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ABSTRACT

Luteolysis is a key event in cattle reproduction. A standard dose of exogenous PGF2α (PGF) will induce full luteolysis in the majority of cows with a matured corpus luteum (CL). However, this will not in cows with a CL < 5 days old. To date it is not known whether a larger dose will have a more potent luteolytic effect in cows during early diestrus. The objective of this study was to characterize the effect of two doses of d-cloprostenol (150 and 300 µg) on the progesterone concentration, luteal diameter and ovulation rate in non-lactating dairy cattle 96 to 132 h post-ovulation. Twenty non-lactating dairy cows were included in the study. Each cow received two treatments of d-cloprostenol in two consecutive cycles: a standard dose of 150 µg and a double dose of 300 µg. The cows were allocated randomly to one of four groups (five cows in each group) according to the age of the CL at the time of treatment: 96, 108, 120 and 132 h. The exact time of ovulation was known within 12 h, because of twice a day ultrasound examination. CL diameter and progesterone concentration were measured before treatment (day 0) and 2 and 4 d after treatment. Within each CL age group, the effect

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of d-cloprostenol dose on luteolysis was determined. More cows treated with double dose tended to have full luteolysis compared with the standard dose (8/10 vs. 4/10, respectively; $P = 0.07$). This effect was only apparent in cows with CLs of 120 and 132 h but not in earlier CLs. The interval from treatment to ovulation was shorter (3.3 ± 0.1 days) in cows treated with double dose than in cows treated with standard dose (4.5 ± 0.4 days) ($P < 0.05$).

INTRODUCTION

The corpus luteum (CL) of cattle is refractory to a single treatment of exogenous native $PGF_{2\alpha}$ (25 mg of dinoprost, manufacturer's recommended dose) within the first four days of the estrous cycle (estrus = day 0) (Beal et al., 1980). Even one day later (Day 5 of the estrous cycle), a similar dose of $PGF_{2\alpha}$ (PGF) failed to cause full luteolysis in treated heifers and cows (Rowson et al., 1972; Henricks et al., 1974). As a result, most PGF-based protocols of ovulation and estrous synchronization in cattle allow at least a period of 5.5 days between the previous ovulation and the administration of PGF (Ovsynch protocol, Pursley et al., 1995) so that the presence of a responsive CL is assured in cows allocated to this type of protocols. However, there is still a percentage of cows (depending on the study 5 to 20%) that fails to undergo full luteolysis following the PGF treatment of the ovsynch protocol (Moreira et al., 2000; Gümen et al., 2003; Brusveen et al., 2009).

An earlier luteolytic response can be obtained by increasing the frequency of PGF administration from one (single treatment) to three treatments 12 h apart (Beltman et al., 2009). In addition, an extra PGF administration 24 h later also increases the percentage of cows with full luteolysis (from 85 to 96%) after the initial PGF treatment of the ovsynch protocol (Brusveen et al., 2009). This phenomenon is relevant to cattle reproduction, since cows with incomplete or partial luteolysis 2 d after PGF treatment have reduced chances of becoming pregnant after fixed time AI in an ovsynch protocol (Souza et al., 2007). Unfortunately, this protocol increases the labor costs resulting from the additional handling of animals required to administer the second PGF treatment.

In addition to the age of the CL and the frequency of the luteolytic treatment, the dose of the luteolytic agent appears to be a factor that may influence the degree of luteolysis. Evidence shows that full luteolysis is achieved in more mares in early diestrus following a single treatment of 500 µg of cloprostenol than after a standard dose of 250 µg (Cuervo-Arango and Newcombe, 2011). Similarly, the administration of 50 mg of dinoprost to dairy cows 3.5 days post-ovulation induced full luteolysis in 22% of treated cows (Cuervo-Arango et al., 2011), time at which the CL is expected to be refractory to a single standard treatment of PGF (Beal et al., 1980). However, to date no study has compared specifically the dose rate effect of a luteolytic agent on the degree of luteolysis in dairy cattle in early diestrus with different CL ages.

The objective of this study was to characterize the effect of two doses of d-cloprostenol (150 and 300 µg) on the progesterone concentration, luteal diameter and ovulation in non-lactating dairy cattle 84 to 132 h post-ovulation. The main hypothesis tested was that the larger dose would induce a greater decrease in progesterone concentration and luteal diameter 2 and 4 d after treatment.

