Effect of the administration of alfaprostol 3 or 6 days after ovulation in jennies: ultrasonographic characteristic and plasma progesterone concentration of corpora lutea

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Abstract: Donkey jenny's corpus luteum (CL) response to PGF2α analogues has not been investigated in depth. Aim of this study was to evaluate the donkey jenny's corpus luteum (CL) ultrasonographic characteristics (diameter, area and vascularized area) by B-Mode, Colour Doppler and serum progesterone concentration ([P4]) after treatment with the prostaglandins F2α analogue alfaprostol at day 3 or day 6 after ovulation (groups PG3 and PG6, respectively). [P4] was positively correlated (P<0.0001) with CL diameter: r^2=0.17; area: r^2=0.21 and vascularized area: r^2=0.54. The interovulatory interval was significantly reduced in the PG6 group (15±1.8 days), compared to the control group (24.5±2.9 days; P<0,05), while there were no significant differences in interovulatory interval between PG3 (21.7±7.9 days) and control or PG6 group. [P4], in the 6 jennies of the PG6 group, dropped under 1 ng/mL within 2 days after treatment, remaining under this concentration until [P4] raised again to levels comparable with those of the control group until spontaneous luteolysis. After alfaprostol administration, one of the 2 remaining PG3 group jennies showed a complete luteolysis, and the other one underwent a partial luteolysis and ovulated in diestrus.

Keywords: donkey jenny, corpus luteum, prostaglandin, Doppler, serum progesterone concentration

Introduction: The correlation between corpus luteum [CL] ultrasounds characteristics and [P4] has been showed in several species, including jennies [1-8]. Physiologic oestrus cycle in the jenny lasts around 24 days and luteolysis occurs usually between the days 15 and 17 after ovulation [9-12]. In horses and cows, exogenous administration of prostaglandins F2α analogues (PGF2α) from day 5 after ovulation induces luteolysis and [P4] drop at basal levels within 2-4 days [13,14]. In the same species, PGF2α treatment before the 5th day after ovulation, usually, is not able to induce luteolysis and the complete structural and functional regression of the corpus luteum, although a reduction of [P4] as well a reduction of the interovulatory interval has been shown in some studies [14,15]. A complete luteolysis and a reduction of interovulatory interval have been obtained in jennies treated by R-cloprostenol as early as 3 days after ovulation [16,17].

Aim of this study was to evaluate the relationship between ultrasonographic characteristics of jenny CLs and [P4] and to evaluate the efficacy of the administration of 3 mg of alfaprostol at day 3 or day 6 after ovulation on interovulatory interval, [P4] and ultrasonographic characteristics of donkey CL (B-Mode and Color Doppler).

Materials and methods: This study has been performed at the Department of Veterinary Sciences of the Pisa University, (43° 41' 00" North, 10° 21' 00" East), between August and November 2015. *Animals and ovarian activity monitoring* - Six pluriparous non-lactating and cyclic Amiata jennies, an Italian donkey breed usually cycling all year long at this latitude[11], between 5 and 10 years of age, with a weight between 300 and 350

kg and 3 of BCS [18] were included in this study. All jennies were in diagnosed in good health and were kept together in an open paddock of around 200 square meters provided with free running water access and fed hay ad libitum.

For 3 complete estrus cycles of each jenny (from ovulation to ovulation) the ovarian activity was monitored daily by trans-rectal ultrasound (MyLab™ 30 Gold machine equipped with a 5.0-7.5 MHz linear probe and a Color Doppler function; Esaote S.p.A, Florence, Italy) and exposed to a jackass stallion to evaluate estrus behaviour.

