EFFECT OF TWO LOW DOSES OF PROSTAGLANDIN $F_{2\alpha}$ ON LUTEOLYSIS IN DAIRY COWS

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In this preliminary study, we determined the effect of a modified method involving the administration of two low doses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at an interval of 24 h on luteolysis in dairy cows, and compared it with the standard single-dose method. Twenty-six cows were assigned to three groups treated with two low doses (TLD group, n = 10), one standard dose (SD group, n = 10), and one low dose (OLD group, n = 6) on day 9 to 10 of the oestrous cycle (day 0 = the day of $PGF_{2\alpha}$ administration). Their serum progesterone (P4) levels and corpus luteum (CL) sizes were measured daily from day 0 to 4 to assess CL regression. The results indicated that the proportion of complete luteolysis, indicating a P4 value $\leq$ 1 ng/mL on day 3, was higher in the TLD group (100.0%) than in the SD (60.0%) and OLD (66.7%) groups. Ultrasonically detected changes in the CL area correlated with the shifts in the P4 values in both the TLD and the SD groups. The remaining CL area was significantly smaller in the TLD group (17.8% ± 3.3%) than in the SD or OLD group on day 4. Thus, we concluded that the proportion of luteolysis in cows was increased with two low doses of $PGF_{2\alpha}$ as compared to a single $PGF_{2\alpha}$ dose, indicating the necessity of the second dose of $PGF_{2\alpha}$. However, further studies with larger sample sizes in the field are required.

Key words: PGF2α, two low doses, standard dose, luteolysis, dairy cows

The luteolytic effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its analogues during the dioestrus period of the oestrous cycle are well documented, and $PGF_{2\alpha}$ has been used widely in the synchronisation protocol (Martins et al., 2011; Chenault et al., 2014). Inadequate regression of the corpus luteum (CL), induced by a standard single dose of $PGF_{2\alpha}$ in the Ovsynch protocol, varies from 10 to 25% (Wiltbank and Pursley, 2014); however, $PGF_{2\alpha}$ at a dose 1.5 times the standard dose results in complete luteolysis in only 90.1% of cows (Giordano et al., 2013). Moreover, the proportion of CL regression is higher with a second $PGF_{2\alpha}$ treatment after the first one in the Ovsynch protocol (Ribeiro et al., 2012; Wiltbank et al., 2015).

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The determination of complete luteolysis in the Ovsynch protocol is based on the P4 profile at two time points, including the administration of $PGF_{2\alpha}$ and the final gonadotropin releasing hormone (GnRH) (Brusveen et al., 2009; Ribeiro et al., 2012); however, the morphologic change of the CL has rarely been described. Furthermore, using the P4 criteria at a single time point might be inadequate to evaluate luteolysis (Nascimento et al., 2014). Thus, the objectives of this study were to evaluate the effects of a modified method involving the administration of two low doses of $PGF_{2\alpha}$ at an interval of 24 h in the middle of the oestrous cycle on luteolysis in dairy cows, and to confirm the necessity of the second $PGF_{2\alpha}$ treatment. The effects of the modified method of $PGF_{2\alpha}$ administration on luteolysis were recognised on the basis of consecutive changes in P4 levels and CL sizes in comparison to the standard single-dose method. We hypothesised that the administration of two low $PGF_{2\alpha}$ doses would lead to a higher proportion of complete luteolysis as compared to a standard single dose, and that the second dose in the modified method is necessary for complete luteolysis.

Materials and methods

Animals and management

The study was conducted in the dairy farm, comprising a total of 50 Holstein dairy cattle, of the National Chung Hsing University in subtropical Taiwan from April to June 2013 and October 2013 to April 2014. All animals were housed in groups in free-stall barns with a slatted floor and sand bed, and equipped with overhead fans and a sprinkler system. Access to an outdoor shaded exercise yard was also available. The cows were fed twice daily with a diet of total mixed ration (TMR). Fresh water was provided *ad libitum*. All lactating dairy cows were milked twice daily and the herd average milk yield was 8500 kg per lactation. All lactating cows exhibited normal uterine involution at 1.5 months postpartum. All cows were subjected to transrectal B-mode ultrasonography three times a week to confirm the presence of functional ovaries within 19 to 22 days of the oestrous cycle. Ultrasonography was performed using a portable scanner equipped with a 7.5-MHz linear-array transducer (SonoSite[®] Ultrasound System, SonoSite Inc., USA). Cows that had exhibited at least one normal oestrous cycle and no ovarian disease, including anovulatory anoestrus and cystic ovarian disease, were enrolled in the experiment. A total of 26 lactating cows with their age (in years), parity, and body condition score ranging from 3 to 8, 1 to 4, and 3.0 to 3.5, respectively, were used in this study. The average number of milking days in the experimental cows was 260. This experiment complied with the current welfare legislation, and all management practices followed the animal care guidelines of the National Chung Hsing University, Taiwan.

