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—Original Article—

Presence of multiple corpora lutea affects the luteolytic response to prostaglandin $F_{2\alpha}$ in lactating dairy cows

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Abstract. Since the 1970s, luteolytic doses used for synchronizing estrus in dairy cattle have remained unchanged. This study aimed to evaluate the dose-response effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which is used for synchronizing estrus, and subsequent fertility in cows with two or more corpora lutea (CL). The study population consisted of 1,683 cows with a single CL (1CL), 501 cows with multiple CL receiving a single dose of $PGF_{2\alpha}$ (2CL1), and 252 cows with multiple CL receiving a 1.5 × $PGF_{2\alpha}$ dose (2CL1.5). Cows with a single CL (n = 1,245) showed estrus significantly (P < 0.01) earlier (3.01 ± 1.23 days; mean ± SD) than cows with multiple CL (n = 287; 3.33 ± 1.69 days). Using 1CL cows as reference, the odds ratio (OR) for the estrus response in 2CL1 cows was 0.13 (P < 0.0001), whereas the ORs for estrus response and pregnancy of 2CL1.5 cows were 1.8 (P = 0.0001) and 1.7 (P = 0.001), respectively. Based on the results for only the 2CL1 cows, the OR for the estrus response was 0.7 (P = 0.01) for cows producing ≥ 45 kg of milk at treatment, compared to the remaining cows producing < 45 kg of milk. Our results showed that the presence of multiple CL reduced the estrus response to that induced by a single $PGF_{2\alpha}$ dose and milk production was inversely associated with this response, whereas an increased $PGF_{2\alpha}$ dose improved the estrus response. Therefore, an increase in the standard $PGF_{2\alpha}$ dose is recommended.
Key words: Double ovulation, Luteolysis failure, Prostaglandin analogues

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The dose-response effect for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its analogues required for synchronizing estrus was established in the 1970s: $PGF_{2\alpha}$, 25 mg; cloprostenol, 500 µg; and fenprostalene, 1 mg [1, 2]. These luteolytic compounds shorten the luteal phase and are only effective in the presence of a functional corpus luteum (plural corpora lutea; CL), during days 5 to 16 of a normal estrus cycle [3]. Fertility during the induced estrus may be similar to [1, 2] or higher than that during spontaneous estrus [4]. The luteolytic doses used in dairy cattle have not been modified in the past 50 years, during which milk production has significantly increased [5]. However, given the higher metabolic clearance rates of steroid hormones and changes in the reproductive endocrinology of cows linked to high milk production [6–8], exogenous prostaglandins are also likely to undergo faster clearance or elicit a lower response than expected, even as milk production shows an increase. It is already known that endogenous $PGF_{2\alpha}$ on reaching the pulmonary circulation is quickly inactivated in the lungs [9]. The clinical implications of increasing the luteolytic dose of $PGF_{2\alpha}$ have also been recently discussed [10]. Multiple ovulations were found to be associated with high milk production, which was highlighted as a primary issue this study. In fact, the incidence of double ovulations in high milk-producing cows may be greater than 20% [11–14], whereas an increased twinning rate of up to 9% was linked to increased milk production in an epidemiological study on 52,362 lactations [15]. In addition, it is likely that multiple ovulations are partly a consequence of gene selection for milk production. In an analysis of data related to 1,324,678 offspring of 37,174 dams, it was shown that dams born after 1990 had a higher incidence of twins than those born before 1980 [16]. The present study evaluated the dose-response effect of $PGF_{2\alpha}$ and subsequent fertility in cows with two or more CL.

