the experiment, a modified ShortSynch protocol was used between 50 and 70 days in milk (DIM). Briefly, after transrectal ultrasonographic examination, cows with a mature CL (≥ 17 mm) and at least one ovarian follicle ≥ 8 mm in diameter were given 375 µg of PGF (cloprostenol sodium, 250 µg/mL; Estrumate, Intervet Deutschland, Unterschleiβheim, Germany) by intramuscular (im) injection, defined as the first day of pre-synchronization (SynPG). An additional 250 µg of PGF was given 24 h later, followed by 250 µg GnRH (gonadorelin, 100 µg/mL; Fertagyl, Intervet Deutschland) given 48 h after SynPG (Figure 1). Luteolysis was confirmed based on serum P_4 concentrations at SynPG and three days later, whereas transrectal ultrasonography was conducted at 72 and 80 h after SynPG to detect ovulation.

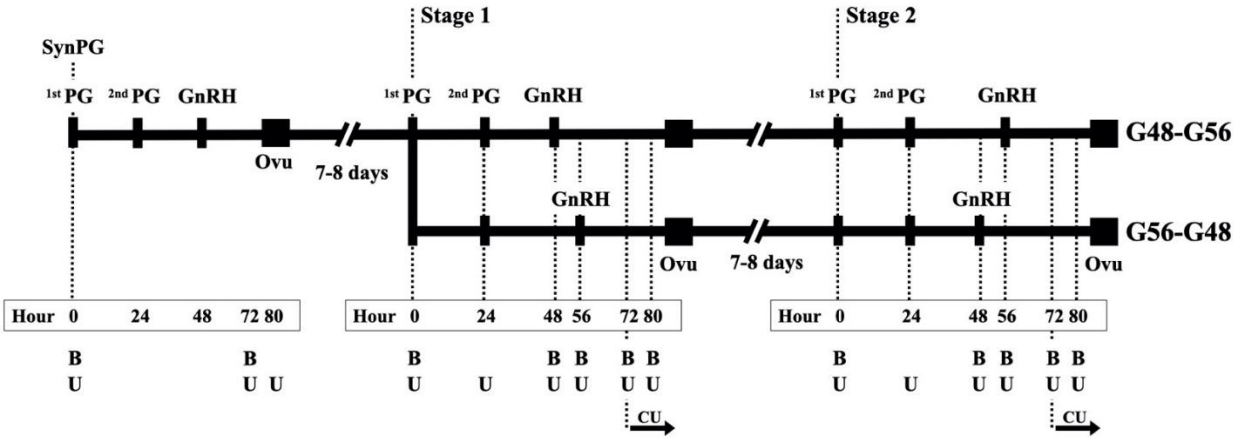

Figure 1 Schematic diagram of treatments performed in individual cows. Blood sampling (B) for progesterone and estradiol; consecutive ultrasound (CU) examination to determine ovulation every 2 h from Hour 72 to 88 until ovulation; 1st and 2nd prostaglandin (PG), administration of 375 µg and 250 µg cloprostenol, respectively. GnRH, 250 µg of gonadorelin; Ovu, observed ovulation time; SynPG, the time of first PGF administration in pre-synchronization; U, ultrasonography for follicle and corpus luteum detection and measurements.

After confirmation of the initial ovulation, a switch-back design (stages 1 and 2, Figure 1) was used to compare the effects of administering GnRH at 48 versus 56 h after PGF. The first PGF treatment (375 µg im) was given 7 to 8 d after ovulation, defined as Hour 0, with a second 250-µg PGF treatment given at Hour 24. Cows were initially randomly allocated by parity to receive 250-µg GnRH either at Hour 48 (G48 group) or Hour 56 (G56 group); once they had received one treatment, following the induced ovulation, they subsequently received the alternate treatment.