Experimental design

The experiment began on day 9 to 10 of the subsequent oestrous cycle in cows with a CL ≥ 2.5 cm and at least one follicle ≥ 1 cm. The CL and follicle diameters were calculated by the average of two measurements (longitudinal and transverse axes) taken at right angles with electronic callipers when the CL and follicle showed maximum diameter in their frozen images (Fig. 1) (Valldecabres-Torres et al., 2012). The cows were randomly divided into three groups: two low doses (TLD group; n = 10), a single standard dose (SD group; n = 10), and one low dose (OLD group; n = 6). The time of first PGF$_{2\alpha}$ administration was defined as day 0. On day 0, the cows in the TLD and OLD groups were administered 375 µg (1.5 mL) PGF$_{2\alpha}$ by intramuscular (IM) injection (cloprostenol sodium; Estrumate®, Intervet Inc., Germany), and the cows in the SD group were administered 500 µg (2 mL) PGF$_{2\alpha}$ IM. After 24 h, the cows in the TLD group were administered 250 µg (1 mL) PGF$_{2\alpha}$ IM and those in the OLD group were administered normal saline (1 mL).
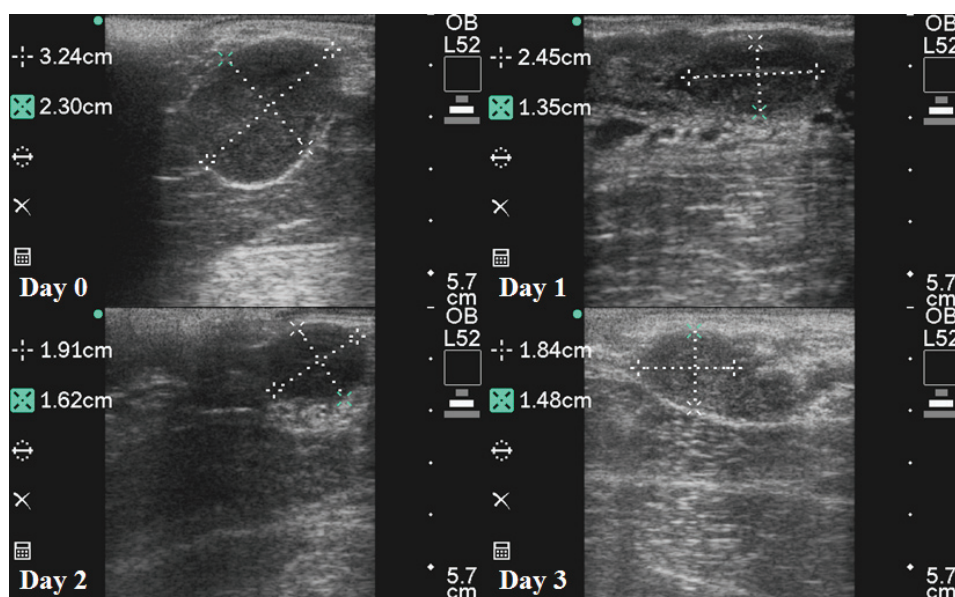

Fig. 1. Corpus luteum (CL) measurement taken at right angles with the electronic callipers when the frozen image of the CL and follicle were maximum. The CL area gradually decreased from day 0 to 3 in a cow of the TLD group

Blood sampling and P4 analysis

Blood sampling was carried out concurrently with ultrasonography between 09:00 and 10:00 a.m. Blood samples for analysing the serum P4 concentrations were collected daily by puncturing the coccygeal vessels of all experi-

mental animals from day 0 to 4 and were immediately refrigerated. The samples were centrifuged at $1300 \times g$ for 10 min, and the serum was collected and stored at −20 °C until assayed for P4. The concentrations of P4 were measured using an enzyme-linked fluorescent assay (VIDAS® Progesterone, BioMérieux, France) with the MiniVidas automated analyser (miniVIDAS®, BioMérieux, France). The measurement range of the VIDAS® Progesterone was 0.25 to 80 ng/mL, and the detection limit was 0.25 ng/mL. The intra- and inter-assay coefficients of variation were 4.0 and 3.1%, respectively. The experimental cows were classified as having complete luteolysis without a functional CL when the P4 concentration was below 1 ng/mL (Ribeiro et al., 2012).