MATERIAL AND METHODS

Animals

This trial was conducted from May to June 2011 at the Veterinary School Research Farm of the Universidad CEU Cardenal Herrera in Naquera, Spain (northern hemisphere). All animal procedures were handled in accordance with the Spanish Department of Agriculture Guide for Care and Use of Animals in Research, and they were approved by the Animal Welfare Committee of the Universidad CEU Cardenal Herrera.

A total of twenty non-lactating Holstein cows were used in the trial. The cows had been dry for at least two years. The cows' age ranged from four to 10 years (mean age of 6.7 ± 1.9 years). There were five nulliparous and 15 multiparous cows. At the beginning of the trial all animals were cyclic and had no apparent uterine or ovarian abnormalities confirmed by ultrasonography. The cows were fed alfalfa hay and cereal concentrate ration calculated for a maintenance diet for dry cows. The mean body condition score was 3.5 ± 0.6 (range 2.5 to 4, scale 1 to 5) and the mean weight was 645 ± 47 kg (range 570 to 680 kg). All the PGF (d-cloprostenol) injections were administered with single-dose syringes in semimembranosus or semitendinosus muscles with 18-gauge 3.5-cm needles.

Experimental design

All cows were administered 25 mg dinoprost (Enzaprost; CEVA Salud Animal S.A. Barcelona, Spain) 14 days apart. The day beginning after the second dinoprost administration, the cows were scanned by rectal ultrasonography every 12 h for the detection of ovulation with an ultrasound scanner (Sonosite 180 Vet Plus; BCF Ultrasound Australasia, Nunawading, VIC, Australia) equipped with an 8-MHz linear-array transducer. Ovulation was detected as per the disappearance of the previously recorded preovulatory follicle and confirmed by the later development of a corpus luteum. Once a cow had ovulated, she was randomly allocated to one of the four CL age groups: A) 84 to 96 h (n =5); B) 96 to 108 h (n = 5); C) 108 to 120 h (n = 5); and D) 120 to 132 h (n = 5). For simplicity the CL age groups are referred to as the oldest possible age (96, 108, 120 and 132 h). The study followed a cross over design: for each CL age group, every cow received two treatments of d-cloprostenol (Dalmazin; Fatro Iberica S.L., Barcelona, Spain) during two consecutive cycles. The treatments consisted of either a standard dose (recommended by the manufacturer) of 150 µg of d-cloprostenol (2 ml of Dalmazin) or a dose of 300 µg of d-cloprostenol (4 ml of Dalmazin; double of the manufacturer’s recommended dose) intramuscularly. After each treatment, the cows were scanned every 12 h until an ovulation was detected or until 7 days later, whatever happened first. If the cow had not ovulated within 7 days of treatment, she was administered 25 mg of dinoprost, and scanned for detection of ovulation every 12 h. After the consecutive ovulation, each cow received the remaining treatment dose of d-cloprostenol at the same interval post-ovulation that during the previous cycle and scanned daily for 7 d. Whether a cow received the standard or the double dose in the first or second cycle was chosen randomly.

Endpoints analyzed

The endpoints analyzed were CL diameter and progesterone concentration just before (Day 0), 2 and 4 d after treatment. And whether the cow ovulated or not spontaneously (no treatment to induce ovulation was used in the study) within 7 d of treatment. If a cystic structure (fluid-filled ovarian structure > 2 cm in diameter) was present 7 d post-treatment, an extra blood sample was taken for progesterone determination and cyst classification: a cow with a luteal cyst had progesterone concentration > 1 ng/mL, whereas < 1 ng/mL with a follicular cyst.

For progesterone analysis, a blood sample was taken from the tail vein on each occasion (0, 2 and 4 d after treatment), in 5 mL heparinized tubes. The tubes were immediately centrifuged during 10 min at 2000 x g. Aliquots of plasma were stored at –20 ºC for later assay determination. Concentrations of plasma progesterone were measured in a single assay, using enzyme-immunoassay kits (Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany) with a sensitivity of 0.04 ng/mL and an intra-assay variation coefficient of 5%. A cow was classified as having full luteolysis when the progesterone concentration was below 1 ng/mL 4 d post-treatment.