Ultrasonography for CL area, follicle diameter and ovulation: Transrectal real-time B-mode ultrasonographic examinations of ovaries were performed with a 7.5-MHz linear-array transducer (SonoSite Ultrasound System; SonoSite, Bothell, WA, USA). Apparent maximal area cross-sections of the CL were frozen and recorded daily at each stage from the first PGF administration (Hour 0) to three days later (Hour 72). Areas of an ovum were calculated, based on the measured longitudinal and perpendicular transverse axes. The CL area at Hour 0 was considered 100% and used as the basis to calculate the remaining CL area (RCLA) percentage at Hours 24, 48 and 72 (Liu, *et al*. 2017). In addition, CL areas were also measured five and eight days after ovulation in Stages 1 and 2. The maximum cross-section of the follicle was recorded at Hours 0, 24, 48 56, 72 and 80 at each stage. Follicle diameter was determined by freezing the image at the maximal apparent size, measuring vertical and horizontal axes, and recording the average. Follicular growth rate was calculated by determining the difference between follicle diameter at Hours 0 and 72 divided by the number three (mm/day). Starting at Hour 72, serial ultrasonographic examinations of preovulatory follicles were conducted at two-hour intervals to confirm ovulation time (no cows ovulated after 36 h of GnRH administration). Ovulation was detected by the disappearance of preovulatory follicles and the diameter of the ovulated follicles closest to the time of ovulation was considered as the preovulatory follicle diameter.

Hormone analyses and luteal regression: Blood samples for P_4 were collected at the first PGF (SynPG, Hour 0) and three days later (Hour 72) in both stages, including pre-synchronization. Post-ovulation P_4 samples were collected five and eight days after ovulation. Blood samples for E_2 were collected at Hours 48, 56, 72 and 80 at both stages. All blood samples were collected by venipuncture of coccygeal vessels and immediately refrigerated. After clotting, samples were centrifuged (1,300 × *g*, 10 min), extracted for serum and frozen (-20℃) for hormone analyses.

Serum P_4 concentrations were determined using an immunoassay system (Siemens ADVIA Centaur® Progesterone (P_4) assay, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) with a detection limit of 0.21 to 60 ng/mL. The intra-assay coefficients of variation (CVs) of low, medium and high controls were 2.6%, 3.9%, and 1.9%, respectively. Inter-assay CVs of low, medium and high controls were 12.4%, 3.7%, and 3.2%, respectively. Cows with either serum P_4 concentration < 1 ng/mL or RCLA < 50% at Hour 72 were considered to have complete luteal regression (Liu, *et al*. 2018).

Serum E_2 concentrations were determined using an immunoassay system (Siemens ADVIA Centaur® Enhanced Estradiol (eE_2) assay, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) with a detection limit of 11.8 to 3000 pg/mL. For low, medium and high controls, intra-assay CVs were 2.0%, 1.9%, and 2.6%, respectively, whereas inter-assay CVs were 11.1%, 5.6%, and 4.1%, respectively.

Statistical analyses: Data analysis was performed using SAS software version 9.4 (SAS Institute, Raleigh, NC, USA). Data is presented as mean ± SEM. Significance was defined as $P < 0.05$, whereas $0.05 \leq P < 0.10$ was defined as a tendency. Treatment order (whether cows had G48 or G56 treatment in stage 1) did not affect the results. Differences between G48 and G56 in the interval from the 1st PGF to ovulation, interval from GnRH to ovulation, follicle diameters at each time points, follicular growth rate (change of

diameter from Hour 0 to 72 per day), serum E_2 concentration at GnRH, and subsequent CL area and serum P_4 concentration on both five and eight days post-ovulation were either assessed by Student's paired *t*-tests or Wilcoxon signed rank test, depending on the univariate analysis results showing whether data was parametric or not. Differences in serum E_2 concentrations, CL areas and RCLAs measured at various time points between groups were determined by repeated-measures ANOVA, and Bonferroni's method was applied to perform multiple comparisons. Correlations between each interval from treatment to ovulation, follicle diameter, preovulatory E_2 and postovulatory CL characteristics were detected by simple linear regression; whereas the relationships between the presence of estrous behavior and these variables were detected using logistic regression.