Treatments, corpus luteum and progesterone monitoring - Each jenny was submitted to a PG3 cycle, to a PG6 cycle and to a control (CTRL) cycle in random order. During the CTRL cycle, the jennies were left untreated, in the remaining two cycles jennies were treated with 3 mg/im of the PGF2α analogue alfaprostol (Gabbrostim®, CEVA, MB, Italy) at day 3 (PG3) or day 6 (PG6) after ovulation (day 0), respectively. Jennies were restrained in a stock, and for every CL, three images were daily taken in color flow mode at a standard depth of 10 cm, frequency of 5.0 MHz, 70% gain, and with a pulse repetition frequency (PRF) of 2.8, at measured maximum cross-sectional diameter (∅). Each ultrasonographic examination took around 5 minutes, and no signs of stress or discomfort was showed by any jenny. Images taken were analyzed using ImageJ 1.52a software (National Institutes of Health, Bethesda, USA). Images were cropped and cross sectional areas (CSA) and vascularized areas (VA) of the CL were measured counting the number of grey and color pixels, for each image taken, respectively [6,19]. Using the same software, the number of pixels resulting from the measurement was converted into cm for ∅ and cm^2 for CSA and VA. Blood samples for [P4] (10 ml each) were collected by jugular venipuncture daily from day 0 to the next ovulation, right after the ultrasonographic examination. The blood collected was immediately submitted to centrifugation, and serum was separated and frozen at -20°C until analysis. Progesterone was evaluated by validated radioimmunoassay as previously described [20]. The sensitivity of the assay was 1.78 pg/tube, and the intra- and inter-assay coefficients of variation were 6.2% and 9.7%, respectively. Cross reactions of other steroids with antiserum raised against P4 were: progesterone (100%), 11a-hydroxyprogesterone (90.9%), 20a-hydroxyprogesterone (1.5%), 17a-hydroxyprogesterone (1.5%), 5a-pregnan-3-20-dione (2.5%), 20a-hydroxy-4-pregnen-3-one (0.9%) and pregnenolone (<0.01%). The results are expressed as ng/mL.

Progesterone concentration < 1 ng/ml was taken as the limit for a non-functional CL: jennies showing P4 concentration < 1 ng/ml were assumed not to have a functional CL at the time of sampling [13,21].

The study was approved by the Organisme for Animal Wellfare of Pisa University with the protocol number 15101/2015.

Statistical analysis - GraphPad Prism 6.00 for Mac Os X (GraphPad Software, 2012, La Jolla California USA, www.graphpad.com), was used to perform the statistical analyses of this study.

Normality of the data included were analyzed by Shapiro-Wilk Normality test. Data were defined normally distributed with P>0.05.

Pearson Correlation was used to evaluate the relationship between [P4] and ∅, CSA and VA.

Differences between cycles CTRL, PG3 and PG6 in inter-ovulatory intervals (considered as the interval, measured in days, between one ovulation to the next) and daily measures of CLs' ∅, CSA and VA and [P4] values, were analyzed by repeated measures One-Way ANOVA and Tuckey's post-hoc test, in case of normal distributions, or repeated measures Friedman test and Dunn post hoc test, in case of not normally distributed data.

CLs' ∅, CSA and VA in case of level of [P4] <1 ng/mL or ≥1 ng/mL were compared with either Student T-Test or Wilcoxon Signed-Rank test depending on data distribution, normal or not, respectively.

For each day of the cycle, the differences between the number of jennies with [P4] ≥ 1 ng/mL in groups (CTRL, PG3 and PG6) were analyzed by the Two-tailed Fisher exact test. Differences have been considered statistically significant when P<0.05.

Results: All the jennies ovulated a single follicle per cycle, and all CLs were highly echogenic and showed a central hyperechoic area, no lacunae have been evidenced in analyzed CLs and no persistent CLs have been observed.

Positive correlations with [P4] were observed for ∅ (r^2: 0.17), CSA (r^2: 0.21) and VA (r^2: 0.54) (P<0.0001).

Data recording the inter-ovulatory intervals of the 3 treatment groups were normally distributed. Inter-ovulatory intervals were affected by treatment groups (P=0.03); in particular, the inter-ovulatory intervals were shortened in PG6 compared to CTRL (15±1.8 and 24.5±2.9 days, respectively; P<0.05), while no differences were observed between PG3 (21.7±7.9; P>0.05) and CTRL or PG6.

CLs' ∅, CSA and VA measured values when [P4] was <1 ng/mL or ≥1 ng/mL were not normally distributed and are reported in the Table 1.