The CL diameter was calculated by the average of two measurements taken at right angles with the electronic calipers when the frozen image of the CL was maximum.

Statistical analyses

For each CL age group, a general linear model of variance with a repeated statement to account for autocorrelation between sequential observations of same individuals (Systat 13; Systat Software Inc., Chicago, IL, USA) was performed. Two models were created, one for progesterone concentration and another for CL diameter. Each model had the same two fixed factors: dose of d-cloprostenol (two levels: standard and double dose) and day relative to treatment (repeated observations: on days 0, 2 and 4 after treatment). If an effect of dose (standard *vs*. double) or an interaction of dose and day were or approached significance, data were examined further by student *t*-test.

In cows with full luteolysis, the intervals from treatment to ovulation of cows receiving the standard dose were compared with those treated with the double dose by unpaired student *t*-test. Frequency data (number of cows with full luteolysis) were analyzed by Fisher's exact test.

A probability of $P < 0.05$ indicated that a difference was significant and probabilities between $P > 0.05$ and $P < 0.1$ indicated that a difference approached significance. Data are presented as mean ± SEM, unless stated otherwise.

RESULTS

No cow ovulated in the two earliest CL age groups (96 and 108 h). However, one cow treated with double dose of d-cloprostenol had full luteolysis followed by the development of a luteal cyst (Table 1). In these two groups, the dose of d-cloprostenol had no effect on the progesterone concentration (Fig. 1; $P > 0.05$). However, the double dose of d-cloprostenol tended to induce a greater decrease in the post-treatment CL diameter in cows treated 108 h post-ovulation. In addition, in this group there was a significant effect of dose by day interaction on the CL diameter. This resulted from a slower increase 2 and 4 d post-treatment in the CL diameter of cows treated with the double dose (Fig. 1).

In the two oldest CL groups (120 and 132 h), the double dose of d-cloprostenol tended ($P = 0.07$) to induce full luteolysis in more cows (8/10) than the standard dose (4/10) (Table 1). Two cows with full luteolysis from the double dose groups did not ovulate but developed cystic ovaries (Table 1). Overall, four and six cows ovulated after treatment with the standard and double dose of d-cloprostenol, respectively ($P > 0.05$). In these ovulating cows, the interval from treatment to ovulation was shorter ($P < 0.05$) in those treated with double dose (3.3 ± 0.1 days) than in cows administered the standard dose (4.5 ± 0.4 days).

For the CL age group of 120 h, the double dose of d-cloprostenol tended to induce a greater decrease in the post-treatment progesterone concentration and CL diameter (Fig. 1; $P < 0.1$). There was an effect ($P < 0.05$) of dose by day interaction on the CL diameter. For the oldest CL group (132 h), the double dose induced a greater decrease in the post-treatment progesterone concentration ($P < 0.05$) and CL diameter ($P < 0.1$; Fig. 1).

For cows treated with double doses of d-cloprostenol, the difference in progesterone concentration between 0 and 2 d post-treatment and with a CL of 120 and 132 h old (1.8 ± 0.59 and 1.7 ± 0.47 ng/mL, respectively) was greater ($P < 0.05$) than that of cows with CLs of 108 and 96 h old (−0.39 ± 0.7 and −0.31 ± 0.1 ng/mL, respectively) (Table 2).

In cows with a CL of 132 h old, the progesterone concentration continued to decrease between 2 and 4 d after a treatment with double dose, but not in cows treated with the standard dose (mean difference in progesterone concentration between 2 and 4 d post-treatment of 0.2 ± 0.1 and −0.6 ± 0.4 ng/mL for double and standard dose groups, respectively; P = 0.09, Table 2).

DISCUSSION

The main hypothesis tested that a larger dose of PGF would induce a greater decrease in progesterone concentration and luteal diameter 2 and 4 d after treatment can be accepted only partially. The larger dose (300 µg) of d-cloprostenol was superior to the standard dose (150 µg) in terms of reducing progesterone concentration within four days of treatment, only in cows with a CL aged between 108 and 132 h (4.5 and 5.5 days) but not earlier. A 12 h gap in the CL age was sufficient to influence whether the dose of d-cloprostenol had an effect on the percentage of cows with full luteolysis. Therefore, the main strength of this study was that the cows were scanned for detection of ovulation every 12 h. This allowed having groups of cows with relatively homogenous CL ages (between 0 and 12 h difference). This, in part overcame the expected individual variation within groups resultant from their relatively small sample size.