Results

Extending proestrus for 8 h between groups of G48 and G56 increased preovulatory E_2 concentration without affecting the interval between GnRH administration and ovulation. All cows had complete luteal regression and ovulated within 32 hours after GnRH administration at each stage. Mean values of ovarian characteristic and hormone analysis between groups are shown in Table 1. The interval from the first PGF administration to ovulation was longer in G56 compared to G48 (84.8 ± 0.4 vs. 76.9 ± 0.5 h; $P < 0.0001$); however, extended proestrus did not influence the interval from GnRH to ovulation ($P > 0.10$; Table 1). The ovulation rate was 100% in both groups, and almost every cow ovulated 28 to 32 h after GnRH injection, except two that ovulated 24 and 26 h after GnRH administration. Preovulatory follicle diameter tended ($P = 0.08$) to be larger in G56 than in G48. Dominant follicle diameters also tended to be larger in G56 at Hour 0 ($P = 0.07$) and at GnRH injection ($P = 0.06$), whereas the follicular growth rate from Hour 0 to 72 showed no difference ($P > 0.1$). There were both treatment ($P < 0.05$) and time effects ($P < 0.001$) on serum E_2 concentrations, which showed reduction after GnRH administration in both G48 and G56 groups (Figure 2). Furthermore, the E_2 concentration at time of GnRH administration was higher for G56 than G48 (41.0 ± 3.3 vs. 33.3 ± 3.9 pg/mL; $P = 0.02$). There was a tendency of positive correlation ($P < 0.1$, $r^2 = 0.1221$) between serum E_2 concentrations and preovulatory follicle diameter, but there was no significant correlation with the occurrence of estrous behavior. There was a negative correlation ($P < 0.01$, $r^2 = 0.2352$) between serum E_2 concentration at GnRH administration and the interval from GnRH administration to ovulation.

In respect of luteal regression, all the cows at Hour 72 showed CL regression to less than 50% of the RCLA with P_4 concentration less than 1 ng/mL; thus, all the cows were considered to have undergone complete luteolysis. Although the CL area during luteal regression did not differ by treatment (Figure 3), there was a treatment effect on RCLA, with G48 showing a lower RCLA than G56. For GnRH-induced CL, neither area nor P_4 concentration differed between groups on 5 or 8 days after ovulation (Table 1). Furthermore, the CL areas and P_4 levels neither correlated to preovulatory follicle nor dominant follicle diameter at GnRH administration but there were positive correlations ($P < 0.05$) between E_2 concentration at GnRH administration and both CL areas on five ($r^2 = 0.1596$) or eight days ($r^2 = 0.2157$).

Table 1 Ovarian characteristics (mean ± SEM) pre- and post-ovulation using modified ShortSynch in groups with proestrus of 48 (G48) and 56 hours (G56).

Item	G48 group (n = 15)	G56 group (n = 15)	*P* value
Time interval from			
1st PGF to ovulation (h)	76.9 ± 0.5	84.8 ± 0.4	< 0.0001
GnRH to ovulation (h)	28.9 ± 0.5	28.8 ± 0.4	0.56
Dominant follicle diameter (mm)			
at Hour 0	14.5 ± 0.7	1.62 ± 0.8	0.07
at GnRH	16.7 ± 0.7	1.81 ± 0.6	0.06
Preovulatory follicle diameter (mm) [a]	17.1 ± 0.7	18.3 ± 0.4	0.08
Follicular growth rate (mm/day) [b]	0.90 ± 0.11	0.70 ± 0.15	0.36
E_2 concentration at GnRH (pg/mL)	33.3 ± 3.9	41.0 ± 3.3	0.02
Synchronization rate (%) [c]	100	100	
CL area (cm^2)			
5 days after ovulation	3.91 ± 0.41	3.59 ± 0.40	0.46
8 days after ovulation	5.41 ± 0.39	5.39 ± 0.44	0.96
P_4 concentration (ng/mL)			
5 days after ovulation	3.64 ± 0.27	3.57 ± 0.19	0.87
8 days after ovulation	6.92 ± 0.63	6.64 ± 0.58	0.72

a. The diameter of the ovulated follicles closest to the time of ovulation was considered as the preovulatory follicle diameter.
b. Follicular growth rate was calculated by determining the difference between follicle diameter at Hour 0 and 72 divided by three (mm/day).
c. Synchronization was defined by the presence of both complete luteal regression and ovulation within 32 hours after GnRH induction.

