CHAPTER V

EXPERIMENT III EFFECTS OF GnRH REPLACEMENT WITH hCG IN TAI PROTOCOL ON OVULATION AND CONCEPTION RATES, CL DEVELOPMENT, AND PROGESTERONE CONCENTRATIONS IN DAIRY COWS

5.1 Introduction

Synchronization of ovulation with TAI is an effective management tool in lactating dairy cows to increase reproductive efficiency (De La Sota et al., 1998). The Ovsynch protocol, which combines GnRH-$PGF_{2\alpha}$-GnRH treatments, has made planned breeding programmes more effective (Pursley et al., 1995; 1997). Although fertilization rates in cattle are reported to be greater than 90% (Diskin and Sreenan, 1980), the majority of embryonic mortality (70 to 80% of the total loss) occurs between days 8 and 16 after insemination (Dunne et al., 2000). Maintenance of pregnancy, in part, is dependent on P4 during early pregnancy (Mehni et al., 2012). A significant proportion of infertility in cattle has been attributed to improper functioning of the CL, which is the major source of P4 in the pregnant cow (Santos et al., 2004). It is generally recognized that plasma P4 >1 ng/ml is an indication of a functional CL (Mann and Lamming, 2001). Poor luteal activity has been associated with infertility in cattle (Mann and Lamming, 2001), and low circulating P4 after breeding may impact embryo development and maternal recognition of pregnancy (De Rensis et al., 2008). Furthermore, recent study reported that animals on day 5, with higher plasma P4 concentrations, had developmentally more advanced and viable embryos (Green et al., 2010).

Various studies reported that gronadotropin-releasing hormone (GnRH) (Howard et al., 2006; Mehni et al., 2012) injection or hCG (Hanlon et al., 2005; Stevenson et al., 2008) on days 5 to 7 after TAI have potential to increase P4 concentration in plasma of cattle at different stages of pregnancy (Dahlen et al., 2010). Previous study suggested that exogenous GnRH administered 5 days after TAI causes ovulation and (or) luteinization of an antral follicle resulting in the formation of an accessory CL (Howard et al., 2006). Similarly, the administration of hCG in the early luteal stage induces the formation of accessory CL, increases the surface area and the volume of the CL and may increase the diameter of CL (Santos et al., 2001). Both GnRH and hCG effectively induced ovulation and increased CL number, but circulating P4 concentrations were only increased in hCG-treated cows (Lonergan, 2011). Finally, the comparison of post-TAI administration of different P4 sources in early lactating dairy cows needs to conduct more investigation (Mehni et al., 2012).

By administering GnRH followed by $PGF_{2\alpha}$ 6 to 7 days later and a second dose of GnRH 24 h later, cows can be subjected to TAI without the need for estrus detection (Pursley et al., 1995; 1997). However, 40 to 60% of treated cows do not become pregnant at the time of TAI. Several factors may lead to the failure of TAI. One such cause could be related to the use of GnRH to induce ovulation (De Rensis et al., 2002). In effect, it has been observed that a single dose of a GnRH-analogue induces an LH surge that lasts for about 5 h (Chenault et al., 1990), which is approximately half the duration of a naturally occurring LH surge (Rahe et al., 1980). This short-lived LH surge may determine the incomplete development of the ovulatory follicle, giving rise to a less functional CL (De Rensis et al., 2008). The effectiveness of hCG in inducing ovulation and the formation of a functional CL has been described by several authors (Longergan, 2011). In the classic Ovsynch protocol, hCG is given to synchronize, induce ovulation (De Rensis et al., 2002), and subsequent plasma P4 concentrations (De Rensis et al., 2008).

Because hCG was more effective than GnRH at stimulating ovulation in dairy cattle (Stevenson et al., 2008), a proper dose of hCG might be a substitute for GnRH in various TAI protocols. It was hypothesised that the longer period of LH-like stimulation of the ovulatory follicle due to the extended half-life of hCG in blood (Schmitt et al., 1996) should better stimulate the ovulatory follicle at concurrent ovulation induction and TAI in Ovsynch strategy. Because poor luteal activity after TAI has been association with deficiency of plasma P4 concentrations and subsequent infertility in cattle (De Rensis et al., 2008; Longergan, 2011), we hypothesized that administering hCG during the final maturation of the preovulatory follicles or administering hCG or GnRH after TAI would affect CL characteristics such as increased number of accessory CL and increased concentrations of plasma P4 in dairy cows. However, relatively few studies have been undertaken to investigate the strategy of GnRH replacement with hCG in TAI protocol to improve ovulation and conception rates and to induce the formation of accessory CL, and subsequent plasma P4 concentrations. Therefore, the objective of this study was to evaluate the effects of GnRH replacement with hCG in TAI protocol on ovulation and conception rates, accessory CL, and subsequent plasma P4 concentrations in dairy cows.

