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Abstract: The ovaries of the cows were examined ultrasonographically during two consecutive estrous cycles. The study followed a crossover design with one treatment cycle in which the cows were treated with a single dose of 50 mg of dinoprost 3.5 days post-ovulation (day 0 = ovulation) and a control untreated cycle. Ultrasound examination and blood samples were performed during the two consecutive cycles. The frequency of examination was twice a day during the peri-ovulatory period to detect ovulation and once daily thereafter. Corpus luteum (CL) diameter and progesterone concentration were compared between control and treated cycles using a SAS mixed statistical procedure. Two of 9 cows (22%) developed full luteolysis with fall in progesterone to below 1 ng/ml within 2 days of PGF treatment and disappearance of a visible CL within 4 days. The remaining 7 (78%) cows during the treated cycle had partial luteolysis with a decrease ($P < 0.05$) in progesterone concentration and CL diameter for 2 and 12 days post-treatment compared with controls, respectively. The inter-ovulatory interval of treated cycles (19.7 ± 2.4 days) was not different ($P > 0.05$) from that of controls (23.8 ± 0.9 days). Two cows developed ovarian cystic degeneration during the PGF-induced cycle. There was no follicular cyst development during control cycles. In conclusion, the treatment of cows with a high dose of PGF 3.5 days post-ovulation induced some degree of luteolysis in all treated cows. This resulted in partial luteolysis with a transient decrease in progesterone concentration and a more prolonged decrease in CL diameter compared to control cycles in 78% of treated cows.

Regression and resurgence of the CL following a single high dose of PGF2α 3.5 days after ovulation in dairy cattle

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Abstract

The ovaries of the cows were examined ultrasonographically during two consecutive estrous cycles. The study followed a crossover design with one treatment cycle in which the cows were treated with a single dose of 50 mg of dinoprost 3.5 days post-ovulation (day 0 = ovulation) and a control untreated cycle. Ultrasound examination and blood samples were performed during the two consecutive cycles. The frequency of examination was twice a day during the peri-ovulatory period to detect ovulation and once daily thereafter. Corpus luteum (CL) diameter and progesterone concentration were compared between control and treated cycles using a SAS mixed statistical

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procedure. Two of 9 cows (22%) developed full luteolysis with fall in progesterone to below 1 ng/ml within 2 days of PGF treatment and disappearance of a visible CL within 4 days. The remaining 7 (78%) cows during the treated cycle had partial luteolysis with a decrease ($P < 0.05$) in progesterone concentration and CL diameter for 2 and 12 days post-treatment compared with controls, respectively. The inter-ovulatory interval of treated cycles (19.7 ± 2.4 days) was not different ($P > 0.05$) from that of controls (23.8 ± 0.9 days). Two cows developed ovarian cystic degeneration during the PGF-induced cycle. There was no follicular cyst development during control cycles. In conclusion, the treatment of cows with a high dose of PGF 3.5 days post-ovulation induced some degree of luteolysis in all treated cows. This resulted in partial luteolysis with a transient decrease in progesterone concentration and a more prolonged decrease in CL diameter compared to control cycles in 78% of treated cows.

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1. Introduction

The manipulation of the estrous cycle in the cow is in part dependant on the ability to terminate the lifespan of the corpus luteum (CL). This can be reliably achieved with a single administration of a luteolytic dose of PGF2α (PGF) on day 6 or more of the estrous cycle [1,2]. However, a single treatment with the manufacturer's recommended dose of PGF2α within the first 4 days is unable to induce luteolysis and regression of the CL [3-5]. Repeated injections of PGF at the recommended dose within the first 4 days of the estrous cycle [3,6,7] or a single dose on day 5 [1,5] reduced the mean progesterone concentration of treated heifers by inducing some degree of partial luteolysis.

The characterization of the luteolysis response of CLs from cattle in early stages of the estrous cycle is relevant to cattle reproduction. The development of protocols to induce research models with low progesterone in cattle have been attempted to study the effect of low progesterone on follicular development, fertility and embryo survival [6,7]. In addition, the success of synchronization protocols to manipulate the estrous cycle is limited to the full luteolytic effect of a single dose of exogenous PGF administered to cattle with a "responsive" mature CL [8].