Materials and Methods

Cattle and herd management

This study was performed on a commercial Holstein-Friesian dairy herd in northeastern Spain (41.13 latitude, 0.24 longitude). Cows were included if they were healthy, with a 2.5–3.5 body condition score on a scale of 1 to 5 [17], produced more than 25 kg of milk per day, and were free of any detectable reproductive disorders and clinical diseases during the study period (days –7 to + 28–34 from insemination). During the study period (January 2009 to April 2016), the mean number of lactating cows in the herd was 1,028 and the mean annual milk production was 11,965 kg per cow. The mean annual culling rate was 29%. Cows were grouped according to number of pregnancies (primiparous *vs*. pluriparous), milked three times daily, and fed complete rations. The feed consisted of cotton seed hulls, barley, corn, soybean, bran, and roughage, which primarily included corn, barley, alfalfa silages, and alfalfa hay. Rations were in line with National Research Council (NRC) recommendations [18]. Dry cows were kept separately and were marked as the "parturition group" 7–25

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days before the expected date of calving, depending on whether or not they were carrying twins [19]. Herd management included the use of fans and water sprinklers. Fans were placed throughout the cubicle and feeding areas, whereas water sprinklers were set up in the feeding area with the water spray directed towards the cows. Fans and water sprinklers were automatically activated when the temperature reached approximately 23°C and 25°C, respectively. All animals were reared within the herd.

Detection of estrus, insemination, and diagnosis of pregnancy

Estrus was detected using a pedometer system (AfiFarm System; SAE Afikim). Walking activity values were recorded at the milking parlor (three times daily) and analyzed automatically using a herd management computer program. Walking activity greater than 80% of the mean activity recorded in the previous two days was taken as the lower limit for a cow to be considered in estrus [20]. If estrus was suspected, it was confirmed by palpation of the rectum using the pedometer system, following which the animals were inseminated. All cows were artificially inseminated, and the herd was maintained on a weekly reproductive health program, as previously described [20, 21]. Briefly, the reproductive tract of each animal was examined by ultrasound to check for normal uterine involution and ovarian structures at the end of the voluntary waiting period on days 30–36 postpartum. Reproductive disorders diagnosed at this time such as endometritis or ovarian cysts were treated until resolved. During the weekly reproductive visit, open cows with more than 60 days in milk (DIM) with no signs of estrus for at least 21 days and in the absence of a CL received a five-day progesterone-based synchronization protocol for fixed-time artificial insemination (FTAI) [21]. If a cow had one or more CL with or without a cavity estimated to be at least 15 mm in diameter (mean of the maximum and minimum measurements), the cow was treated with $PGF_{2\alpha}$. The cows that failed to show signs of estrus during the seven days following the FTAI protocol. Cows with a small CL were either inseminated following a spontaneous estrus during the 7-day period between the exams or at the next visit. All cows were inseminated by one of four technicians with frozen-thawed semen from 32 bulls. Sex-sorted sperm was not used during the study period. If a cow returned to estrus, its status was confirmed by a rectal examination, and the animal was recorded as non-pregnant. In the remaining cows, pregnancy diagnosis was performed by ultrasound 28–34 days post-AI. The final study population consisted of cows with one or more CL receiving $PGF_{2\alpha}$ treatment (n = 2436 treatments on 1948 cows).

All gynecological examinations were performed by the author using transrectal ultrasonography with a portable B-mode ultrasound scanner equipped with a 7.5 MHz transducer (Easy Scan, BCF™ Technology Ltd., Scotland, UK) between 2009 and 2012 and with a 5–10 MHz transducer (E.I. Medical IBEX LITE; E.I. Medical Imaging, Loveland CO, USA) from 2013 to 2016. Each ovary was scanned in several planes by moving the transducer along the surface of the corresponding body region to identify luteal structures, and the number and location of corpora lutea were recorded. Scanning was also performed along the dorsolateral surface of each uterine horn for diagnosing pregnancy.

Experimental design

During the reproductive visit, cows with one CL were treated with a single $PGF_{2\alpha}$ dose (25 mg dinoprost intramuscular ; Enzaprost, CEVA Santé Animale, Barcelona, Spain) and assigned to the group 1CL (control cows; n = 1,683). Cows with two or more CL were chronologically assigned to the following groups: 2CL1, treated with a single $PGF_{2\alpha}$ dose; or 2CL1.5, treated with a 1.5 × $PGF_{2\alpha}$ dose (37.5 mg dinoprost). For every two cows included in the 2CL1 group (n = 501), one cow was included in the 2CL1.5 group (n = 252). All procedures were approved by the Ethics Committee on Animal Experimentation of the University of Lleida.