5.2 Materials and Methods

5.2.1 General

This experiment was conducted in Khon Kaen, Thailand at 77 16° 26' N latitude and 102° 50' E longitude. The cows formed part of a semi-commercial dairy farm located in Khon Kaen and were housed in free-stall barns, provided with shade and were fed a total mixed ration (TMR) at the ratio of 40:60 (roughage:concentrate) according to the nutrient requirement of NRC (2001). The cows were scored for body condition using quarter-point scale from 1 to 5, where 1=emaciated and 5=obese (Ferguson et al. 1994). Only cows scoring 2.5-3.5 were considered to be in good condition (Gearart et al., 1990) and included in the experiment. The voluntary waiting period was 60 days after calving.

5.2.2 Animals and treatments

The GnRH agonist was purchased from Intervet (Receptal, Auckland, New Zealand). The PGF was purchased from Pfizer Animal Health (Lutalyse, New York, USA). The hCG was purchased from Intervet (Chorulon, Auckland, New Zealand). Nonpregnant Holstein-Friesian cows (n=36) were randomly allocated to three groups as follows.

Group 1: modified Ovsynch (GPH; De Rensis et al., 2008), cows (n=12) received a GnRH agonist (100 µg, i.m.) on day -10, PGF (25 mg, i.m.) on day -3, hCG (3000 IU, i.m.) on day -1, and were timed inseminated on day 0 or 16 h after the final hCG (Figure 5.1).

Group 2: Ovsynch+hCG (Hanlon et al., 2005), cows (n=12) received a GnRH agonist (100 µg, i.m.) on day -10, PGF (25 mg, i.m.) on day -3, GnRH (50 µg, i.m.) on day -1, were timed inseminated on day 0 or 16 h after the second GnRH, and 3000 IU hCG (i.m.) on day 5 after TAI (Figure 5.1).

Group 3: Ovsynch+GnRH (Howard et al., 2006), cows (n=12) received a GnRH agonist (100 µg, i.m.) on day -10, PGF (25 mg, i.m.) on day -3, GnRH (50 µg, i.m.) on day -1, were timed inseminated on day 0 or 16 h after the second GnRH, and 100 µg GnRH (i.m.) on day 5 after TAI (Figure 5.1).

5.2.3 P4 concentrations

Blood samples were collected according to the hormonal treatments from the coccygeal vein into heparinized vacutainers. Blood samples were centrifuged at 3,000 X g for 20 min at 4 ^{o}C. Plasma samples were stored at -20 ^{o}C until assayed for P4. Concentrations of P4 were determined by competitive ELISA (Crane et al., 2006). Goat anti-mouse IgG (H+L) was made in mouse by using a P4-horse radish peroxidase conjugate. Intraassay coefficient of variation was 8.75% and assay sensitivity was 0.025 ng/ml.

5.2.4 Ultrasound of ovarian structures

The ovaries of cows were examined by transrectal ultrasonography (7.5-MHz linear array transducer, Pie medical, Maastricht, Netherlands) on day -10, day -8, day -3, day -1, day 0, 20 to 48 h after TAI, day 5, day 8, and day 12 (Figure 5.1) and all ovarian structures were mapped to monitor changes in CL and follicles in response to treatment. To determine luteal status and luteal response to $PGF_{2\alpha}$ administration, blood samples were collected by venipuncture of the coccygeal vein immediately before 24 h after $PGF_{2\alpha}$ administration on day -3 (all cows). Ovulatory response to GnRH injections administered on day -10 (all cows) and day -1 (Group 2 and 3 cows) and hCG injection on day -1 (Group 1 cows) was conducted as described previously (Fricke et al., 1998; Navanukraw et a., 2004). Briefly, ovarian structures (antral follicles >8 mm in diameter and CL) were monitored using the ultrasound machine and transducer described above. Follicle diameter was measured using the digital calipers of the ultrasound machine, and ovulatory follicle diameter was calculated as the mean of the vertical and horizontal diameter measurements (mm) for each follicle. Synchronized ovulation rate was calculated as the number of cows that ovulated a follicle within 48 h of the first and second GnRH injection (Group 2 and 3) or 48 h of GnRH and hCG injection (Group 1), expressed as a percentage of the total number of cows receiving the synchronization protocol (Fricke et al., 1998; Navanukraw et a., 2004).