The revision of different protocols on inducing luteolysis of cows with early CLs has provided evidence that CL responsiveness to PGF is dependent on the duration of the luteolytic stimuli (repeated treatments) and on the age of the CL. However, there is a lack of dose-response (amplitude of stimulus) studies which test the effect of different PGF dose rates at known ages of early CLs. To our knowledge all studies on luteolysis of early CLs have used the manufacturers' recommended dose for the native (25 mg dinoprost) or synthetic PGF (500 µg cloprostenol).

In other domestic species such as the mare, a single administration of the recommended dose of native PGF, three to 4 days post-ovulation, induced partial luteolysis with a significant reduction in progesterone concentration in all treated mares [9]. However, when the PGF was administered to mares at the double of the recommended dose at the same time period, more than 50% of treated mares developed full CL regression and estrous signs within 5 days of treatment [10]. In cattle, one study looked at the effect of two doses of PGF, 25 or 35 mg of dinoprost (recommended luteolityc dose of 25 mg) on progesterone concentration and CL cross sectional area [11]. There was no difference between doses, although the CLs of cows given the two doses of PGF were mature with > 17 mm in diameter. In other study, PGF treatment was administered to cows at random stages of the estrous cycle with CLs of unknown

ages but with progesterone of > 1 ng/ml [12]. In the latter study, cows treated with lower than the recommended dose of a synthetic PGF (cloprostenol, 125 µg) had a longer interval from treatment to estrus compared with cows treated with the recommended dose (500 µg) [12]. Therefore, it seems that not only the age of the CL but also the dose of PGF are critical factors that influence the luteolytic response of cows with early CLs. However this has not been tested critically. Probably because many studies defined day 0 of the estrous cycle as the day on which the cows or heifers showed signs of standing heat but not according to the day of ovulation [3,6,7]. Unfortunately the interval from the beginning of standing estrus to ovulation in dairy cattle is highly variable with a mean of 30 h and a range of 18.5 to 45.5 h [13]. Corpora lutea with a difference of more than 24 h in age are likely to respond differently to a similar dose of PGF in terms of luteolysis.

Evidence in other species shows that in early stages of the estrous cycle (less than 5 days), the CL sensitivity to an exogenous treatment of PGF is highly dependant on the age of the CL (measured from the point of ovulation). Such is the relevance of the CL age that a difference less than 24 h can increase significantly the luteolytic response rate of mares to the same PGF dose [10]. In the latter study, 500 µg of cloprostenol (double of the recommended luteolytic dose for mares) administered to mares with CLs aged 80 to 88 h induced full luteolysis in 58% of treated mares compared with a 100% response rate if the same dose was administered to mares with a CL aged 96 to 108 h [10].

The relationship between progesterone production and changes in luteal diameter after a PGF treatment have been investigated in cattle with mature CLs [11] but they have not in dairy cows with early CLs which are assumed to be unresponsive or to show partial luteolysis after a single dose of exogenous PGF. The present study

was design to examine the physiology and ultrasonic morphology of the CL in response to a single administration of native PGF 3.5 days after ovulation. The functional and structural regressive changes of the CL in treated cycles were compared to control untreated cycles in the same cows. In addition, the effect of PGF administration to cows with early CLs on the inter-ovulatory interval and on the incidence of anovulatory conditions was characterized.

2. Materials and Methods

2.1. Animals

Seven multiparous and two nulliparous Holstein cyclic cows aged 5.7 ± 2.2 (range 2.5 to 9) years old with no apparent uterine or ovarian diseases confirmed by ultrasonography were used in the study. The cows were part of the research herd of the Veterinary School of the Univeristy CEU-Cardenal Herrera. The multiparous cows had been dry for a period of ≥ 2 years. The cows were fed on alfalfa hay and cereal concentrate ration calculated for a maintenance diet for dry cows. The mean body condition score was 3.5 ± 0.7 (range 3 to 4, scale 1 to 5) and the mean weight was 647 ± 45.7 kg (range 580 to 690, calculated with a measuring tape). All animal procedures were handled in accordance with the Spanish Department of Agriculture Guide for Care and Use of Animals in Research.