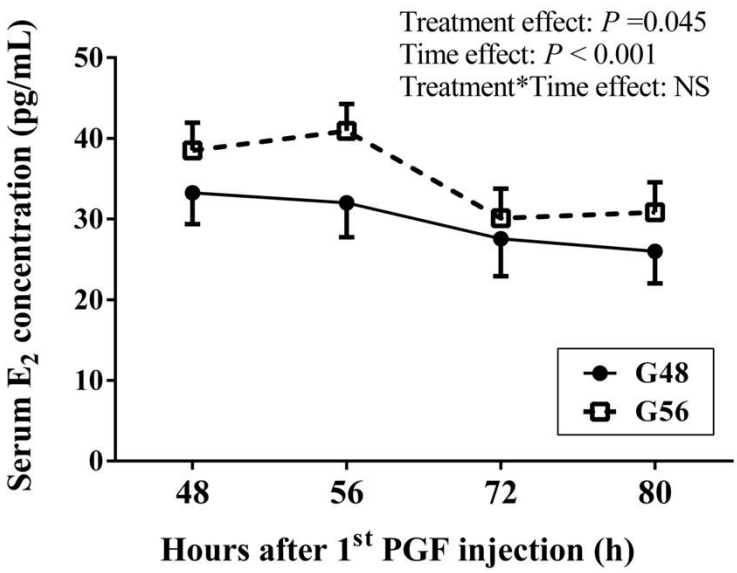

Figure 2 Comparative changes in mean E_2 concentration (mean ± SEM) of G48 (n = 15) and G56 (n = 15) between Hour 48 to 72 after PGF administration.

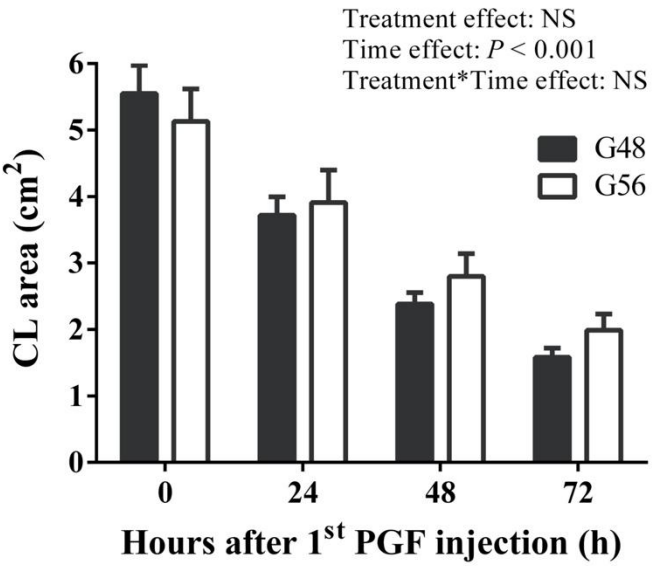

Figure 3 Comparative changes in mean CL area of G48 (n = 15) and G56 (n = 15) between Hour 0 to 72 after PGF administration. Values are mean ± SEM of each point.

Discussion

The present study aimed to demonstrate the feasibility of extending proestrus for 8 h to increase E_2 concentration without affecting the synchrony of induced ovulation. Our results suggest that the 8-hour extension of proestrus from 48 to 56 hours led to a significant increase in E_2 levels along with a predictable occurrence of ovulation within 28 to 32 hours after GnRH administration. This supports our first and second hypotheses and proves that modified ShortSynch protocols could achieve high synchronization rate and have benefits for pregnancy establishment in dairy cattle.

Previous studies have reported that both Ovsynch and ovulation induction protocols enabled ovulation to occur between 28 to 32 hours after GnRH administration (Liu, *et al*. 2018; Pursley, *et al*. 1995); however, these results were only examined under a 48-hour fixed proestrus. Although the longer proestrus resulted in greater follicle steroidogenic activity and improved uterine gene expression (Bridges, *et al*. 2012; de la Mata, *et al*. 2018), the unpredictable time of ovulation could be unfavorable to timed AI (Nunez-Olivera, *et al*. 2020). For instance, Cosynch protocols had a proestrus of 72 hours but were often reported to yield lower pregnancy rates when compared to Ovsynch (Brusveen, *et al*. 2008; Geary, *et al*. 1998). This could have resulted from the design concept of Cosynch, in which GnRH administration and AI were performed simultaneously to reduce labor. However, in cases of ovulation stimulated by GnRH administration, wherein the ovulation took place 28 hours later, more than 90% of the post-thawed spermatozoa would have lost viability at 24 hours (Nagata, *et al*. 2019), making fertilization difficult (Dalton, *et al*. 2001). On the other hand, if AI could match with the spontaneous LH surge, concurrent