Additionally, on day 5 after TAI, ultrasonographic examinations of the ovaries were performed to confirm the occurrence of ovulation, as evidenced by the presence of a CL. In addition, the location and number of CL and follicles greater than 8 mm in diameter was recorded. After the normal saline or hCG or GnRH injections on day 5, ultrasonographic examinations of the ovaries were performed on day 8 and day 12 to confirm the occurrence of ovulation, as evidenced by the presence of additional CL (Figure 5.1).

5.2.5 Pregnancy and conception rate

Visualization of a fluid-filled uterine horn and the presence of a conceptus were used as positive indicators of pregnancy at 42 d after TAI using ultrasound machine equipped with a transrectal 8 MHz linear–array transducer. The number of cows diagnosed pregnant expressed as a percentage of cows within that treatment group was defined as the conception rate (Navanukraw, 2003).

5.2.6 Statistical analysis

Categorical data (ovulatory responses to GnRH or hCG) were analyzed using the LOGISTIC procedure of SAS (SAS Inst. Inc., Cary, NC). The statistical model included treatment and parity (primiparous vs. multiparous), with DIM and BCS at TAI as regression variables and all 2-way interactions with treatment. The effect of AI sire was not included in this model but sires were distributed evenly between treatments. All possible interactions between treatment and variables were tested before the final model was chosen. Treatment interactions (DIM and BCS) were then reanalyzed using Chi-square analysis. Continuous data (dominant follicle size on the first GnRH, size of preovulatory follicle, size and number of CL on day 5, day 8, and day 12 after TAI) were analyzed using procedure GLM of SAS. When the *F*-test was significant ($P<0.05$), differences among means were evaluated by using the Duncan's New Multiple Range Test (Steel et al., 1997). P4 concentrations were analyzed with a nested analysis of variance with treatment, animal (treatment), and day included in the model, and differences between specific means were evaluated by least significant difference (Navanukraw et al., 2004).