2.2. Ultrasonography and heat detection

All cows were examined by transrectal B-Mode ultrasonography with a portable scanner ultrasound (Sonosite 180 Vet Plus®, Sonosite Inc. Australia) equipped with an 8 MHz linear-array transducer. At each examination, the ovaries were scanned and all follicles ≥ 3 mm in diameters were recorded. Follicular diameter was obtained from the

125 average of 2 linear measurements of the antrum taken at right angles when the image of
126 the follicle was maximum using the electronic callipers. Ovulation was detected as per
127 the absence of the previously recorded follicle within a given ovary and confirmed by
128 the later presence of a developing CL in the same ovary. All cows were examined twice
129 daily (at 8:00 and 20:00 h) for the detection of ovulation. The CL was measured
130 following the same technique from the average of 2 linear measurements of the cross
131 sectional surface of the CL's body at right angles (**Fig. 1**). Signs of standing heat were
132 detected by means of a heat detector aid (Estrotec®, Estrotec Inc., Madrid, Spain)
133 which was examined every 12 h (8:00 and 20:00 h).
134
135 *2.3. Experimental protocol*
136 The study was carried out between May 5th and July 6th 2010 in Valencia, Spain
137 (northern hemisphere). At the beginning of the study, all cows were administered 25 mg
138 dinoprost (Enzaprost®, CEVA Salud Animal S.A., Barcelona, Spain). Following the
139 PGF treatment, each cow was scanned once daily until they had follicles of ≥ 10 mm.
140 After that time each cow was scanned twice daily for detection of ovulation. Therefore
141 the exact time of ovulation was known with ± 6 h. Regardless of the hour of ovulation,
142 the day of ovulation (day 0) was set as the first day in which a cow had ovulated by 8:00
143 h in the morning.
144 The experimental protocol had a crossover design: each cow was followed for a
145 control and treatment cycle. Once a cow was diagnosed as having ovulated in the
146 morning of day 0 (8:00 h), she was administered 50 mg of dinoprost (Enzaprost®,
147 CEVA Salud Animal S.A., Barcelona, Spain) or left untreated on day 3.5 (at 20:00 h of
148 day 3). Therefore at the moment of PGF treatment, some cows (n = 5) had a CL aged 90
149 ± 6 h and the rest (n = 4) had a CL of 102 ± 6 h old. Before the PGF treatment each cow

was taken a blood sample and the CL was measured. Twelve hours after the PGF treatment (day 4 at 8:00 h) each cow was bled and the CL measured. Subsequently, CL measurements and blood samples were performed once daily until the next ovulation. In the control cycle, blood samples and CL measurements were performed at the equivalent time points. Only cows with a solid CL were used in the study. Whether the first cycle of each cow was a treated or a control cycle, was randomly chosen: five cows received the PGF treatment during the first cycle and 4 cows during the second cycle.

A full luteolysis occurred when the progesterone concentration decreased below 1 ng/ml within 48 h of treatment and remained < 1 ng/ml until a new ovulation took place. Partial luteolysis occurred when the progesterone concentration decreased by 12 h of treatment or the expected post-ovulatory rise was delayed, then it remained lower than that of the control cycle during a certain period of the luteal phase but then increased to values of untreated controls.

Two cows that were administered PGF in the first cycle did not have a control cycle since both of them developed cystic follicles (one with active production of progesterone). In these two cows the daily ultrasonographic examinations and blood collection were continued for 40 days after the PGF treatment.

2.4. Blood collection and progesterone determination

Blood samples were collected from the middle caudal vein into heparinised vacutainer 5 ml tubes. The tubes were immediately centrifuged during 10 min at 2000 ***g***. Aliquots of plasma were stored at –20 ºC for later assay determination.

Concentrations of plasma progesterone were measured in a single assay, by 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%.

2.5. Statistical analyses

Sequential data on progesterone concentration and CL diameter were analysed by SAS mixed procedure with a repeated statement to account for autocorrelation between sequential observations (Version 9.1.3; SAS Institute, Cary NC, USA). If an effect of group (control *vs*. treatment) or an interaction of group and day were significant, data were examined further by paired Student's *t*-test within days. Frequency data were analysed by Fisher's exact test: difference in full luteolysis rate between cows with CLs aged 90 ± 6 h and 102 ± 6 h. A probability of $P \leq 0.05$ indicated that a difference was significant and probabilities between $P > 0.05$ and $P \leq 0.1$ indicated that a difference approached significance. Data are given as mean ± S.E.M., unless stated otherwise.

3. Results

There were 7 cows followed during two consecutive cycles (control and treatment), whereas the remaining two were followed only during the treatment cycle for 40 days. One of these cows, after PGF treatment 102 ± 6 h post-ovulation had partial luteolysis and developed a follicular cyst that luteinised and collapsed spontaneously at a later stage (**Fig. 2**). The other cow was treated at 102 ± 6 h too, had full luteolysis with fall in progesterone concentration below 1 ng/ml within 48 h of treatment and then entered a period of at least 40 days with progesterone concentration < 1 ng/ml despite the development of follicular waves with follicular structures of up to 21 mm in diameter.