Table 1: Differences between CLs' ∅ (cm), CSA and VA (cm^2) when [P4] was <1 ng/mL or ≥1 ng/mL. Data are expressed as median (25%/75% percentile)

	CLs' diameter	**CLs' cross sectional area**	**CLs' vascularized area**
[P4] <1	2.0 (1.8/2.3) [a]	3.5 (2.8/4.5) [a]	0.03 (0.0/0.2) [a]
[P4] ≥1	2.7 (2.5/3.0) [b]	6.2 (5.2/7.3) [b]	1.3 (0.6/2.0) [b]

In between the same column: [a≠b]: *P<0,001*

In the PG3 cycle, an evident decline of [P4] was observed, but only in 2 cases [P4] dropped under 1 ng/mL the day after alfaprostol administration. One of these 2 jennies showed heat and ovulated at the 11th day of the cycle, while in the other one the [P4] returned over 1 ng/ml and ovulation occurred the 20th day of the cycle. One more jenny, during the PG3 cycle, ovulated the 13th day after the previous ovulation with [P4] still above 1 ng/ml and without showing estrus behaviour. In the PG6 cycle, 6/6 jennies reached [P4] values < 1 ng/ml in 4 days after alfaprostol administration [P4] remained low and all of them ovulated in estrus within 16 days after the previous ovulation.

Differences in number of jennies with [P4] > 1 ng/mL at each day in the studied cycles are described in Figure 1.

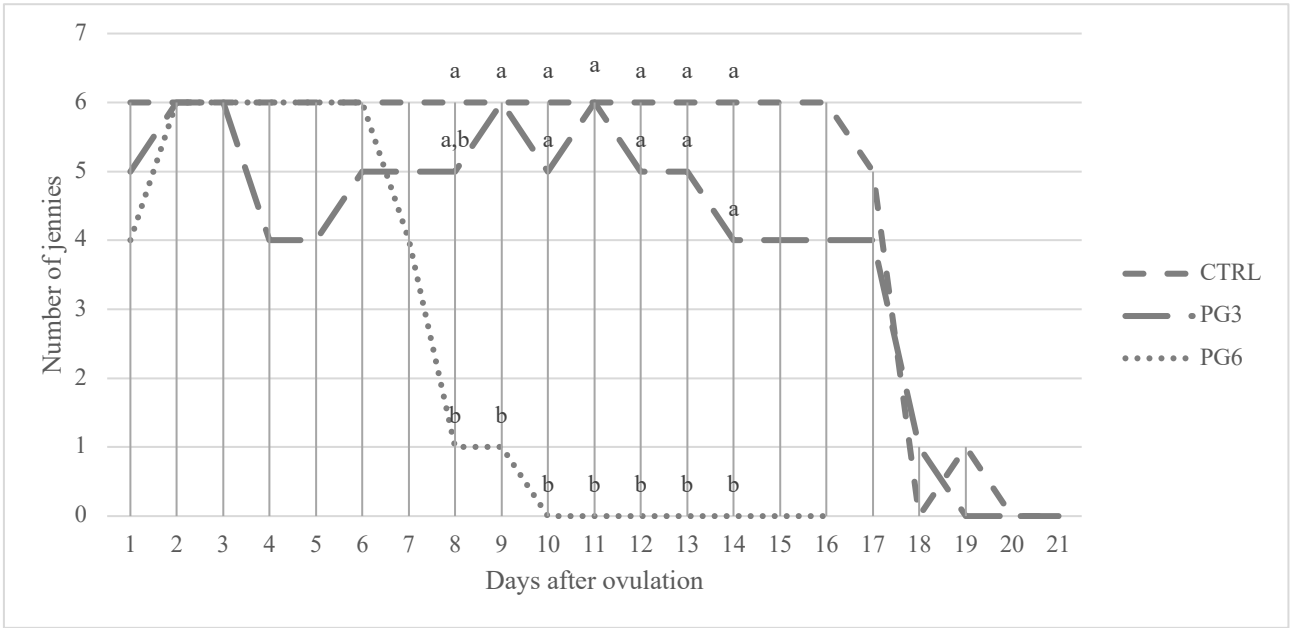

Figure 1: Number of jennies with progesterone > 1 ng/mL per each day of the control cycle (CTRL) and the treated cycles. PG3 = treatment with alfaprostol 3 days after ovulation. PG6 = treatment with alfaprostol 6 days after ovulation. *a≠b: P<0.05*

The daily values of [P4], ∅, CSA and VA in the 6 jennies were normally distributed in each treatment group. [P4], ø, CSA and VA resulted statistically different among treatments and differences per day are described in figures 2-5.