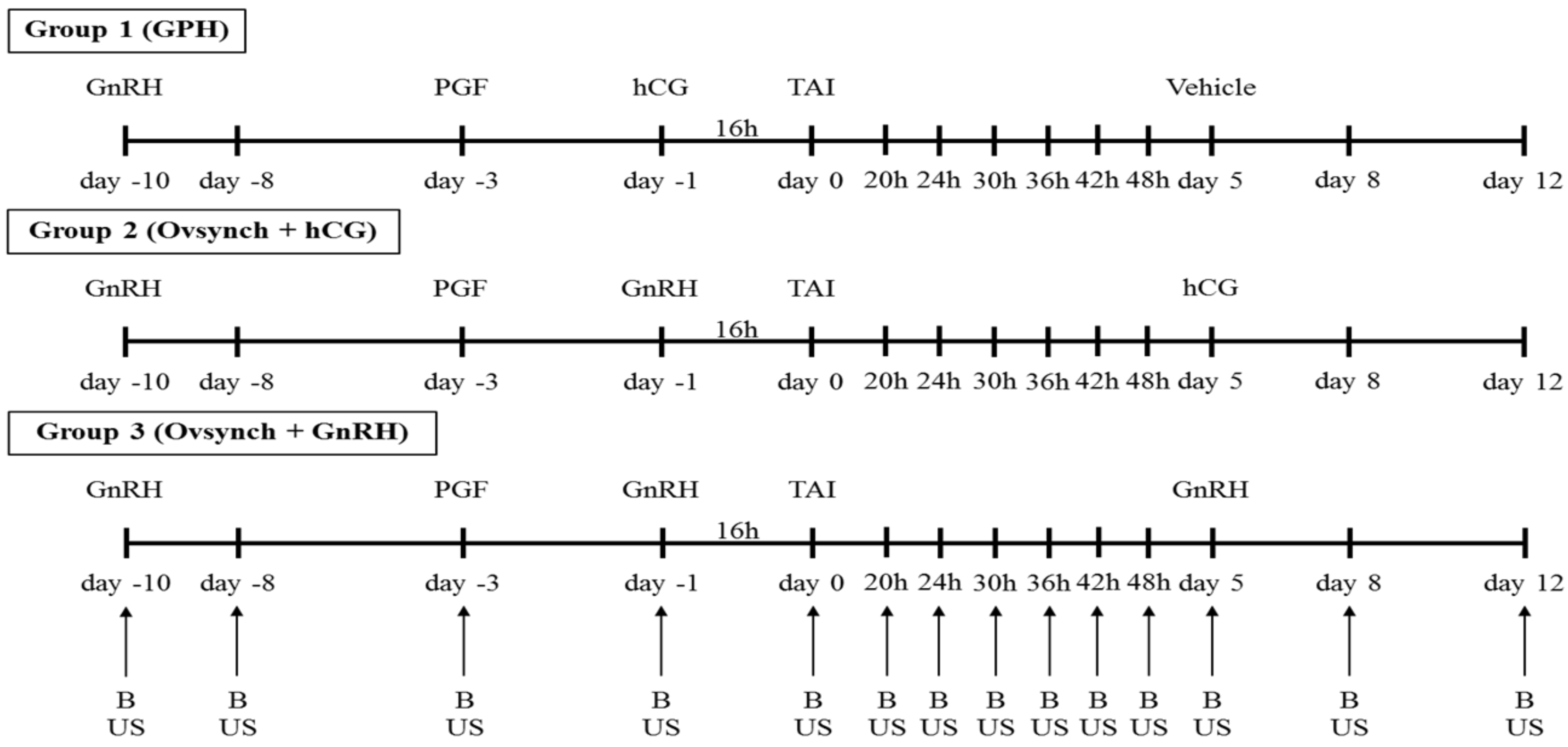

Figure 5.1 Description of timing of injections for the three synchronization protocols. GnRH, gonadotropin releasing hormone (Receptal®); PGF, prostaglandin $F_{2\alpha}$ (Lutalyse®); hCG, human chorionic gonadotropin (Chorulon®); TAI, timed artificial insemination. Abbreviations for the bottom individual: blood samples taken (B) or ultrasound evaluation of the ovaries (US) for all treatment groups.

5.3 Results

5.3.1 Characteristics of dairy cows

At the initiation of the experiment three groups were similar in parity (2.2±0.3, 2.4±0.2, and 2.5±0.3 for GPH, Ovsynch+hCG, and Ovsynch+GnRH groups), milk yield (18.2±1.0, 19.4±1.1, and 19.2±1.0 kg/day for GPH, Ovsynch+hCG, and Ovsynch+GnRH groups), DIM (87.5±2.8, 82.6±3.5, and 81.7±4.2 days for GPH, Ovsynch+hCG, and Ovsynch+GnRH groups), and BCS (2.8±0.1, 2.8±0.1, and 2.7±0.1 for GPH, Ovsynch+hCG, and Ovsynch+GnRH groups).

5.3.2 Follicular dynamic, size and number of CL, and synchronized ovulation and conception rates

The results are shown in Table 5.1. The dominant follicle size at the time of the first GnRH treatment was not different (P>0.05) among the GPH, Ovsynch+hCG, and Ovsynch+GnRH groups. Similarly, at the time of the second GnRH or the final hCG treatment, there was no significant (P>0.05) difference in size of preovulatory follicle when compared with the GPH, Ovsynch+hCG, and Ovsynch+GnRH groups. The percentage of CL responsive of $PGF_{2\alpha}$ was not difference (P>0.05) in all treatment groups. Additionally, the percentages of cows ovulating to the first and second GnRH injections or the final hCG injection were not different (P>0.05). Conception rate to TAI was not differed (P>0.05) for cows receiving GPH (50.0%), Ovsynch+hCG (58.3%), and Ovsynch+GnRH (50.0%) groups.