Data collection and statistical analysis

Estrus response rate was defined as the percentage of cows (of total number of cows in the group), showing estrus and pregnancy rate was defined as the percentage of cows (of total number of cows in the group) that became pregnant after AI. The following data were recorded for each animal: parturition and treatment dates; lactation number (parity; primiparous *vs*. pluriparous); number of CL; treatment (1CL, 2CL1, or 2CL1.5); milk production at treatment (mean production during the three days before treatment; low producers < 45 kg *vs*. high producers ≥ 45 kg); DIM at treatment (DIM; < 90 days postpartum *vs*. ≥ 90 days postpartum); estrus response after $PGF_{2\alpha}$ treatment (presence *vs*. absence); and days from treatment to AI (continuous), AI technician, semen-providing bull, and pregnancy 28–34 days post-AI for cows showing estrus. The threshold for milk production was derived from the median value of the production of primiparous cows. The threshold value of 90 DIM was useful in a previous study on possible factors influencing double ovulation [12]. Treatment dates were used to assess the effects of season on subsequent reproductive performance. In the study geographical region, there are only two clearly differentiated weather periods: warm (May to September) and cool (October to April) [22, 23].

The overall reproductive performance of the three treatment groups was evaluated using the chi-square test (percentages). The effects of treatment on the interval (in days) from $PGF_{2\alpha}$ treatment to estrus was assessed by ANOVA and Tukey post hoc tests.

The effects of treatment on rates of estrus response and pregnancy were analyzed using binary logistic regression. A total of four regression analyses were performed using the estrus response after $PGF_{2\alpha}$ treatment as the dependent variable for all cows and then for each treatment group. Further, binary logistic regression was performed using pregnancy 28–34 days post-AI as the dependent variable. The factors entered in the models as independent variables were: year and season of treatment, parity, milk production, and DIM at treatment. For the dependent variables of estrus response for all cows and pregnancy rate for inseminated cows, the treatment was added as an independent variable. For the dependent variable of pregnancy rate, days from $PGF_{2\alpha}$ treatment to AI (categorical variable), AI technician, and semen-providing bull were added as independent variables, and only inseminated cows were included in the analysis. Possible interactions between treatment and the variables of season and parity and between CL number and milk yield were also examined. For the dependent variables of estrus response in cows with two or more CL (groups 2CL1 and 2CL1.5), the presence of three or more CL was added as a factor. Regression analyses were conducted according to the Hosmer and Lemeshow method [24] using the PASW Statistics for Windows Version 18.0 (SPSS Inc., Chicago, IL, USA). Significance was set at $P < 0.05$. Values were expressed as mean ± standard deviation (S.D.).

Results

Among the 2,436 cows included in the study, 1,683 (69.1%) had one CL and 753 (30.9%) had two or more CL: 718 (95.4%), 2 CL; 32 (4.2%), 3 CL; and 3 (0.4%), 4 CL. Mean milk production at the time of treatment, DIM at AI, and number of lactations were 47.4 ± 9.9 kg (26–86 kg), 112 ± 73 days (48–554 days), and 2.9 ± 1.7 lactations (1–9 lactations), respectively (mean ± SD, ranges between parentheses). The independent variables recorded for each of the three treatment groups and the effects of the different treatments on each dependent variable are shown in Table 1. Estrus was detected in 1,532 cows (62.9%), all of which were inseminated, with 645 (42.1%) becoming pregnant following AI. Estrus response rate showed a significant decrease ($P < 0.0001$) in the 2CL1 group (15.6%) and a significant increase ($P < 0.001$) in the 2CL1.5 (82.9%) group compared to the 1CL group (74%). The conception rate was significantly higher ($P < 0.01$) in the 2CL1.5 group (53.6%) than in the 1CL group (40%). Cows with a single CL (n = 1245) showed estrus significantly ($P < 0.01$) earlier (3.01 ± 1.23 days) than cows with two or more CL (n = 287; 3.33 ± 1.69 days).