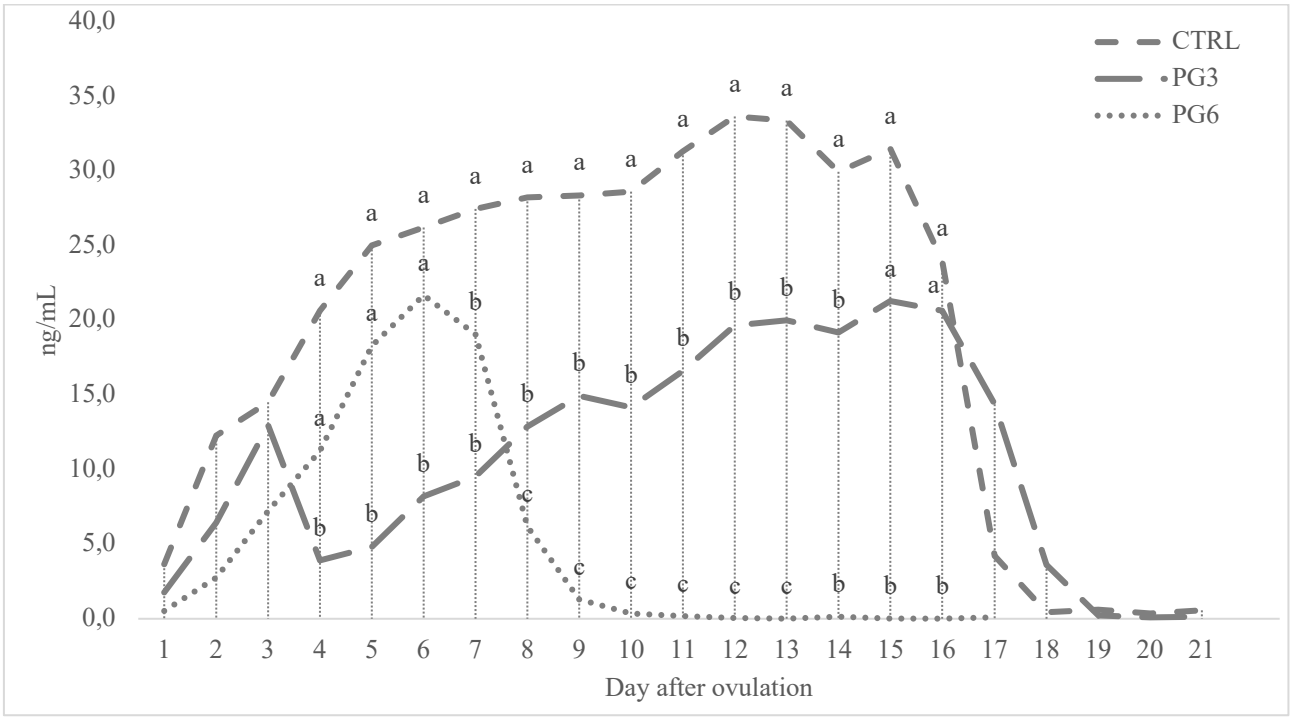

Figure 2: progesterone levels during the control cycle (CTRL) and the treated cycles (PG3 = treatment with alfaprostol 3 days after ovulation; PG6 = treatment with alfaprostol 6 days after ovulation). *a≠b≠c: P<0.05*