Size of CL on day 5 after TAI was the greatest (P<0.05) in the Ovsynch+hCG group compared with the GPH group but was not different when compared with the Ovsynch+GnRH group. Nevertheless, sizes of CL on day 8 and day 12 was not different (P>0.05) among all treatments (P<0.05). There were no significant different (P>0.05) in numbers of CL on day 5, day 8, and day 12 among the GPH, Ovsynch+hCG, and Ovsynch+GnRH groups. In addition, on day 5 after TAI, 34 of 36 cows of all treatments (data not shown) had a CL present (Figure 5.2a). Subsequently, on day 12 after TAI, 29 of 36 cows of all treatments (data not shown) had developed an accessory CL (Figure 5.2b).

Table 5.1 Follicular dynamic, size and number of CL, and synchronized ovulation rate of postpartum dairy cows received GPH (modified Ovsynch), Ovsynch+hCG, and Ovsych+GnRH protocols.

Items	GPH	Ovsynch+hCG	Ovsych+GnRH
Size of dominant follicle at first GnRH (mm)	12.1±0.5	10.8±0.7	10.5±0.7
Size of preovulatory follicle (mm)	13.1±0.5	13.6±0.5	12.6±0.5
CL responsive of $PGF_{2\alpha}$ (%)	75.0	91.7	75.0
(no./no.)	(9/12)	(11/12)	(9/12)
Synchronized ovulation rate (%)			
After the first GnRH	83.3	91.7	75.0
(no./no.)	(10/12)	(11/12)	(9/12)
After the second GnRH	-	100.0	100.0
(no./no.)		(12/12)	(12/12)
After the final hCG	100.0	-	-
(no./no.)	(12/12)		
Conception rate (%)	50.0	58.3	50.0
(no./no.)	(6/12)	(7/12)	(6/12)
Size of CL on D5 after TAI (mm)	12.9±0.7[a]	14.8±0.5[b]	13.3±0.5[ab]
Size of CL on D8 after TAI (mm)	14.6±0.9	14.9±1.0	13.2±0.9
Size of CL on D12 after TAI (mm)	16.9±0.7	18.7±1.0	16.5±0.6
Number of CL on D5 after TAI (no.)	0.8±0.1	1.0±0.0	1.0±0.0
Number of CL on D8 after TAI (no.)	1.7±0.1	1.8±0.1	1.8±0.1
Number of CL on D12 after TAI (no.)	1.7±0.1	1.8±0.1	1.8±0.1

Note: Different letters (a, b) in the same row are different ($P<0.05$).