GnRH administration would be redundant and increase costs. According to previous research, the earliest ovulation in protocols without GnRH occurs at 84 h after PGF administration (Liu, *et al*. 2018), suggesting that 56 hours after PGF administration would be an ideal proestrus interval to avoid the spontaneous LH surge 28 hours prior to ovulation. The results of the present study agree with the first hypothesis: group G56 showed no difference in the period between GnRH and ovulation compared to G48, indicating both predictable and synchronous ovulation time by using GnRH.

Another goal of the present study was to elevate the preovulatory E_2 concentration, which was strongly associated with the pregnancy-establishment process, including fertilization (Jinks, *et al*. 2013), uterine mRNA expression in the subsequent cycle (Bridges, *et al*. 2012), as well as embryonic growth and placental attachment (Madsen, *et al*. 2015). Although modified protocols that involve addition of exogenous dosage (Martins, *et al*. 2017) or prolonging the interval of proestrus (de la Mata, *et al*. 2018; Nunez-Olivera, *et al*. 2020) can successfully elevate E_2 levels with increased fertility, the latter would still be the only option in countries with restrictions over the use of exogenous E_2. Different methods of E_2 detection could have explained the difference in E_2 levels between the present and previous studies (McLean, et al. 2022). Nevertheless, with an 8-h extension of proestrus, the present study showed a relatively increased preovulatory E_2 level in cows with 56-hour of proestrus, which could explain the merit and trends of using Ovsynch-56 protocols in the past decades (Brusveen, *et al*. 2008; Wiltbank and Pursley 2014). On the other hand, the prolonged proestrus seemed to have little effect on the size of the preovulatory follicle, since there were only differences of tendency ($P < 0.1$) in diameter between groups with a similar ($P > 0.1$) follicular growth rate. Interestingly, Bridges *et al*. (2010), also managed to achieve higher E_2 concentrations by adjusting the length of proestrus and reported no difference in follicle diameter. In that study, the interval of follicular growth (from follicle ablation to GnRH administration) was fixed at 6.75 days, whereas the length of proestrus was controlled by timing PGF administration (Bridges, *et al*. 2010). Thus, the length of proestrus, rather than the length of follicular growth, has a major influence on follicle steroidogenic capacity. Considering the fact that most protocols were programmed with fixed proestrus, the induced follicles might not be able to achieve their maximum potential since the growth of the follicle would cease soon after the LH surge, thus causing a decline of circulating E_2 levels (Chenault, *et al*. 1975). For instance, ovulation-synchronized cows that did not exhibit estrus were reported to have lower peak E_2 concentrations (Perry, *et al*. 2014); however, a fixed proestrus of 48 hours could be halting the growth of potentially improvable follicles, given that the E_2 concentration was still increasing after Hour 48 in the G56 group in the present study. In contrast to the effects of a lower preovulatory E_2, the influence of higher E_2 levels on the time and peak of the induced LH surge is also worth noticing (Madsen, *et al*. 2015). There was a negative correlation ($P < 0.05$) between E_2 levels at GnRH administration and the time of ovulation (interval from GnRH to ovulation), indicating that cows with high preovulatory E2 levels could ovulate sooner than those with low E2 levels.

Ovulated follicles with higher steroidogenic capacity were also believed to produce CLs with better function to support pregnancy (Nunez-Olivera, *et al*. 2020), but neither the CL area nor P_4 concentration differed between G48 and G56 in the present study. The postovulatory P_4 concentrations on either five or eight days after ovulation did not correlate with preovulatory E_2; however, the CL areas on both these days had positive relationships ($P < 0.05$) with serum E_2 on GnRH administration. One plausible explanation is that the benefit of a higher E_2 steroidogenic capacity was in preventing early luteolysis through uterine programming (Bridges, *et al*. 2013); therefore, the differences in the subsequent circulating P_4 levels occurred from 7 to 12 days after ovulation (de la Mata, *et al*. 2018) and were thus difficult to recognize in the present study.