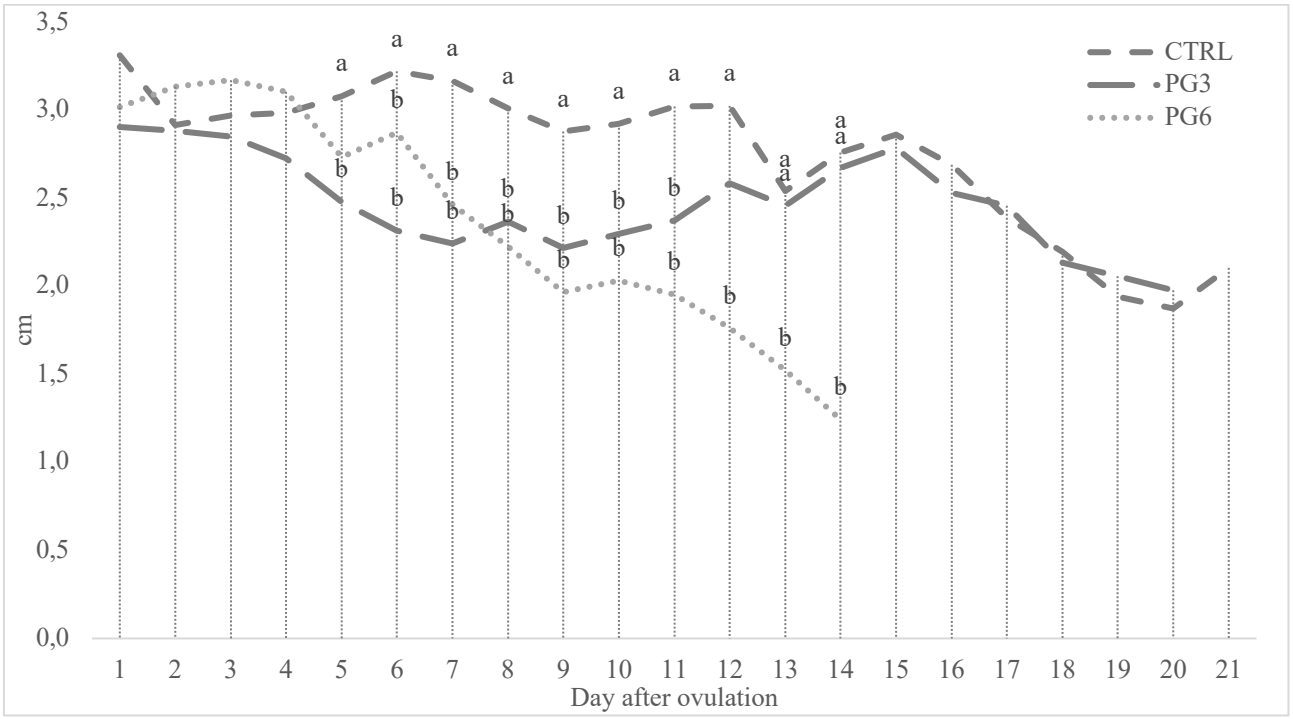

Figure 3: CL's ∅ (cm) during the control cycle (CTRL) and the treated cycles (PG3 = treatment with alfaprostol 3 days after ovulation; PG6 = treatment with alfaprostol 6 days after ovulation). *a≠b: P<0.05*