Table 2 summarizes the estrus response rate, odds ratio (OR), and 95% confidence interval for all cows with two or more CL at the time of PGF2α treatment. The final model included the effect of the warm period of the year and treatment. Year, parity, milk production, and DIM were not significant and were not included in the final model. Associations between treatment and season, treatment and parity, or CL number and milk yield were not found. The OR for estrus response was 0.5 ($P = 0.004$) during the warm period of the year. Using 1CL cows as reference, the ORs for estrus response of 2CL1 and 2CL1.5 cows were 0.13 ($P < 0.0001$) and 1.8 ($P = 0.0001$), respectively.

For the analysis of each treatment group, the final model included the effect of season and milk production, even if there was no significant effect of these variables. On analyzing 1CL cows, it was found that the effect of the warm period of the year was the only factor influencing the estrus response rate with an OR of 0.5 ($P < 0.001$) for cows treated during this period (Table 2). In 2CL1 cows, the OR for the estrus response was 0.7 ($P = 0.01$) for cows producing ≥ 45 kg of milk at treatment, compared to the remaining cows producing < 45 kg of milk (Table 2). No factors could be associated with the estrus response rate of 2CL1.5 cows. In 2CL1 and 2CL1.5 cows, the presence of three or more CL was not significant. Among the 22 cows with three or more CL (21 cows with three CL and one cow with four CL) in the 2CL1 group, estrus was detected in two cows with three CL, whereas among the 13 cows with three or more CL (11 cows with three CL and two cows with four CL) in the 2CL1.5 group, estrus was detected in eight cows with three CL and two cows with four CL.

As seen in Table 3, the OR for pregnancy of pluriparous cows was 0.7, compared to primiparous cows ($P = 0.009$), and was 1.7 for 2CL1.5 cows, compared to 1CL cows ($P = 0.001$). Year, milk production, DIM, days from $PGF_{2\alpha}$ treatment to estrus, AI technician, and sire were not significant and were not included in the final model. No interactions were found between the treatment and the variables of season and parity.

Discussion

To the best of our knowledge, this study is the first to examine the estrus response and subsequent fertility of lactating cows with two or more CL subjected to $PGF_{2\alpha}$ treatment. The following findings were pertinent in this study: 1) the interval from treatment to estrus was increased in cows with two or more CL, with no effect on fertility; 2) the presence of two or more CL significantly reduced the estrus response to that induced by a single $PGF_{2\alpha}$ dose, particularly in high milk producers; and 3) 1.5 × $PGF_{2\alpha}$ dose increased both the estrus response and fertility of cows with two or more CL, compared with cows with a single CL that received a single $PGF_{2\alpha}$ dose.

Table 1. Independent variables recorded at treatment and effects of the different treatments [a] on each dependent variable (n = 2,436)

	1CL (n = 1,683)	2CL1 (n = 501)	2CL1.5 (n = 252)
Independent variables [b]			
Parity (pluriparous)	1301 (77.3%)	406 (81%)	212 (84.1%)
Milk production (≥ 45 kg)	941 (55.9%)	302 (60.3%)	153 (60.7%)
Days in milk (≥ 90 days)	976 (58%)	306 (61.1%)	151 (59.9%)
Season (warm period: May–September)	572 (34%)	160 (31.9%)	81 (32.1%)
Three or four CL		22 (4.4%)	13 (5.2%)
Dependent variables [c]			
Estrus response [d]	1245/1683 (74%) *	78/501 (15.6%) **	209/252 (82.9%) ***
Conception rate [e]	498/1245 (40%) *	35/78 (44.9%) *,**	112/209 (53.6%) **
Days (mean ± SD) [f]	3.01 ± 1.23 *	3.47 ± 1.69 **	3.28 ± 1.25 **