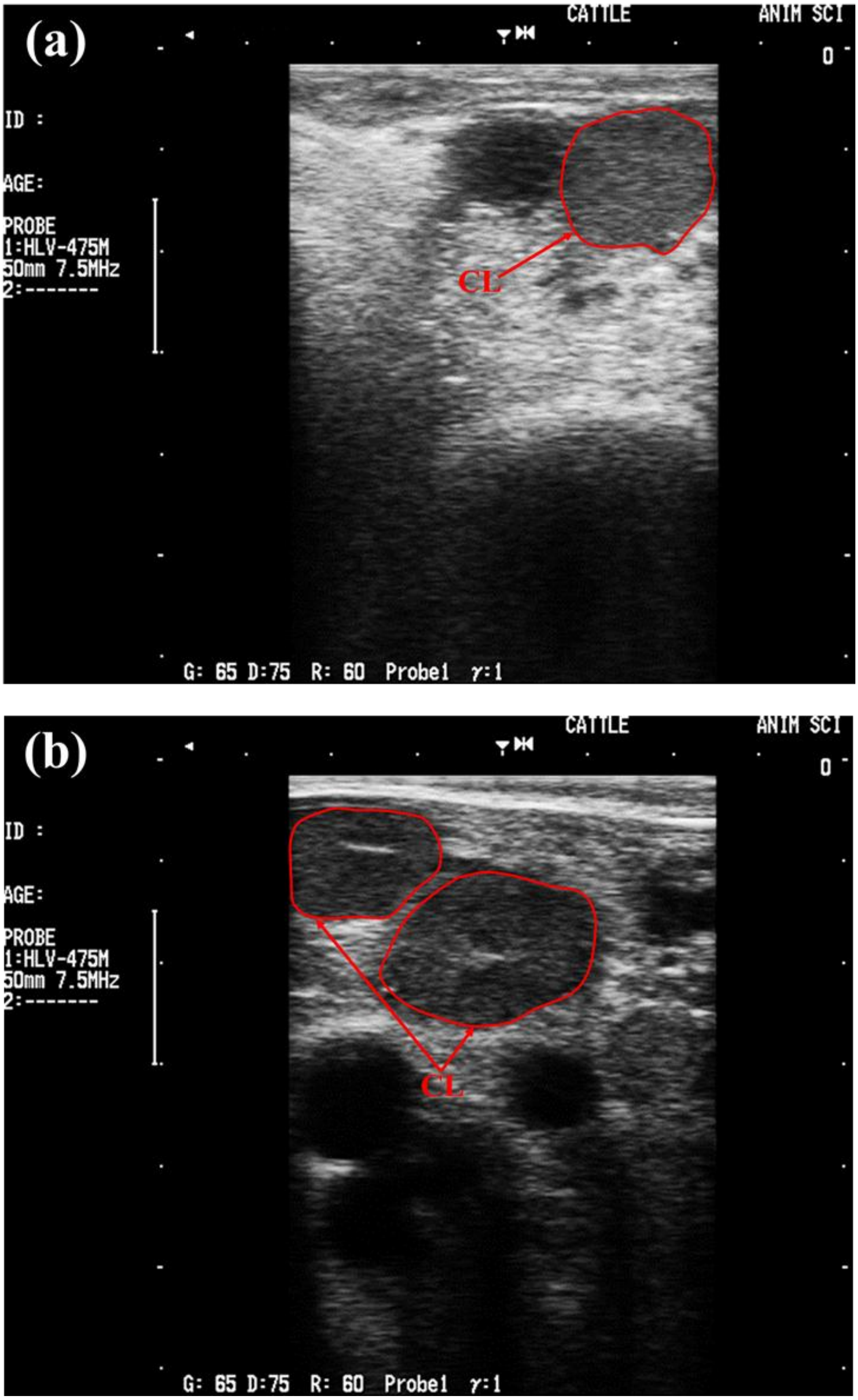

Figure 5.2 Sonogram images of a CL on day 5 after AI (a) and two CL side by side (b) on the ovary of a hormone-treated cow on day 12 after AI.

5.3.3 P4 concentration

There were no difference (P>0.05) in the mean plasma P4 concentrations at the time of the first and second GnRH or final hCG injections, on day 5, and day 8 after TAI in all groups in this study (Figure 5.3). However, on day 12 after TAI the P4 concentrations were highly greater (P<0.05) in the Ovsynch+hCG treatment compared with the Ovsynch+GnRH treatment but was not different when compared with the GPH treatment (5.5±0.2, 5.8±0.2, and 5.1±0.2 ng/ml for the GPH, Ovsynch+hCG, and Ovsynch+GnRH groups; Figure 5.3).