It appears that the effect of dose on progesterone concentration becomes more apparent as the CL gets older: the double dose had no effect in cows with a CL aged between 96 and 108 h, this became a tendency in 120 h and finally had a significant effect when the CL was 132 h old. This finding may have clinical implications since the cows enrolled in an ovsynch protocol have a CL aged between 132 and 144 h at the time of the PGF treatment (Pursley et al., 1995). In this line, a large field study with lactating dairy cows involving ovsynch-based synchronization programs with different doses of a luteolytic agent should be carried out. Then it would be possible to elucidate whether increasing the dose of PGF is beneficial to achieve full luteolysis in a greater proportion of cows and so improve pregnancy rates (Souza et al., 2007; Martins et al., 2011).

The CL's natural resistance to exogenous induced-luteolysis during early diestrus has been widely studied in ruminants (Tsai and Wiltbank, 1997; Tsai and Wiltbank, 1998; Skarzynski and Okuda, 1999; Mamluk et al., 1999; Silva et al., 2000; Sayre et al., 2000; Levy et al., 2000), and yet it is not completely understood. Possible explanations are reduced availability of endothelin-1 (Levy et al., 2000) and increased availability of prostaglandin dehydrogenase (Silva et al., 2000) in early CLs compared with mature CLs. Endothelin-1 is a proteinaceous vasoconstrictor and steroidogenic cell modulator produced by endothelial cells in response to PGF that alters progesterone production in cattle (Girsh et al., 1996), whereas prostaglandin dehydrogenase (PGDH) metabolizes PGF to its inactive form, 15-keto- $PGF_{2\alpha}$ in ewes (Silva et al., 2000). It is possible that providing a higher dose of exogenous PGF would override to some extent the anti-luteolytic effect resultant from the increased availability of PGDH and reduced concentration of ET-1 in the early CL.

The results of a similar study in mares (Cuervo-Arango and Newcombe, 2011), also showed a significant effect of d,l-cloprostenol dose on the percentage of mares with full luteolysis when it was administered at different stages of early diestrus. The difference became more apparent as the mares' CL became older. In the latter study, three doses of d,l-cloprostenol were attempted: 250 (standard), 500 and 750 µg. The double dose induced a greater percentage of mares with full luteolysis than did the standard dose but it was similar to that induced with 750 µg. This effect was only significant in mares with CLs aged 96 to 104 h but not earlier. Therefore, it seems that there is also a threshold upon which an increase in PGF dosage does not result in an equivalent luteolytic effect.

The CL morphology seemed to be affected by the dose of PGF to a greater extent and at an earlier stage than its functionality. The diameter of the CL and its ability to secret progesterone do not always correlate well (Herzog et al., 2010). In a recent study (Cuervo-Arango et al., 2011), the CL diameter was reduced to a greater extent and for longer than the progesterone concentration in cows with PGF-induced partial luteolysis compared with non-treated cows.

The reason why the interval between treatment and ovulation in cows treated with double dose was shorter than that of cows treated with the standard dose is unknown. A possible explanation might be that the greater reduction in progesterone concentration resultant from the larger dose, allowed a more rapid increase in LH with the subsequent advance in follicular maturation and ovulation. The negative effect that progesterone exerts on LH has been shown (Hannan et al., 2010). This theory on the increased LH concentration might also account for the greater occurrence of ovarian cystic degeneration in cows treated with a large dose of PGF at early stages of diestrus. This association between high dose of PGF and ovarian cyst development has been observed previously (Cuervo-Arango et al., 2011) and should not be left unnoticed.

CONCLUSIONS

Treatment with double dose of d-cloprostenol (300 µg) induced a greater proportion of full luteolysis in cows with a CL aged 120 to 132 h than the standard dose (150 µg). This effect was not apparent in cows with earlier CLs. The CL diameter tended to be smaller in cows treated with double than standard dose whet hay had a CL of 108 to 132 h old. A difference of as little as 12 h in the CL age was sufficient to influence the effect of d-cloprostenol dose on the CL morphology and functionality. Cows treated with double dose of d-cloprostenol ovulated a day earlier than ovulating cows treated with the standard dose.