Measurement of CL area

Daily ultrasound examination was performed from day 0 to 4 in order to determine the CL diameter. The CL area was calculated using the following formula of oval area: ½ longitudinal axis × ½ transverse axis × π (Kastelic et al., 1990). In addition, the CL area on day 0 in each cow was defined as 100%, and the remaining CL area on day 1 to 4 was expressed as the percentage of this area.

Ovulation detection

The disappearance of a dominant follicle was also determined by daily ultrasonography. Ovulation was defined by the disappearance of a dominant follicle (≥ 1 cm) followed by a new CL development at the same site of the ovary. If the dominant follicle remained until day 5, it was considered a non-ovulatory follicle.

Statistical analyses

All data were analysed using SAS (version 9.3, SAS Institute Inc., USA), and a $P < 0.05$ was considered significant. The P4 concentration, CL area, and remaining CL area among groups at any time point and across time within each group were evaluated by one-way analysis of variance (ANOVA) using PRO GLM of SAS. Proportions of complete luteolysis and ovulation were evaluated using the Mantel extension test using PRO FREQ. The correlations between complete luteolysis and a remaining CL area of < 50% on day 2 or 3 were evaluated by the chi-square test with PRO FREQ.

Results

Changes in P4 concentrations

The concentrations of P4 decreased in a stepwise manner from day 0 to 2 ($P < 0.05$) in the TLD group. The mean P4 values showed pronounced changes (9.79 ± 1.02 to 0.51 ± 0.07 ng/mL) from day 0 to 4. On day 3, all values in the

TLD group were < 1 ng/mL, and the mean value was 0.56 ± 0.07 ng/mL, indicating the occurrence of complete luteolysis (Table 1). In the SD group, the P4 concentrations decreased from day 0 to 4 (10.54 ± 0.74 to 2.90 ± 1.13 ng/mL), and the mean value was 1.68 ± 0.61 ng/mL on day 3, when four values were above 1 ng/mL. A significant decrease in the P4 value was only observed between day 0 and 1. In the OLD group, the P4 concentrations decreased from day 0 to 4 (13.40 ± 2.44 to 5.03 ± 2.99 ng/mL), and the mean value on day 3 was 4.43 ± 2.60 ng/mL, which included two P4 concentrations greater than 1 ng/mL. No significant difference in the P4 concentrations was observed from day 0 to 4 within the OLD group.

Table 1

Changes in serum progesterone (P4) concentration (mean $\pm$ SE) after prostaglandin F$_{2\alpha}$ administration in each of the three groups

P4 concentration (ng/mL)	Treatments		
	TLD	SD	OLD
Day 0	9.79 ± 1.02^{A}	10.54 ± 0.74^{A}	13.40 ± 2.44
Day 1	3.04 ± 0.20^{B}	2.52 ± 0.32^{B}	4.70 ± 1.80
Day 2	0.79 ± 0.07^{C}	1.56 ± 0.46^{B}	4.31 ± 2.39
Day 3	0.56 ± 0.07^{C}	1.68 ± 0.61^{B}	4.43 ± 2.60
Day 4	0.51 ± 0.07^{C}	2.90 ± 1.13^{B}	5.03 ± 2.99

TLD: two low doses group; SD: single standard dose group; OLD: one low dose group; $^{A-E}$Different upper case superscript letters indicate difference across time in a group

Proportion of complete luteolysis

The proportions of complete luteolysis on day 3 in the TLD, SD, and OLD groups were 100.0, 60.0, and 66.7%, respectively. There was a trend effect of different PGF$_{2\alpha}$ doses on complete luteolysis (P = 0.0385; Table 2).

Table 2

Proportion of complete luteolysis and ovulation after prostaglandin F$_{2\alpha}$ administration among three groups in cows with a corpus luteum on day 9 to 10 of the oestrous cycle

Item	Treatment[1]			
	TLD, % (n/total)	SD, % (n/total)	OLD, % (n/total)	P value
Complete luteolysis[2]	100 (10/10)	60 (6/10)	66.7 (4/6)	0.0385
Ovulation[3]	90 (9/10)	60 (6/10)	0 (0/6)	0.0003

[1]TLD: two low doses group; SD: single standard dose group; OLD: one low dose group; [2]P4 concentration < 1 ng/mL after the first PGF$_{2\alpha}$ administration on day 3; [3]Disappearance of the dominant follicle ($\geq$ 1 cm) followed by a new CL development at the same site

Changes in CL area

The CL area in the TLD group decreased from day 0 to 4 (5.04 ± 0.17 to 0.90 ± 0.16 cm^2). In addition, the CL area decreased significantly from day 0 to 2 (Table 3; Fig. 1). In the SD group, the CL area diminished from day 0 to 4 (5.18 ± 0.31 to 1.66 ± 0.40 cm^2), and a significant difference was observed between day 0 and 1. In the OLD group, the CL area decreased daily without significant differences within the group.