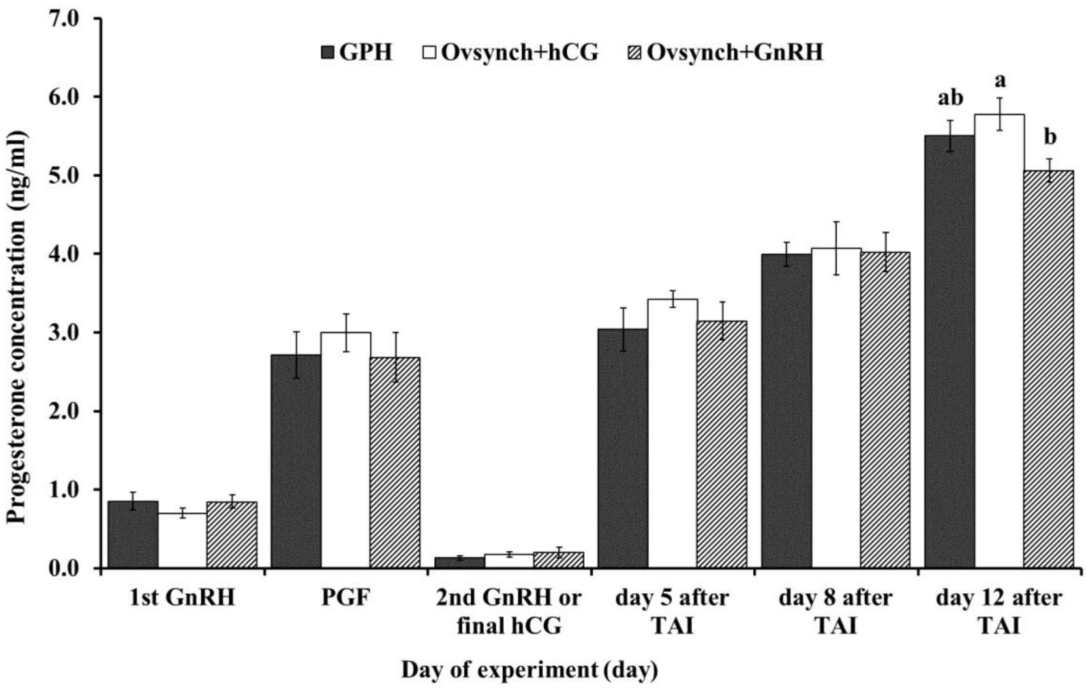

Figure 5.3 Mean plasma P4 concentrations (ng/ml) determined in dairy cows after receiving GPH (black bars), Ovsynch+hCG (white bars), or Ovsynch+GnRH (hatched bars). [a, b] Proportion was differed (P<0.05).

5.4 Discussion

Data from this study are consistent with the hypothesis that hCG administration within a TAI or hCG or GnRH given on day 5 after TAI induced ovulation resulting in formation of an accessory CL thereby increasing endogenous P4 concentrations on day 12. In effect, hCG administration has been shown to reduce the number of small luteal cells (Farin et al., 1988), increasing the number of large luteal cells along with the diameter, surface area and volume of the CL (Santos et al., 2001). All these effects will have a positive effect on plasma P4 concentrations (Halon et al., 2005; Howard et al., 2006). In several studies reported that administrations of hCG or GnRH on day 5 after ovulation initiate ovulation or luteinization of that dominant follicle (Khoramian et al., 2011) and subsequent formation of a CL (De Rensis et al., 2010).

In a previous study suggested that the positive effect of hCG treatment at the time of AI is mainly achieved through improved CL function (De Rensis et al., 2008). As reported previously, treatment of cows with hCG on the time of AI resulted in larger CL on day 7 after AI (Rajamahendran and Sianangama, 1992). Although most of the effect of hCG on total luteal area in the current study was caused by the presence of multiple CL, treatment with hCG may also have increased the size of the CL originating from the spontaneous ovulation (Santos et al., 2001). Some cows in the hCG-treated group might have had a spontaneous double ovulation during estrus prior to hCG treatment, since multiple CL were observed in 23.2% of the control cows (Santos et al., 2001). However, hCG use in synchronization protocol during the breeding period has been limited. One major limitation has been the incidence in twin pregnancy as a result of double ovulation (Santos et al., 2004). Additionally, treatment with hCG during the periovulatory period enhances the subsequent luteal activity of the primary CL (De Rensis et al., 2008). Administrative hCG given at the time of AI increases plasma P4 levels from day 5 to day 12 of the estrous cycle in cows with no accessory CL (Johson et al., 2010). In fact, hCG can increase plasma P4 levels through two possible mechanisms: a direct effect on the primary CL and/or by developing accessory CL (De Rensis et al., 2010). Therefore, the increase in original CL function and/or the increase in CL number are likely responsible for the increased P4 concentration on day 12 after TAI in the GPH group. In the present study, injection of hCG or GnRH on day 5 after TAI consistently induced formation of accessory CL and subsequently increased concentrations of plasma P4 on day 12 after TAI in dairy cows. Similarly, previous studies reported that treatments with hCG and GnRH on day 5 to days 7 after TAI have similar effects on the ovary (Halon et al., 2005; Howard et al., 2006), inducing ovulation (Santos et al., 2001; Khoramian et al., 2011) and the formation of accessory CL (Schmitt et al., 1996; Garcia-Ispierto and Lopez-Gatius, 2012), with a significant increase in plasma P4 concentrations achieved 7 to 13 days after treatment (Halon et al., 2005; Howard et al., 2006). However, the half-life and therefore the LH-like effect of hCG on the ovarian cells may last for 30 h after treatment (Schmitt et al., 1996); in contrast, administration of GnRH increases LH concentrations in serum for approximately 5 h (Chenault et al., 1990). Main and accessory CL diameter and average plasma P4 concentration were higher for hCG treated cows compared with GnRH treatments (Binelli et al., 2001). As hCG has a longer half-life than GnRH, it can persist in the circulation longer. Therefore, besides inducing formation of accessory CLs, hCG may exert luteotropic effects by directly stimulating P4 production (Shabankareh et al., 2010). Our data demonstrated that the Ovsynch+hCG treatment significantly increased greater P4 concentration on day 12 after TAI than in Ovsynch+GnRH treatment. Similarly, our previous studies clearly showed that hCG treatment usually elevates P4 concentrations more than dose GnRH treatment (Schmitt et al., 1996; Stevenson et al., 2008). Moreover, the CL formed after ovulation induced by GnRH may not be fully functional (Diaz et al., 1998; Santos et al., 2001). Administration of hCG or GnRH after insemination at specific times coincident with the presence of the dominant follicle of the first and second follicular waves may stimulate CL function, induce accessory CL formation, increase P4 and reduce E2 production with a consequent positive effect on embryo survival (Thatcher et al., 2003; Khoramain et al., 2011).