[a] 1CL: cows with one corpus luteum (CL) receiving a single $PGF_{2\alpha}$ dose (25 mg dinoprost); 2CL1: cows with two or more CL receiving a single $PGF_{2\alpha}$ dose; 2CL1.5: cows with two or more CL receiving a 1.5 × $PGF_{2\alpha}$ dose (37.5 mg dinoprost). All cows showing estrus were inseminated. [b] No significant differences were detected using the chi-square test. [c] Values with different superscripts within rows denote significant differences detected using the chi-square test (percentages) or ANOVA and Tukey post-hoc tests (means ± SD). [d] In all cows (*–**, **–***: $P < 0.0001$; *–***: $P < 0.001$). [e] In inseminated cows (*–**: $P < 0.01$). [f] Days from $PGF_{2\alpha}$ treatment to estrus, ranging from 1 to 7 days (*–**: $P < 0.01$).

Table 2. Odds ratios for estrus response rate calculated using the final logistic regression model for all cows (n = 2,436) [a]; for 1CL cows (n = 1,683) [b]; for 2CL1 cows (n = 501) [c]; and for 2CL1.5 cows (n = 252)

Factor	Class	n	% Estrus response	Odds ratio	95% Confidence interval	P
All cows [a]						
Season [d]	Cool	1087/1623	67	Reference		
	Warm	445/813	54.7	0.5	0.3–0.8	0.004
Treatment [e]	1CL	1245/1683	74	Reference		
	2CL1	78/501	15.6	0.13	0.08–0.2	< 0.0001
	2CL1.5	209/252	82.9	1.8	1.2–2.5	0.0001
1CL cows [b]						
Season [d]	Cool	858/1111	77.2	Reference		
	Warm	366/572	64	0.5	0.4–0.8	< 0.001
Milk production	< 45 kg	543/742	73.2	Reference		
	≥ 45 kg	702/941	74.6	1.1	0.7–1.2	0.3
2CL1 cows [c]						
Season [d]	Cool	55/341	16.1	Reference		
	Warm	23/160	14.4	0.9	0.8–1.2	0.5
Milk production	< 45 kg	40/199	20.1	Reference		
	≥ 45 kg	38/302	12.6	0.7	0.4–0.9	0.01
2CL1.5 cows						
Season [d]	Cool	143/171	83.6	Reference		
	Warm	66/81	81.5	0.7	0.5–1.1	0.7
Milk production	< 45 kg	81/99	81.8	Reference		
	≥ 45 kg	128/153	83.7	1.1	0.8–1.4	0.5

[a] Hosmer and Lemeshow goodness-of-fit = 26.6; 3 df; P = 0.93. R^2 Nagelkerke = 0.67. [b] Hosmer and Lemeshow goodness-of-fit = 25.6; 3 df; P = 0.90. R^2 Nagelkerke = 0.10. [c] Hosmer and Lemeshow goodness-of-fit = 25.6; 3 df; P = 0.90. R^2 Nagelkerke = 0.10. [d] Cool period: October–April; warm period: May–September. [e] 1CL: cows with one CL receiving a single $PGF_{2\alpha}$ dose (25 mg dinoprost); 2CL1: cows with two or more CL receiving a single $PGF_{2\alpha}$ dose; 2CL1.5: cows with two or more CL receiving a 1.5 × $PGF_{2\alpha}$ dose (37.5 mg dinoprost).