Table 3

Changes in corpus luteum (CL) area (mean ± SE) after prostaglandin $F_{2\alpha}$ administration in the three groups

CL area (cm^2)	Treatments		
	TLD	SD	OLD
Day 0	5.04 ± 0.17^A	5.18 ± 0.31^A	4.78 ± 0.37
Day 1	3.12 ± 0.20^B	3.52 ± 0.27^B	3.33 ± 0.62
Day 2	1.90 ± 0.16^C	2.70 ± 0.21^{BD}	2.90 ± 0.62
Day 3	1.35 ± 0.17^{CE}	2.04 ± 0.36^{CD}	2.69 ± 0.73
Day 4	0.90 ± 0.16^{DE}	1.66 ± 0.40^{CD}	2.40 ± 0.81

TLD: two low doses group; SD: single standard dose group; OLD: one low dose group;
$^{A–E}$Different upper case superscript letters indicate difference across time in a group

Remaining CL area

The remaining CL area in the TLD, SD, and OLD groups was diminished to 17.8 ± 3.5, 33.1 ± 8.1, and 46.6 ± 11.7%, respectively, on day 4 (Table 4). Significant decreases in the remaining CL area were observed between day 0 and 1 and day 1 and 2 in the TLD group, but only between day 0 and 1 in the SD group and between day 1 and 2 in the OLD group. The remaining CL area was greater in the OLD group than in the TLD group on day 2 (P = 0.0112) or 3 (P = 0.0455).

Table 4

Remaining corpus luteum (CL) area (mean ± SE) after prostaglandin $F_{2\alpha}$ administration in each of the three groups

Remaining CL area (%)	Treatments		
	TLD	SD	OLD
Day 0	100^A	100^A	100^A
Day 1	62.1 ± 3.6^B	68.0 ± 3.4^B	68.1 ± 8.5^{AB}
Day 2	37.7 ± 2.8^{C,a}	52.2 ± 3.5^{BD,ab}	59.3 ± 8.9^{B,b}
Day 3	27.1 ± 3.6^{CE,a}	39.9 ± 7.0^{CD,ab}	53.4 ± 10.2^{B,b}
Day 4	17.8 ± 3.5^{DE}	33.1 ± 8.1^{CD}	46.6 ± 11.7^B

TLD: two low doses group; SD: single standard dose group; OLD: one low dose group;
$^{A–E}$Different upper case superscript letters indicate difference across time in a group;
$^{a–b}$Different lower case superscript letters indicate difference between groups

Correlations between P4 concentration and remaining CL area

Based on the P4 concentrations on day 3, all animals were pooled and then reassigned into two groups, one for complete luteolysis (n = 20) and the other for partial luteolysis (n = 6). Of the 20 cows with complete luteolysis, the number of cows that showed ≤ 50% remaining CL area on days 2 and 3 was 15 (75.0%) and 19 (95.0%), respectively. On the other hand, among six cows with partial luteolysis, five cows (83.3%) showed > 50% remaining CL area on days 2 and 3. This result demonstrated a significant correlation between the complete luteolysis and the remaining CL area (≤ 50%) on day 2 (P = 0.0184) and day 3 (P < 0.001).

Proportion of ovulation

The proportions of ovulation were 90.0, 60.0, and 0.0% in the TLD, SD, and OLD groups, respectively. Significant trend effects of the different PGF$_{2\alpha}$ doses on ovulation (P = 0.0003) were observed (Table 2).

Discussion

In this study, cows with a mature CL showed appropriate response to two low doses of PGF$_{2\alpha}$. The proportions of complete luteolysis in the TLD, SD, and OLD groups were 100.0, 60.0, and 66.7%, respectively, which indicated that the effect of two low doses of PGF$_{2\alpha}$ on luteolysis was better than those of the standard single dose or one low dose. Similar results were reported by Brusveen et al. (2009) and Giordano et al. (2013), which demonstrated the failure of luteolysis in dairy cows following the single dose of PGF$_{2\alpha}$ in the Ovsynch protocol (Brusveen et al., 2009; Giordano et al., 2013). A half dose of PGF$_{2\alpha}$ would induce 30.4% of the cows with partial luteolysis (Meira et al., 2006). Based on the results of consecutive monitoring of the serum P4 concentrations and CL sizes from day 0 to 4 in all experimental cows, we suggested that the second treatment of PGF$_{2\alpha}$ was needed to persistently enhance the regression of CL. Although a similar study by Brusveen et al. (2009) found that the proportion of complete luteolysis in cows could be elevated after a second treatment with a standard dose of PGF$_{2\alpha}$ 24 h after the first treatment (Brusveen et al., 2009), the cost of using two low doses of PGF$_{2\alpha}$ in the present study was less than that of the two standard doses. However, the mechanism by which the second dose enhanced luteolysis is not fully clear.