The effect of a single hCG dose at the time of TAI on fertility is variable and several studies have reported an improved conception rate to first service (De Resis et al., 2008; 2010) or no improvement (Helmer and Britt, 1986). In dairy cows, several studies have achieved improved conception rates using hCG on days 5 to 7 after TAI (Santos et al., 2001). Conversely, although hCG treatment at pregnancy diagnosis (from days 29 to 42 after AI) induced the development of accessory CL and increased

P4 plasma levels for 4 wk, it did not reduced embryo losses compared with GnRH-treated or control cows (Stevenson et al., 2008). The latter observation is consistent with previous reports of no differences in conception rates between control and treated cows receiving hCG at AI (Helmer and Britt, 1986) or on day 5 post-AI (Schmitt et al., 1996; Hanlom et al., 2005). In the present study, conception rate was not different among the GPH, Ovsynch+hCG, and Ovsynch+GnRH treatments. Conception rate seemed to be higher in Ovsynch+hCG group than in GPH and Ovsynch+hCG groups, but the difference did not reach statistical significance. The likelihood of detecting treatment effects in conception rate was limited in the present study because only the 36 cows enrolled into the study and because of the small difference in conception rate observed among treatment groups (Steel et al., 1997). In addition, a limited number of cows and different protocols and dosage regimens were utilized, which might have affected the ability to detect any difference between treatment groups (Santos et al., 2001). Also, accessory CL and plasma P4 concentrations on day 12 after TAI in cows synchronized based on the results of experiment. This is consistent with other studies on the treatment with hCG at the time of TAI (De Resis et al., 2008; Johnson et al., 2010) and with hCG (Rizos et al., 2003; Hanlon et al. 2005) or GnRH on day 5 after TAI (Howard et al., 2006; Garcia-Ispierto and Lopez-Gatius, 2012). Additionally, average costs of hormonal treatment were 838, 935, and 612 Baht/head for the GPH, Ovsynch+hCG, and Ovsynch+GnRH treatments, respectively (data not shown).

5.5 Conclusion

In conclusion, there is sufficient evidence to suggest that administration of GnRH replacement with hCG in TAI protocol offers an opportunity to induce the formation of an accessory CL, and increase plasma concentrations of P4 after TAI. The effectiveness of hCG (GPH and Ovsynch+hCG groups) in inducing ovulation of the dominant follicle, development of original CL or accessory CL, and increasing P4 concentration are similar to that observed in GnRH administration (Ovsynch+GnRH group). Nevertheless, as hCG has a longer half-life and produces a direct effect on the ovary, hCG instead of GnRH should be considered in TAI protocol targeted at postpartum dairy cows. Applying hCG instead of GnRH as the second gonadotrophic dose in the Ovsynch protocol led to elevated plasma P4 levels on day 12 post-TAI, reduced the calving-conception interval, and gave rise to similar conception rates as those recorded in cows receiving Ovsynch+hCG and Ovsynch+GnRH groups. This conception rate indicates economic opportunity for improvement in dairy cattle reproduction. Improvements in reproduction typically translate in increased conception rate and increased profitability. Profitability is changed as a result of changes in herd demographics and the direct cost of the reproductive program.