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Table 1. Effect of d-cloprostenol dose and age of CL on ovulation and luteolysis

CL age (h)	d-CLO dose	% cows P4 < 1 ng/mL		% cows P4 < 0.5 ng/mL		Ovulating cows
		2 d	4 d	2 d	4 d	
96	standard	0.0	0.0	0.0	0.0	0/5
	double	0.0	0.0	0.0	0.0	0/5
108	standard	0.0	0.0	0.0	0.0	0/5
	double	20.0	20.0	0.0	20.0	0/5*
120	standard	20.0	20.0	0.0	20.0	1/5
	double	40.0	60.0	40.0	60.0	2/5[#]
132	standard	60.0	60.0	40.0	40.0	3/5
	double	80.0	100.0	60.0	80.0	4/5*

The CL age groups refer to the interval from ovulation to treatment (age of CL). 2 d and 4 d: blood samples were taken two and four days after treatment. Double and standard doses are 300 and 150 µg of d-cloprostenol, respectively. An asterisk (*) indicates that a cow developed a luteal cyst with progesterone concentration of > 1 ng/mL seven days after treatment. One cow developed a follicular cyst (#) with progesterone concentration < 1 ng/mL seven days after treatment.

Table 2. Effect of CL age and d-cloprostenol dose on rate of progesterone decrease over time

CL age (h)	d-CLO dose	P4: 0 d – 2 d (ng/mL)	P-value	P4: 2 d – 4 d (ng/mL)	P-value
96	standard	−0.6 ± 0.3	NS	−1.8 ± 0.8	NS
	double	−0.3 ± 0.1[a]		−1.9 ± 0.4	
108	standard	−0.6 ± 0.5	NS	−1.0 ± 1.1	NS
	double	−0.4 ± 0.7[a]		−0.5 ± 1.1	
120	standard	0.6 ± 0.9	NS	−0.8 ± 0.7	NS
	double	1.8 ± 0.6[b]		−0.2 ± 0.6	
132	standard	1.9 ± 0.6	NS	−0.6 ± 0.4	0.09
	double	1.7 ± 0.5[b]		0.2 ± 0.1	

The corpus luteum (CL) groups refer to the age of the CL in hours at the time of treatment with a double (300 µg) or standard (150 µg) dose of d-cloprostenol. P4 0 d – 2 d: difference in progesterone concentration between just before and two days after treatment. In this column, different letters (a, b) indicate that the rate of decrease is different (P < 0.05) in cows treated with double dose (effect of CL age). P4: 2 d – 4 d: difference in progesterone concentration between two and 4 days after treatment. Within CL age groups, the rate of progesterone decrease tended (P = 0.09) to be greater in cows with CLs of 132 h treated with double dose.