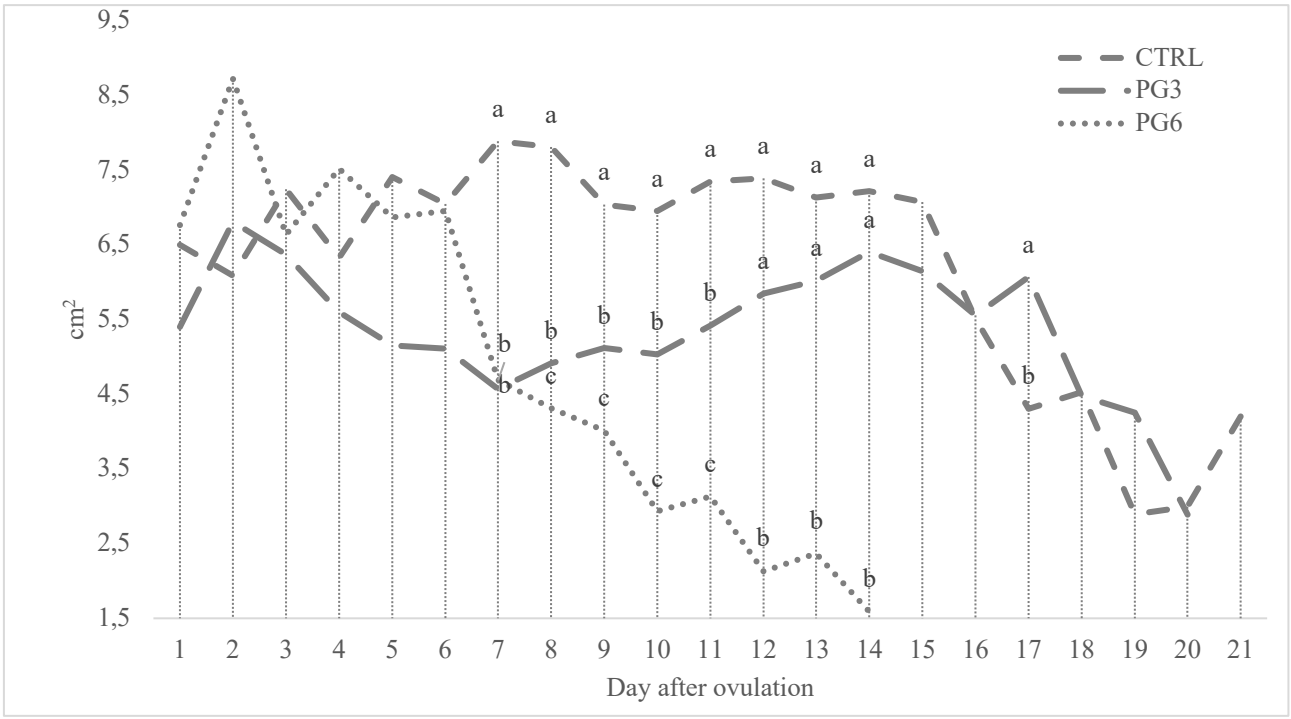

Figure 4: CL's cross-sectional area (cm^2) during the control cycle (CTRL) and the treated cycles (PG3 = treatment with alfaprostol 3 days after ovulation; PG6 = treatment with alfaprostol 6 days after ovulation). *a≠b≠c: P<0.05*

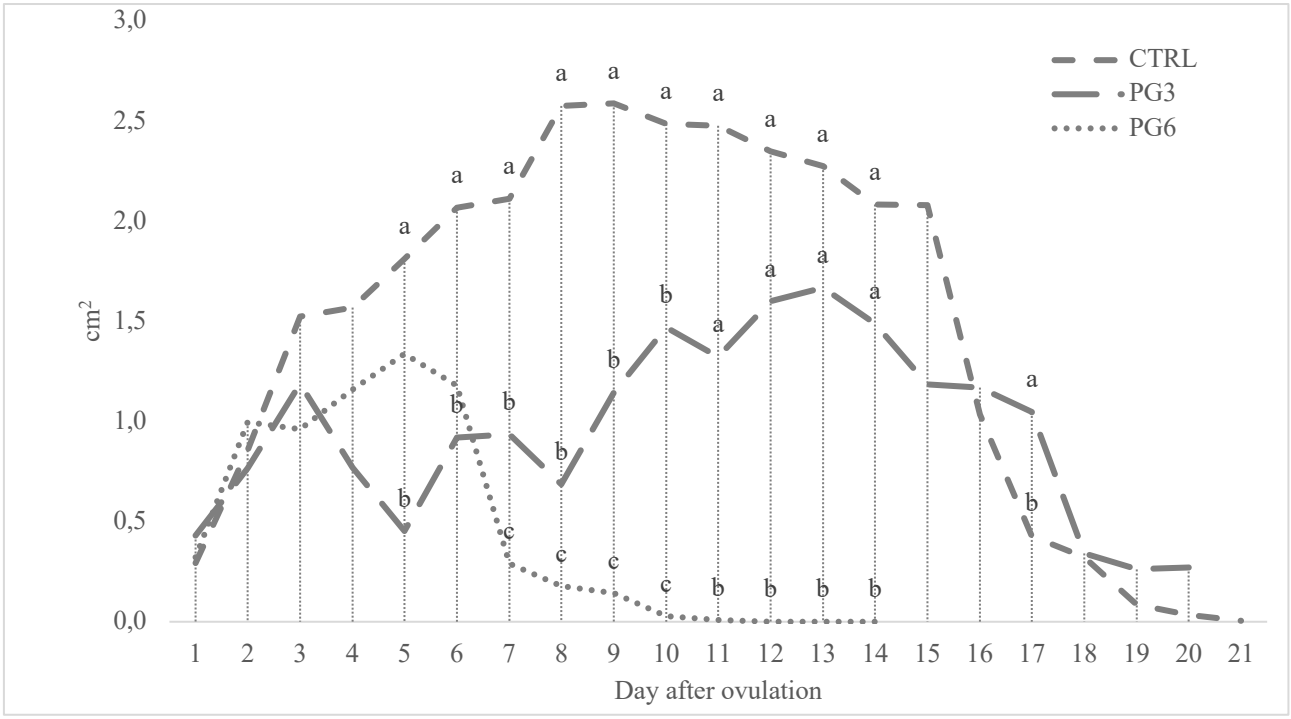

Figure 5: CL's vascularized area (cm^2) during the control cycle (CTRL) and the treated cycles. PG3 = treatment with alfaprostol 3 days after ovulation. PG6 = treatment with alfaprostol 6 days after ovulation. *a≠b≠c: P<0.05*

Discussion: The average jennies' cycle characteristics observed in the control group were comparable to what reported in literature [10-12,22-28]. A single ovulation occurred in all the cycles monitored, similarly to what reported in some studies [22,12]). Others observed an incidence of multiple ovulations in 15% [11,23,29] to 50% of the cycles [26,28,30-33] on jennies of different breeds, at different latitudes and submitted to different management. Corpora lutea ultrasounds appearance was analogue to what described in literature, as well as the absence of any anechoic areas [8,26].