198 Overall, the treatment of cows on day 3.5 post-ovulation (day 0 = day of
199 ovulation) with 50 mg of dinoprost induced full luteolysis in 2 of 9 cows (22%) and
200 partial luteolysis in the remaining 7 cows (78%). All treated cows had a CL diameter of
201 < 17 mm just before the administration of PGF (**Table 1**). The CL of cows with full
202 luteolysis regressed completely and became undetectable after 4 days of treatment (**Fig.**
203 **3**). In cows with partial luteolysis, the effect of PGF treatment, day, and treatment by
204 day interaction on the CL diameter was significant. The CL of cows with partial
205 luteolysis decreased significantly ($P < 0.05$) in diameter by 12 h after treatment,
206 remained smaller than the control counterparts for 12 days post-treatment but was not
207 different between groups thereafter (**Fig. 4**). There was a significant effect of day of
208 cycle ($P < 0.01$) and PGF treatment ($P < 0.05$) but not of treatment by day interaction (P
209 > 0.05) on progesterone concentration in cows with partial luteolysis (**Fig. 4**). In these
210 cows, progesterone concentration decreased ($P < 0.05$) 12 h after treatment, remained
211 lower than in the control cycle for 36 h post-treatment ($P < 0.05$) and tended to be lower
212 3 and 5 to 7 days post-treatment ($P < 0.1$).

213 The inter-ovulatory intervals of treated (19.7 ± 2.4 days) and control estrous
214 cycles (23.8 ± 0.9 days) were not significantly different (**Table 1**). The percentage of
215 cows with full luteolysis in those with a CL aged 102 ± 6 h (2/4, 50%) tended to be
216 higher ($P = 0.1$) than in cows with a CL aged 90 ± 6 h (0/5, 0%). The interval between
217 the first evidence of mounting behaviour to ultrasonographic detection of ovulation was
218 highly variable in both treated (12 to 48 h) and control (12 to 96 h) cycles (**Table 1**).

219

220 **4. Discussion**

221 The main objective of the current study was to characterise the effect of a single
222 administration of exogenous PGF at a high dose, on the progesterone concentration and

CL diameter in cows with early CLs. The CLs of all 9 cows were affected to some extent by the single administration of PGF, two of them undergoing full luteolysis. This finding is in contrast with a previous report [3]. The latter study reported no effect of a single dose of 25 mg of dinoprost on day 4 of the cycle. However it is difficult to make a reliable comparison with the previous study, since the exact age of the CL was unknown. In the reported study, day 0 of the cycle was allocated when the animals were detected in standing estrus. Assuming that the heifers ovulated 24 h after the onset of estrus and knowing that the heifers were observed for estrous signs twice a day, a day 4 heifer could have a CL aged between 72 and 84 h approximately. Probably, at that early stage of the estrous cycle, the CL was not responsive to a standard dose of PGF but might have been to higher doses. In the current study a 12 h difference in CL age seemed to be enough to make a single high dose of PGF sufficient to cause full luteolysis in half of treated cows. The two cows that underwent full luteolysis had a CL diameter before PGF treatment of 16 and 15 mm respectively. This is below the threshold value of $\geq$ 17 mm considered for a mature CL [11,12]. In spite of being immature, the CLs underwent a rate of reduction in diameter similar to that observed in mature CLs after treatment with a luteolytic dose of PGF [11]. Furthermore the progesterone concentration of these two cows fell below 1 ng/ml within 36 h of treatment which is in agreement with the progesterone profiles of cows with induced-luteolysis in mid cycle with mature CLs [11].

Although this should be tested further with a dose-rate study involving CLs at different ages, it seem that both CL age and PGF dose are important for the luteolytic response rate especially with CLs aged < 120 h and < 17 mm in diameter.