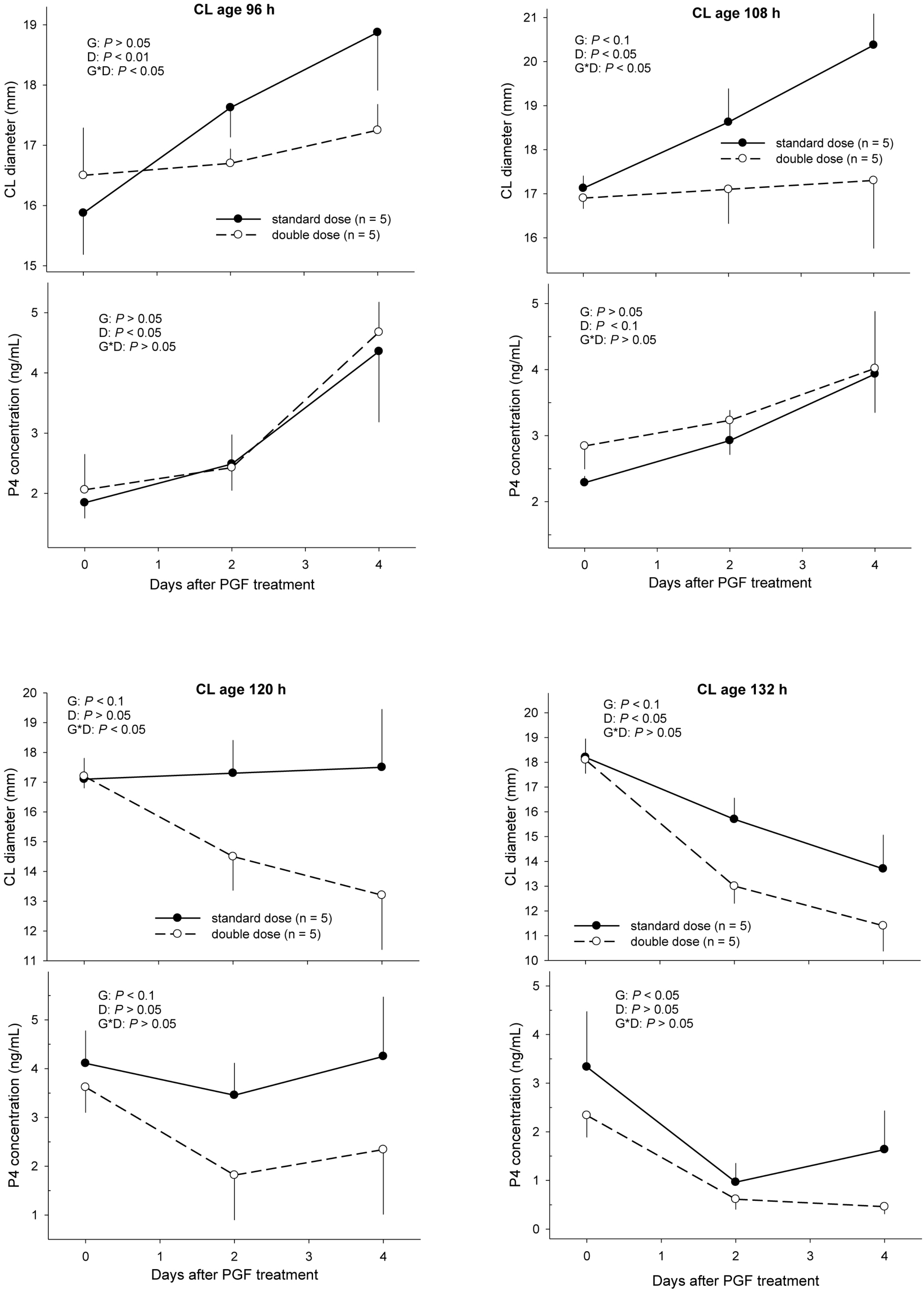
CL age 96 h
G: P > 0.05
D: P < 0.01
G*D: P < 0.05
CL diameter (mm)
standard dose (n = 5)
double dose (n = 5)
G: P > 0.05
D: P < 0.05
G*D: P > 0.05
P4 concentration (ng/mL)
Days after PGF treatment
CL age 108 h
G: P < 0.1
D: P < 0.05
G*D: P < 0.05
CL diameter (mm)
standard dose (n = 5)
double dose (n = 5)
G: P > 0.05
D: P < 0.1
G*D: P > 0.05
P4 concentration (ng/mL)
Days after PGF treatment
CL age 120 h
G: P < 0.1
D: P > 0.05
G*D: P < 0.05
CL diameter (mm)
standard dose (n = 5)
double dose (n = 5)
G: P < 0.1
D: P > 0.05
G*D: P > 0.05
P4 concentration (ng/mL)
Days after PGF treatment
CL age 132 h
G: P < 0.1
D: P < 0.05
G*D: P > 0.05
CL diameter (mm)
standard dose (n = 5)
double dose (n = 5)
G: P < 0.05
D: P > 0.05
G*D: P > 0.05
P4 concentration (ng/mL)
Days after PGF treatment

Figure 1. Effect of d-cloprostenol dose on progesterone concentration and corpus luteum (CL) diameter. The CL and progesterone data are arranged in four groups according to the age of the CL at the time of treatment (96, 108, 120 and 132 h). The double and standard doses correspond with 300 and 150 µg of d-cloprostenol, respectively. Within each CL age group, the probabilities for the effect of group (G: double *vs.* standard dose), day (D: 0, 2 and 4 d after treatment) and group by day interaction (G*D) are shown.