In the present study, the failure of CL regression in the SD group was similar to that described previously (Valldecabres-Torres et al., 2012). Our results indicated that the proportion of complete luteolysis in the SD group was even lower than that in the OLD group. Nevertheless, no significant difference in luteolysis was observed between these two groups. It has been reported that the

bovine CL develops a luteolytic capacity on day 5 to 5.5 of the oestrous cycle (Valldecabres-Torres et al., 2012). In this study, all cows received $PGF_{2\alpha}$ on days 9 or 10 of the oestrous cycle, a period sufficient for the development of the luteolytic capacity in cows; however, some cows showed a failure of CL regression. Our results provided evidence that the second treatment of $PGF_{2\alpha}$ would improve the problem of partial luteolysis. Thus, we suggest that further, more detailed studies are required for better understanding the failure of CL regression in the process of luteolysis.

The P4 concentration decreased significantly from day 2 in the TLD group, which indicated the occurrence of functional luteolysis, while a delayed occurrence of structural luteolysis was indicated by the decrease in the CL area. Until day 4, the value of the remaining CL area was significantly lower than that on day 2. It was likely that an appreciable lag time was required for the apoptosis of luteal cells. A previous report revealed that P4 concentration significantly decreased 8 h after $PGF_{2\alpha}$ administration, but the apoptosis of luteal cells, as indicated by oligonucleosome formation, was not apparent until 24 h after the treatment (Juengel et al., 1993), indicating that P4 decreased because of reduced blood flow in the CL and subsequent luteal cell apoptosis with structural regression.

According to the previous studies, the determination of CL regression was mainly based on a single measurement of serum P4 concentration (Santos et al., 2010; Giordano et al., 2013) or a decrease in the CL diameter (Dirandeh et al., 2015) at 24–72 h after $PGF_{2\alpha}$ administration. In the present study, the P4 concentrations and the remaining CL area were monitored throughout the experimental period, and we provided evidence that the P4 concentration in cows with partial luteolysis rebounded on days 2 or 3 accompanied by changes in the CL area. Interestingly, a regrowth of CL was observed by continual ultrasonography in some cases of luteolytic failure. Some studies also demonstrated that the P4 concentrations rebounded 2 or 4 days after $PGF_{2\alpha}$ treatment with either a single or two standard doses (Valldecabres-Torres et al., 2012; Nascimento et al., 2014). Thus, we suggest that after $PGF_{2\alpha}$ treatment, consecutive detections of the function or structure of CL are required to evaluate luteolysis instead of a single detection.

The proportions of ovulation were higher in the TLD group, and a trend effect was more often noticed in the TLD group as compared to the SD and OLD groups. It was interesting to note that five cows, which showed complete luteolysis accompanied by at least one follicle ≥ 1 cm, had not ovulated by day 5 in the TLD and OLD groups, among which 3 cows ovulated more than 5 days after the first administration of $PGF_{2\alpha}$ (data not shown). It has been reported that the dominant follicles have the capacity for ovulation with a size greater than 10 mm in diameter in dairy cows (Sartori et al., 2001). In this study, the ultrasonography confirmed that the dominant follicles in cows with complete luteolysis failed to ovulate. The prerequisite for spontaneous ovulation are luteolysis and a subsequent surge in luteinising hormone (LH) (Mihm et al., 2002); however, some

studies revealed that the subluteal P4 concentrations might elevate the LH pulse frequency (Kojima et al., 2003) and prolong the period of dominance (Savio et al., 1993). We speculated that the five cows that did not ovulate by day 5 might experience a delayed ovulation or a prolonged follicular phase; however, more studies are needed to demonstrate whether any interaction exists between ovulatory failure and P4 concentrations, even when P4 is < 1 ng/mL.

We concluded that the modified method involving the administration of two low doses of PGF$_{2\alpha}$ at an interval of 24 h in cows might contribute to a higher proportion of complete luteolysis than the standard single dose, and that the second dose of PGF$_{2\alpha}$ was essential for complete luteolysis. In addition, the use of this modified protocol increased the proportion of ovulation within 5 days after the first treatment. Further studies with larger sample sizes are needed to prove the benefits of this modified protocol in the field.

Acknowledgement

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