The cows with partial luteolysis had a significant but transient decrease in progesterone concentrations compared with the control cycles. This progesterone profile

is similar to that reported previously after repeated (twice daily) administration of PGF between day 3 and 4 of the cycle [6,7]. In the latter studies, CL diameter measurements were not performed, but in the current study the effect of a single treatment with PGF on day 3.5 appeared to affect the size of the CL to a greater extent than its progesterone secretory ability. In the treated cycle, the CL diameter was significantly smaller from 12 h after PGF treatment until 12 days post-treatment reaching a maximum mean diameter of approximately 18 mm not before 17 days post-ovulation. This lack of correlation between the resurgence in CL diameter and its ability of secreting progesterone following PGF treatment is in agreement with the results of a previous study in mares [9]. In the study of mares, a standard dose of PGF was administered to mares on day 3 of the cycle (day 0 = day of ovulation), all mares underwent partial luteolysis, with an initial decrease in progesterone concentration and CL diameter. The progesterone concentration had resurgence from 2 to 5 ng/ml but not the CL diameter which remained reduced to an ultrasonographically undetectable size within 6 days of treatment. All mares with partial luteolysis had a shortened inter-ovulatory interval in contrast to the cows of the current study. The difference in the inter-ovulatory interval between mares and cows with PGF-induced partial luteolysis is not surprising since the equine species is unique in that is able to ovulate with progesterone concentrations above 1 ng/ml [14] without showing behavioural signs of estrus.

It is worth noting the abnormal outcomes in terms of anovulatory waves observed in two of the PGF-induced estrous cycles. One cow entered a stage of anovulatory anestrus (progesterone < 1 ng/ml) following the PGF-induced full luteolysis that lasted for at least 40 days with recurrent anovulatory follicular waves with follicles of 16 to 21 mm in diameter. The other cow had a partial luteolysis with resurgence in progesterone concentration after 2 days post-treatment to fall below 1

ng/ml by 15 days post-ovulation, after which time the growing follicle developed into a follicular cyst reaching a maximum diameter of 33 mm, eventually the cyst luteinised and collapsed but continued producing progesterone. It is plausible that the PGF-induced low concentration of progesterone during early stages of follicular development may have interfered with the ovulatory process by altering the LH profile. Treatment with high doses of PGF to mares early in the estrous cycle has been linked to an increased incidence of hemorrhagic anovulatory follicles [15]. This observation was confirmed and explained by an increased LH concentration during early stages of follicular development as a result of the removal of the negative feedback that progesterone exerted on LH [16]. The association between sub-luteal progesterone [17-19] and increased LH [20,21] concentrations during early stages of follicular development and the occurrence of ovarian cystic disease in cattle has also been shown.

In conclusion the treatment of cows with a single administration of 50 mg of native PGF 3.5 days after ovulation induced partial and full luteolysis in 78 and 22% of treated cows, respectively. The CL diameter was significantly reduced for most of the estrous cycle in PGF-treated cows that underwent partial luteolysis in spite of a higher resurgence in progesterone than in CL diameter. This protocol seems a good model to induce low progesterone concentrations during the early stages of follicular development which could be used as an alternative to protocols that use repeated administrations of PGF. Although some cows developed full luteolysis and CL regression, the occurrence of anovulatory follicular waves in some of the treated cows renders this protocol to induce early luteolysis, unadvisable for clinical practice. Finally, CL age between 84 and 108 h hours post-ovulation appears to be a critical window of time for the degree of responsiveness to a single administration of exogenous PGF. Therefore it is recommended to know accurately the CL age within 8 to 12 h difference

298 if a study on the luteolytic effect of exogenous PGF in early stages of the estrous cycle
299 is to be done.
300

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Tables

Table 1
Estrous cycle characteristics of cows during a control and a PGF-induced estrus

Group	n	CL age PGF (h)	CL d3.5 (mm)	P4 d3.5 (ng/ml)	CL d5 (mm)	P4 d5 (ng/ml)	2 Ov (n)	+ Heat to ov (h) (range)	IOI (days)
CON	7	96.7 ± 2.1	14.8 ± 0.6	2.5 ± 0.3	19.0 ± 0.9	5.6 ± 1.2	1	48.0 ± 16.1 (12-96 h)	23.8 ± 0.9
PGF	9	95.3 ± 2.1	14.9 ± 0.5	2.9 ± 0.9	13.1 ± 0.9	1.5 ± 1.1	0	32.5 ± 3.4 (24-48 h)	19.7 ± 2.4
P value		NS	NS	NS	0.001	0.03	NS	NS	NS
Luteolysis									
Full	2	102 ± 0.0	15.5 ± 0.5	4.1 ± 1.3	11.5 ± 1.0	0.8 ± 0.2	0		7.0
Partial	7	93.4 ± 2.2	14.7 ± 0.4	2.2 ± 0.6	13.8 ± 0.8	1.8 ± 0.8	0		22.0 ± 1.2