Unlike classical Ovsynch protocols, ShortSynch avoided the usage of the first GnRH administration to synchronize cattle; instead, ultrasonography was applied to determine the suitable time of ovulation induction using PGF and GnRH (Funakura, *et al*. 2018). In actual practice, there are two major considerations for the timing of this ovulation induction protocol: 1) the maturity of the CL and 2) the age of the ovulatory follicle. Induction in the presence of an unmatured CL (age less than 5 days) results in incomplete luteal regression (Nascimento, *et al*. 2014), which could lead to a lower conception rate than in those having complete luteolysis (Carvalho, *et al*. 2018; Giordano, *et al*. 2012). This could be related to the compromised ciliary motility of the oviduct attributed to the higher P_4 concentration (Bylander, *et al*. 2010; Li, *et al*. 2018). On the other hand, embryo quality was thought to be affected by over-matured oocytes produced by aged follicles (Cerri, *et al*. 2009). Therefore, the induction of complete luteolysis in this study was performed seven to eight days after ovulation, which could avoid occurrences of both the incomplete luteal regression and possible over-matured follicles in our previous study (Liu, *et al*. 2019). Although it is not clear why G56 had a higher RCLA than G48 in the present study, both groups had RCLA lower than 50% at Hour 72 without affecting preovulatory follicles and ovulation process. To our knowledge, there have been no reports comparing the fertility outcomes of both G48 and G56 protocols; nevertheless, ShortSynch protocols with 56 hours of proestrus have achieved a reasonable pregnancy rate of 60.4% in beef cattle (Funakura, *et al*. 2018) and have been applied to resynchronization strategies after non-pregnancy diagnosis with a pregnancy rate of 31.6% in dairy farms (Wijma, *et al*. 2018). Although it is impossible to precisely interpret the exact day of the estrous cycle for the start of the synchronization, the inference of an applicable timing can still be achieved by collecting information on estrus behavior, size of the CL, and the diameter of dominant follicle. With the usage of the second dose of PGF, ovulation induction could be applied as early as seven days post-ovulation with the premise of a medium-sized follicle (Liu, *et al*. 2019; Wiltbank and Pursley

2014). Furthermore, follicular blood flow could also be evaluated using color doppler ultrasonography to identify and avoid the induction of possible atretic follicles, which would have a lack of detectable blood flow (Bollwein, *et al*. 2016). Since the absence of estrous signs in cows may be attributed to lower E_2 concentration (Perry, *et al*. 2014), induced proestrus of at least 56 hours would be suggested if cows did not exhibit estrous behavior after administering PGF.

In conclusion, the feasibility of a ShortSynch protocol with 56 hours of proestrus was confirmed with the benefit of higher E_2 concentrations along with a predictable time of ovulation at 28 to 32 hours after GnRH. Cows with higher preovulatory E_2 did have a larger postovulatory CL, but P_4 steroidogenic capacity was not correlated eight days after ovulation. On-farm application of this protocol should include the second dosage of PGF to avoid incomplete luteal regression of CLs at early diestrus, as well as the extended proestrus to reach sufficient E_2 concentration in cows without obvious estrous signs.

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References

Atanasov B, Dovenski T, Celeska I, and Stevenson JS 2021. Luteolysis, progesterone, and pregnancy per insemination after modifying the standard 7-day Ovsynch program in Holstein-Friesian and Holstein cows. J Dairy Sci. 104: 7272-7282.

Bello NM, Steibel JP, and Pursley JR 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of ovsynch in lactating dairy cows. J Dairy Sci. 89: 3413-3424.

Bó GA and Cedeño A 2018. Expression of estrus as a relevant factor in fixed-time embryo transfer programs using estradiol/progesterone-based protocols in cattle. Animal Reproduction. 15: 224-230.

Bollwein H, Heppelmann M, and Lüttgenau J 2016. Ultrasonographic Doppler Use for Female Reproduction Management. Vet Clin North Am Food Anim Pract. 32: 149-164.

Borchardt S, Schuller L, Wolf L, Wesenauer C, and Heuwieser W 2018. Comparison of pregnancy outcomes using either an Ovsynch or a Cosynch protocol for the first timed AI with liquid or frozen semen in lactating dairy cows. Theriogenology. 107: 21-26.

