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Effect of aglepristone (RU534) administration during follicular phase on progesterone, estradiol-17β, and LH serum concentrations in bitches

[a]Troisi A, [b]Polisca A*, [b]Cardinali L, [c]Orlandi R, [d]Brecchia G, [b]Menchetti L, [b]Zerani M, [b]Maranesi M, [e]Di Mari W, [f]Verstegen JP.

a *School of Biosciences and Veterinary Medicine, University of Camerino, Via Circonvallazione 93/95 - 62024 Matelica, Italy*

b Department of Veterinary Medicine, University of Perugia. Via S. Costanzo 4, 06126 Perugia, Italy

c Tyrus Clinica Veterinaria, Via Aldo Bartocci, 1G, 05100 Terni, Italy

d Department of Veterinary Medicine, University of Milano, Via dell'Università, 6, 26900 Lodi, Italy

e Comando Generale Guardia di Finanza, Viale Ventuno Aprile, 00162 Roma, Italy

f TherioExpert LLc. and College of Veterinary Medicine University of Nottingham United Kingdom

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*Corresponding author. Department of Veterinary Medicine, University of Perugia Via S. Costanzo 4, 06126 Perugia, Italy Phone: +39755857623 Fax: +390755857624

e-mail address: angela.polisca@unipg.it (A. Polisca)

Abstract

Aglepristone was administered in bitches during the follicular phase to evaluate its effects on progesterone, estradiol-17β and LH serum concentrations. Ten German Shepherds were divided into two groups (treated n = 5; control n =5). Treated bitches received 10 mg/kg BW of aglepristone subcutaneously during the early follicular phase, 24 hours after and then 7 days later. The control group was injected, at the same time periods, with saline solution (0.3 ml/kg BW). For the steroid evaluations, blood was collected daily from the onset of proestrus until the first day of cytological diestrus. For LH base-line serum determination, blood was also collected every 20 min for 2 hours at the onset of proestrus. For LH surge identification, blood was collected daily (every 6 hours) starting from the day of the first administration of aglepristone or saline solution until the first day of diestrus. All animals ovulated but the treated group presented longer ovulation-diestrus intervals than the control group (5.2 ± 2.2 days $p < 0.05$). Serum concentrations of the evaluated hormones were similar between experimental animals except for serum LH. Indeed, no LH peaks were detected in the treated group while LH surges were clearly observed in the control group (9±1 days after the beginning of proestrus. In particular, the area under the curve for LH was significantly lower in treated than control animals (12 ± 4 ng/ml x Day; $p = 0.01$). In conclusion, administrations of aglepristone during the follicular phase of the bitch does not affect the steroid hormone patterns but does prevent the occurrence of a LH surge. This work raises significant questions and opens perspectives concerning the mechanisms of ovulation in bitches.

1. Introduction

The estrous cycle of the female dog is characterized by many specificities that make it unique. While most species have a short follicular phase typically starting during the previous cycle, bitches show a long estrous phase (Senger, 2015) that initiates during the previous anestrus with progressive and slow pulsatile changes of FSH and LH basal concentrations (Concannon, 2011; Verstegen et al., 1997). These late anestrus changes control, in turn, gonadal control function, maturation of follicles, and production of estradiol. In mammals, ovulation is initiated by an LH surge secreted by the pituitary, the so-called pre-ovulatory gonadotropin surge, that superimposes upon or temporarily replaces the pulsatile LH secretion pattern of low intensity and variable frequency (Knobil, 1995). The LH surge, characterized by high intensity and long duration, stimulates the pre-ovulatory follicles to produce local mediators that coordinate complex intra- and extra-cellular events leading to ovulation (Choi et al., 2017). In humans and ewes, estradiol exerts continuous positive feedback on the pre- ovulatory LH surge that usually begins when the plasma estradiol concentrations are still at their maximum (Liu and Yen, 1983; Karsch et al., 1997; Evans et al., 1997). In bitches, in contrast, Onclin et al. (2002) reported that the plasma estradiol concentration reached a maximum 24–48 hours before the pre-ovulatory LH surge and the following decrease is associated with a significant increase in plasma progesterone concentrations over basal values (Concannon et al., 1975). Significant progesterone changes over 0.5 ng/ml are observed as early as 48 hours in some bitches (Onclin et al. 2002) or concomitantly with the LH surge initiation in other dogs (Kooistra and Okkens, 2001). At the time of the LH surge, circulating progesterone is significantly higher than in any other species with values over 1 to 3 ng/ml and concentration over 5 ng/ml at ovulation. For these reasons, the exact initiators of ovulation are still not yet clear even if, for Concannon and others, the change in the serum estradiol/progesterone ratio initiates the pre-ovulatory LH surge