Table 3. Association between pregnancy and independent variables included in the final logistic regression model for inseminated cows (n = 1532) [a]

Factor	Class	n	% Pregnancy	Odds ratio	95% Confidence interval	P
Parity	Primiparous	175/363	48.2	Reference		
	Pluriparous	470/1169	40.2	0.7	0.5–0.9	0.009
Treatment [b]	1CL	498/1245	40	Reference		
	2CL1	35/78	44.9	1.2	0.8–2.0	0.3
	2CL1.5	112/209	53.6	1.7	1.3–2.4	0.001

[a] Hosmer and Lemeshow goodness-of-fit = 27.6; 3 df; P = 0.91. R^2 Nagelkerke = 0.09. [b] 1CL: cows with one CL receiving a single $PGF_{2\alpha}$ dose (25 mg dinoprost); 2CL1: cows with two or more CL receiving a single $PGF_{2\alpha}$ dose; 2CL1.5: cows with two or more CL receiving a 1.5 times $PGF_{2\alpha}$ dose (37.5 mg dinoprost).

Stages of the estrus cycle and differences in the size of the dominant follicle influence the duration of the interval from $PGF_{2\alpha}$ treatment to estrus, which mostly ranges from two to five days [1, 2, 4]. However, the study results may indicate that another factor affects this interval. Cows with multiple CL at treatment may show an increase in the mean interval to estrus. This delay to display estrus would be expected; the incidence of multiple ovulations in heifers is very low [7], and the interval to estrus after $PGF_{2\alpha}$ is significantly shorter for heifers than for cows [25]. Similarly, the percentages of double ovulation for first, second, and third or more lactations were 6.7%, 16.6%, and 25%, respectively, in an extensive study on 1917 inseminations in the same geographical area [12]. Therefore, an increased mean interval to estrus following treatment in older cows, which show a higher incidence of multiple ovulations, should be expected.

The estrous response was very high (82.9%) for 2CL1.5 cows and very low (15.6%) for 2CL1 cows, compared to 1CL cows (74%). This highly significant effect of the number of corpora lutea and treatment could mask possible interactions. This was why the analysis was performed for each group after completing the analysis for all cows, despite no significant interactions between treatment and season as well as treatment and milk production. These subsequent analyses revealed the significant effects of season (1CL group) and milk production (2CL1 group).

The presence of two or more CL significantly reduced ($P < 0.0001$) the responsiveness to that induced by a single $PGF_{2\alpha}$ dose. This low response could be further exacerbated by increased milk production. However, the underlying mechanisms for these results were not explored herein, but could be associated with the metabolic stress placed upon high-milk producing dairy cows when luteolytic processes are induced. Such metabolic stress would be expressed as an endocrine imbalance, compromising full luteolysis and subsequent estrus response. However, the $1.5 \times PGF_{2\alpha}$ dose proposed here had positive impacts on both the estrus response and fertility. Increased fertility of cows with multiple CL is easier to interpret. Cows show significant recurrence of multiple ovulations [26, 27], which has been linked to higher fertility [11, 28]. Regarding the estrus response, an increased $PGF_{2\alpha}$ dose reduced other types of stress, such as heat stress, aside from the metabolic stress linked to increased milk production. Any factors that influenced the very high estrus response (82.9%) could not be identified when the analysis included only the 2CL1.5 cows, whereas the analysis of the 1CL cows revealed that the estrus response was significantly lower ($P < 0.0001$) during the warm period. Additionally, it was important to note that an increased dose may favor luteal regression of a single minimally responsive CL such as a young CL [29–31]. The fact that a second $PGF_{2\alpha}$ treatment 24 hours after the first used in breeding synchronization protocols improves luteal regression and subsequent fertility reinforces this assertion [32].

Cows are predominantly monovular, but over the last 30 years, the incidence of multiple ovulations has considerably increased, and it is expected that in the coming years the multiple ovulation rate will increase in parallel to milk production [7, 26, 33]. Therefore, the recommended luteolytic dose of $PGF_{2\alpha}$ in dairy cattle should be increased [10]. Our results suggest that the presence of multiple CL reduces the luteolytic response to that induced by a single $PGF_{2\alpha}$ dose, particularly in high milk producers, and increases the interval from treatment to estrus, whereas an increased $PGF_{2\alpha}$ dose improves the estrus response.

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