Nine cows were administered 50 mg dinoprost (n = 9) or left uintreated (n = 7) 3.5 days after ovulation (day 0 = ovulation) during two consecutive estrous cycles. CL age PGF: interval in hours between detection of ovulation and PGF administration (PGF group) or equivalent control. CL d3.5 and d5: CL diameter (mm) 3.5 and 5 days after ovulation, respectively. P4 d3.5 and P4 d5: progesterone concentration 3.5 and 5 days after ovulation. 2 Ov: number of double ovulations; + Heat to ov: interval from positive detection of the activated heat detector aid (Estrotec®) to ultrasonographic diagnosis of ovulation (hours). IOI: inter-ovulatory intervals (in the PGF group only 6 cows were analysed for the IOI since one cow with full luteolysis never ovulated during the 40 days-period of the study).

Figure

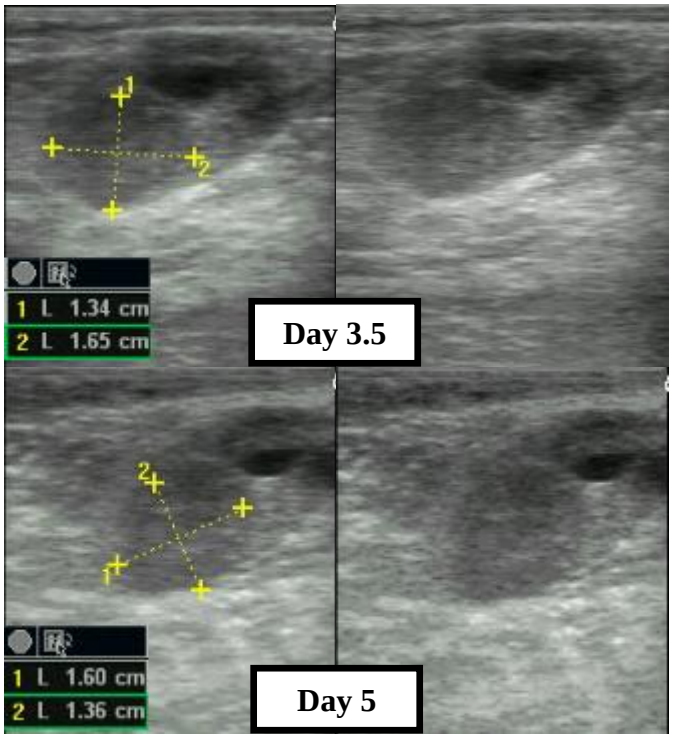

Fig.1. Sonogram series of the corpus luteum of one cow during the PGF treated cycle. 50 mg of dinoprost were administered on day 3.5 (Ovulation = day 0), (102 ± 6 h after ovulation) just after the image "Day 3.5" was taken. Note the reduced size of the CL (< 17 mm) still on day 5 (138 ± 6 h post-ovulation). The single administration of PGF induced partial luteolysis with decrease in progesterone concentration and delay in corpus luteum growth.