Bridges GA, Day ML, Geary TW, and Cruppe LH 2013. Triennial Reproduction Symposium: deficiencies in the uterine environment and failure to support embryonic development. J Anim Sci. 91: 3002-3013.

Bridges GA, Mussard ML, Burke CR, and Day ML 2010. Influence of the length of proestrus on fertility and endocrine function in female cattle. Anim Reprod Sci. 117: 208-215.

Bridges GA, Mussard ML, Helser LA, and Day ML 2014. Comparison of follicular dynamics and hormone concentrations between the 7-day and 5-day CO-Synch + CIDR program in primiparous beef cows. Theriogenology. 81: 632-638.

Bridges GA, Mussard ML, Pate JL, Ott TL, Hansen TR, and Day ML 2012. Impact of preovulatory estradiol concentrations on conceptus development and uterine gene expression. Anim Reprod Sci. 133: 16-26.

Brusveen DJ, Cunha AP, Silva CD, Cunha PM, Sterry RA, Silva EP, Guenther JN, and Wiltbank MC 2008. Altering the time of the second gonadotropin-releasing hormone injection and artificial insemination (AI) during Ovsynch affects pregnancies per AI in lactating dairy cows. J Dairy Sci. 91: 1044-1052.

Bylander A, Nutu M, Wellander R, Goksor M, Billig H, Larsson DG 2010. Rapid effects of progesterone on ciliary beat frequency in the mouse fallopian tube. Reprod Biol Endocrinol. 8: 48.

Carvalho PD, Santos VG, Giordano JO, Wiltbank MC, Fricke PM 2018. Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. Theriogenology. 114: 165-172.

Cerri RL, Rutigliano HM, Chebel RC, Santos JE 2009. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reproduction. 137: 813-823.

Chenault JR, Thatcher WW, Kalra PS, Abrams RM, Wilcox CJ 1975. Transitory Changes in Plasma Progestins, Estradiol, and Luteinizing Hormone Approaching Ovulation in the Bovine1, 2. Journal of Dairy Science. 58: 709-717.

Dalton JC, Nadir S, Bame JH, Noftsinger M, Nebel RL, Saacke RG 2001. Effect of Time of Insemination on Number of Accessory Sperm, Fertilization Rate, and Embryo Quality in Nonlactating Dairy Cattle. Journal of Dairy Science. 84: 2413-2418.

de la Mata JJ, Nunez-Olivera R, Cuadro F, Bosolasco D, de Brun V, Meikle A, Bó GA, and Menchaca A 2018. Effects of extending the length of pro-oestrus in an oestradiol- and progesterone-based oestrus synchronisation program on ovarian function, uterine environment and pregnancy establishment in beef heifers. Reprod Fertil Dev. 30: 1541-1552.

DeJarnette JM and Marshall CE 2003. Effects of pre-synchronization using combinations PGF(2alpha) and (or) GnRH on pregnancy rates of Ovsynch- and Cosynch-treated lactating Holstein cows. Anim Reprod Sci. 77: 51-60.

Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G 1989. A Body Condition Scoring Chart for Holstein Dairy Cows. Journal of Dairy Science. 72: 68-78.

Funakura H, Shiki A, Tsubakishita Y, Mido S, Katamoto H, Kitahara G, and Osawa T 2018. Validation of a novel timed artificial insemination protocol in beef cows with a functional corpus

luteum detected by ultrasonography. J Reprod Dev. 64: 109-115.

Geary TW, Whittier JC, Thrift FA, and Dolezal SL 1998. Effects of a Timed Insemination Following Synchronization of Ovulation Using the Ovsynch or CO-Synch Protocol in Beef Cows. The Professional Animal Scientist. 14: 217-220.

Giordano JO, Wiltbank MC, Guenther JN, Pawlisch R, Bas S, Cunha AP, and Fricke PM 2012. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. J Dairy Sci. 95: 639-653.

Jinks EM, Smith MF, Atkins JA, Pohler KG, Perry GA, Macneil MD, Roberts AJ, Waterman RC, Alexander LJ, and Geary TW 2013. Preovulatory estradiol and the establishment and maintenance of pregnancy in suckled beef cows. J Anim Sci. 91: 1176-1185.