In this study, the [P4] levels resulted correlated with the CL's ∅, CSA and VA. Especially VA and [P4] curves resulted similar, confirming what previously reported in sheep [1], cow [2-4], mare [5-7], and jenny [8]. The low number of cycles studied didn't allow to extrapolate cutoff values for ∅, CSA and VA when [P4] was <1 ng/ml, as described for the bovine and the equine species [7,34,35].

It was reported that the administration of 0.075 mg/im of the PGF analogue cloprostenol at day 3 after ovulation resulted in a complete luteolysis in nearly all jennies treated [16, 17]; and the time from treatment to return in estrus and estrus length were similar when treating jennies at days 3, 5, 7 and 9 after ovulation [16]. In only 1/22 jennies treated with cloprostenol at day 3, it was observed a decline in [P4] followed by a rise to normal diestrus levels. In the present study, on the contrary: a decline and resurgence of [P4], as response to PGF injected at day 3 after ovulation, was observed in 5/6 jennies, while a complete luteolysis occurred in only 1/6 animals. These results are more similar to what reported in the mare, where administration of the PGF analogue dinoprost trimethamine at day 3 after ovulation, after an initial [P4] decrease resulted in 3 response groups: with [P4] major resurgence (6/16), with [P4] minor resurgence (6/16), and with no [P4] resurgence (4/16) [14]. Similarly, it was reported that several PGF administration are needed to induce complete luteolysis before the fifth day after ovulation, in the mare [36, 37]. Differences in luteolytic effect of the two different PGF analogues, or the breeds of jennies studied, could be responsible of the different results between alfaprostol, used in this study, and cloprostenol [16,17], when employed 3 days after ovulation. Moreover we cannot exclude that a variability in CL sensitivity exist in jennies and mares, probably due to a subjective CL's vessels endothelial cells maturation rate and vascularization development [14,38].

There is consensus in the findings for administration of PGF analogues from day 5 after ovulation in the jenny: the administration of alfaprostol at day 6 of this study, cloprostenol at days 5, 7 and 9 [16] and dinoprost trimethamine at day 10 [8] always resulted in complete luteolysis and return to estrus.

In the present study, the ultrasonographic characteristics of the CL's after alfaprostol administration 3 days post ovulation followed the progesterone profile and underwent a reduction of mean values if compared to the control group. In the PG3 group, compared to the CTRL, the [P4] levels remained significantly lower between day 4 and day 14; while values of ∅, CSA and VA were lower on day 5 to day 11, days 7 to 11 and days 5 to 10, respectively, to successively reach values comparable to those of the CTRL group. These phenomena seem to indicate, once again, the good correlation between the ultrasounds evidences and the CL functionality.

In the present study, 1/6 PG3 jennies ovulated in diestrus with [P4]>1 ng/ml and no estrus behavior; thus observation is similar to what described in the mare and in the synchronized cattles for incomplete induced luteolysis [39,40].

Serum progesterone concentration and CL's patterns observed in this study in the control and PG6 groups followed what already described in jennies [16,17 8], mares [7,41] and cattle [34,35].

Conclusions: This study confirms the positive correlation existing between jenny CL's diameter, area and, in particular, vascularized area with plasma progesterone concentration. No direct quantitative measurement of vascularization is today present in the ultrasonographic machines, but when available, will be possible, for practitioners, to evaluate the CL function. The administration to jennies of 3 mg of alfaprostol at day 3 after ovulation does not shorten the interovulatory interval and results in incomplete luteolysis in most of the jennies, although a temporary quantitative reduction in serum progesterone concentration was clear. The administration of alfaprostol at 6 days following ovulation induces a complete luteolysis and shortens the interovulatory interval. It is thus better to treat jennies with this alfaprostol from day 6, and not from day 3 after ovulation if induction of luteolysis is required. More studies are needed to investigate reasons for the different response observed in jennies to cloprostenol [16,17] and alfaprostol (this study).

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