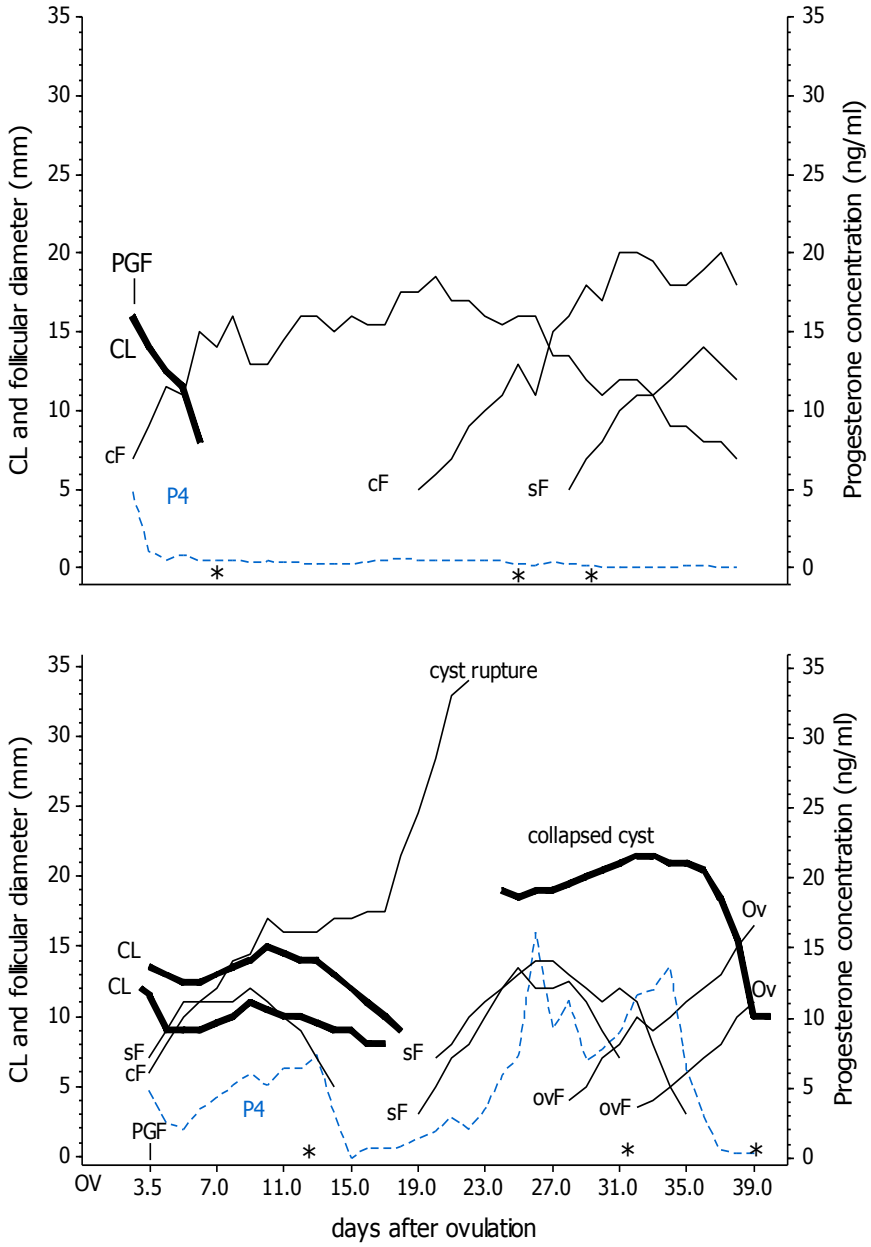

Fig. 2. Follicular dynamics of two cows with ovarian cystic disease during an induced cycle with 50 mg of dinoprost administered 3.5 days after ovulation. Ov: ovulation; cF: follicular cyst; sF: subordinate follicle; ovF: ovulatory follicle; CL: corpus luteum; collapsed cyst: solid structure that developed after spontaneous rupture of a luteal cyst (lower panel). P4: progesterone concentration. An asterisks (*) indicates an episode of standing heat evidenced by positive activation of the heat detector aid (Estrotec®).

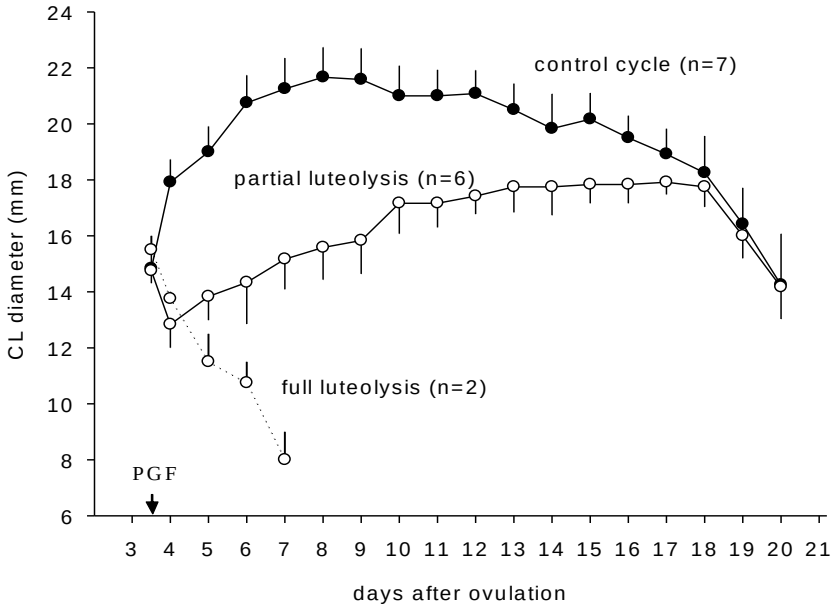

Fig.3. Mean ± S.E.M diameter of CLs from 8 cows during a PGF-treated cycle (n = 8) 3.5 days after ovulation and during their subsequent control cycle (n = 7). Two cows underwent full luteolysis with inter-ovulatory intervals of 7 and > 40 days (cystic follicles) respectively. Six cows underwent partial luteolysis. One cow with two CLs and a cystic follicle during the treatment cycle was not included in the analysis.