Kasimanickam R, Day ML, Rudolph JS, Hall JB, and Whittier WD 2009. Two doses of prostaglandin improve pregnancy rates to timed-AI in a 5-day progesterone-based synchronization protocol in beef cows. Theriogenology. 71: 762-767.

Li C, Zhang H-Y, Liang Y, Xia W, Zhu Q, Zhang D, Huang Z, Liang G-L, Xue R-H, Qi H, He X-Q, Yuan J-J, Tan Y-J, Huang H-F, and Zhang J 2018. Effects of Levonorgestrel and progesterone on Oviductal physiology in mammals. Reproductive biology and endocrinology : RB&E. 16: 59-59.

Liu TC, Chiang CF, Ho CT, and Chan JP 2018. Effect of GnRH on ovulatory response after luteolysis induced by two low doses of PGF2alpha in lactating dairy cows. Theriogenology. 105: 45-50.

Liu TC, Ho CT, Li KP, Chang CC, and Chan JP 2019. Human chorionic gonadotropin (hCG)-induced ovulation occurs later but with equal occurrence in lactating dairy cows: comparing hCG and gonadotropin-releasing hormone protocols. J Reprod Dev. 65: 507-514.

Liu TC, Ho CY, and Chan JP 2017. Effect of two low doses of prostaglandin F2alpha on luteolysis in dairy cows. Acta Vet Hung. 65: 105-114.

MadsenCA, Perry GA, Mogck CL, Daly RF, MacNeil MD, and Geary TW 2015. Effects of preovulatory estradiol on embryo survival and pregnancy establishment in beef cows. Anim Reprod Sci. 158: 96-103.

Martins T, Talamoni JP, Sponchiado M, Maio JRG, Nogueira GP, Pugliesi G, and Binelli M 2017. Impact of estradiol cypionate prior to TAI and progesterone supplementation at initial diestrus on ovarian and fertility responses in beef cows. Theriogenology. 104: 156-163.

McLean MK, Geary TW, Zezeski AL, Smith MF, Spencer TE, Pohler KG, Reese ST, and Perry GA 2022. Impact of preovulatory estradiol concentrations on subsequent luteal function in beef cattle. Syst Biol Reprod Med. 68: 286-297.

Nagata MB, Egashira J, Katafuchi N, Endo K, Ogata K, Yamanaka K, Yamanouchi T, Matsuda H, Hashiyada Y, and Yamashita K 2019. Bovine sperm selection procedure prior to cryopreservation for improvement of post-thawed semen quality and fertility. J Anim Sci Biotechnol. 10: 91.

Nascimento AB, Souza AH, Keskin A, Sartori R, and Wiltbank MC 2014. Lack of complete regression of the Day 5 corpus luteum after one or two doses of PGF2alpha in nonlactating Holstein cows. Theriogenology. 81: 389-395.

Nunez-Olivera R, Cuadro F, Bosolasco D, de Brun V, de la Mata J, Brochado C, Meikle A, Bó GA, and Menchaca A 2020. Effect of equine chorionic gonadotropin (eCG) administration and proestrus length on ovarian response, uterine functionality and pregnancy rate in beef heifers inseminated at a fixed-time. Theriogenology. 151: 16-27.

Perry GA, Swanson OL, Larimore EL, Perry BL, Djira GD, and Cushman RA 2014. Relationship of follicle size and concentrations of estradiol among cows exhibiting or not exhibiting estrus during a fixed-time AI protocol. Domest Anim Endocrinol. 48: 15-20.

Pursley JR, Mee MO, and Wiltbank MC 1995. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology. 44: 915-923.

Souza AH, Ayres H, Ferreira RM, and Wiltbank MC 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology. 70: 208-215.

Stevenson JS 2016. Synchronization and Artificial Insemination Strategies in Dairy Herds. Vet Clin North Am Food Anim Pract. 32: 349-364.

Wijma R, Pérez MM, Masello M, Stangaferro ML, and Giordano JO 2018. A resynchronization of ovulation program based on ovarian structures present at nonpregnancy diagnosis reduced time to pregnancy in lactating dairy cows. J Dairy Sci. 101: 1697-1707.

Wiltbank MC and Pursley JR 2014. The cow as an induced ovulator: timed AI after synchronization of ovulation. Theriogenology. 81: 170-185.