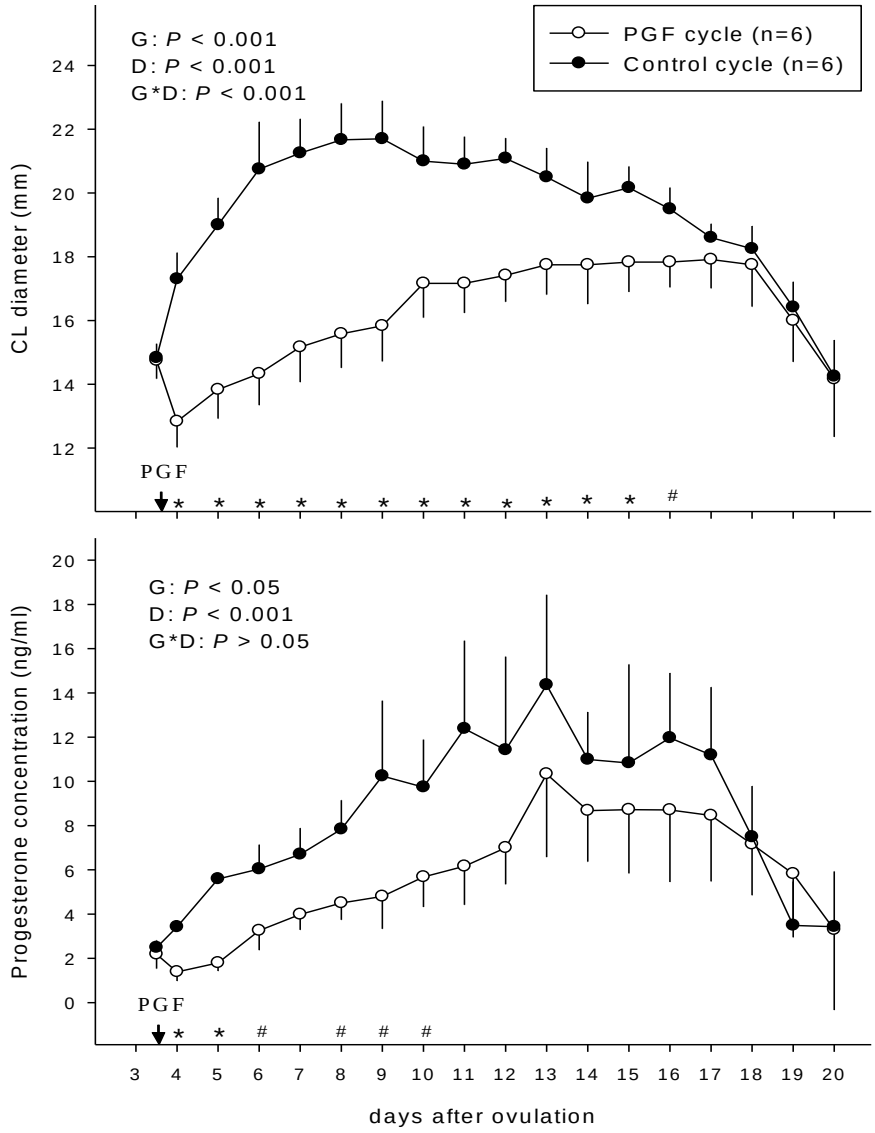

Fig.4. Mean ± S.E.M diameter of CLs (upper panel) and progesterone concentration (lower panel) of cows that underwent partial luteolysis following administration of 50 mg of dinoprost 3.5 days after ovulation (n = 6) and during a control untreated cycle (n = 6). Each same cow had a control and a treated cycle. Cows with full luteolysis or those that did not have a control cycle were not included in the analysis. Treatment with PGF (G) and day of cycle (D) had a significant effect on the progesterone concentration and CL diameter. The group by day (G*D) interaction was only significant for the CL diameter. A symbol (*) indicates that the difference in progesterone concentration or CL diameter between the control and treatment cycle at a given day is significant ($P < 0.05$) or approached (#) significance ($P < 0.1$).

***Author Agreement**

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