(Concannon et al., 1975; Kooistra and Okkens, 2001; Concannon, 2009; Smith and McDonald, 1974 ; Olson et al., 1982). Progesterone receptor antagonists, like aglepristone, are known to bind specifically with high affinity to progesterone receptors (PR) without inducing any progesterone-like activities (Cadepond et al., 1997; Manothaiudom et al., 1995; Hoffman and Schuler, 2000). These molecules, if administered after the LH surge when the corpus luteum (CL) is fully active, will prevent uterine and/or embryonic progesterone effects and induce embryonic resorption, abortion or premature parturition when given in early, mid or late pregnancy, respectively (Gogny and Fieni, 2016). However, the CL, being relatively independent, is not affected and will continue to produce progesterone for an extended period (Polisca et al., 2010). When aglepristone was administered during the follicular phase in dogs no effects on ovulation were observed in a study by Raynaud et al. (2015). However, they observed delayed oocyte maturation, and reduced intra-uterine and intra-oviductal transit of spermatozoa. These findings differ from what was observed in primates where administrations of PR antagonist during the follicular phase inhibit follicular development with a consequent delay or inhibition of the LH surge and ovulation (Chang and Jaffe, 1978; Liu et al., 1987; Batista et al., 1992; Ledger et al., 1992; Spitz et al., 1994). To our knowledge, the effects of aglepristone administrations to the bitch during the follicular phase on gonadotrophin secretion and estrus have never been evaluated *in vivo*. Therefore, the aim of this present work is to evaluate the effects of aglepristone administered in the early follicular phase on follicular and CL development and on plasma progesterone, estradiol, and LH dynamics.

2. Materials and Method

2.1. Animals Ten healthy female German Shepherd dogs, aged from 3 to 6 years and weighing 29.1 ± 1.02 kg (mean ± SEM), were included in the study. The privately-owned dogs were

followed by the service of Obstetrics and Gynecology of the Veterinary Teaching Hospital of the University of Perugia (Italy) as described below. The study was approved by the Institutional Animal Care and Use Committee of the University of Perugia and performed with owner consent in accordance with Italian laws and EU directives.

2.2. Experimental procedure

The animals were randomly divided into two groups of 5 animals each. From the first appearance of vulvar serous sanguineous discharges (onset of proestrus) until the first day of cytological diestrus, sexual behavior was observed, and vaginal smears were performed daily. The first day of cytological diestrus was defined as the day where vaginal smears presented all types of epithelial cells (from basal to superficial), numerous WBC, and typical foam or metestrus cells (Johnston et al., 2001).

The treated group received subcutaneous administrations of aglepristone (Alizin®, Virbac Laboratories, Carros, France). Treated bitches received the first injection (10 mg/kg BW) during the follicular phase when progesterone serum concentration was still below 1 ng/ml, and vaginal cytology presented about 30% of superficial cells and an abundance of RBC (early to mid-proestrus). The next injections were performed at 24 hours and at 7 days later. The control group (n = 5) was injected subcutaneously, at the same periods, with saline solution (0.3 ml/kg BW).

2.3. Blood sampling

At every time point, 2 ml of blood were collected by venipuncture of the radial vein. The samples, drawn into tubes without anticoagulant, were centrifuged (3000 X g for 15 min) within 30 min of collection and sera stored at -20 °C until hormonal assay. For the steroid evaluations, blood was collected daily from the first appearance of vulvar serous sanguineous discharges (onset of proestrus) until the first day of cytological diestrus, as assessed by vaginal

smears. Individual dog results are all evaluated centered on the day of ovulation as identified by sonography. For LH base-line serum determination blood was collected every 20 min for 2 hours at the first day of proestrus in addition to the above blood samples. For the LH surge identification, blood was collected every 6 hours, starting from the day of the first administration of aglepristone or saline solution until the first day of diestrus. Only the samples collected 4 days before and 1 day post ovulation, identified retrospectively when the progesterone values were greater than 10 ng/ml, were evaluated

2.4. Ultrasound scanning

Ultrasonographic examinations were performed daily using a My Lab 30 Gold ultrasound scanner (Esaote, Genoa, Italy) equipped with a 5.5 to 7.5 MHz micro-convex probe for B-mode. The bilateral scans were performed as described by Polisca et al. (2013). Ovulation or follicular disappearance (day 0) was determined when a clear transformation of the ovaries image was recorded compared to the last ultrasound scanning and at least some of the follicular image was lost to be replaced by an increasingly echogenic structure. . Corpora lutea appeared as structures with hypoechoic lumen, surrounded by thick walls and protruding from the surface of the ovaries. Day 0 was subjectively defined as the day when typical density and structure changes were observed with sonography and progesterone values increased over 5 ng/ml (Polisca et al., 2013).

2.5. Measurements of serum progesterone, estradiol-17β and LH concentrations

Serum progesterone concentrations were determined by RIA using a specific antibody (Sigma-Aldrich, St Louis, MO, USA) according to the procedure reported by Boiti et al. (2004). Progesterone was extracted from corresponding 0.5 ml plasma samples with ethyl ether and each sample was assayed in duplicate. The assay sensitivity and intra- and inter-assay coefficients were 10 pg/ml, 6%, and 11%. The highest point of the calibration curve used for

the calculation of the progesterone results was 36.00 ng/ml. Estradiol-17β concentrations were assayed by RIA as previously reported (Gobetti et al., 1992). Estradiol-17β was extracted from corresponding samples with ethyl ether and each sample was assayed in duplicate. Intra- and inter-assay coefficients of variation and minimum detectable doses were 8.2%, 12.7%, and 12 pg/ml respectively. Serum LH concentrations were determined by ELISA using the validated canine LH ELISA kit (Abnova – Walnut, CA, USA Catalog Number KA2292 http://www.abnova.com/products/products_detail.asp?catalog_id=KA2292). The minimal detectable concentration of LH was 1 ng/ml. For each bitch, a LH peak was identified when, at minimum, 3 consecutive values significantly over the maximum value observed during the proestrus were detected.

2.6. Statistical analysis

Progesterone and estradiol17-β concentrations were analyzed by the linear mixed model procedure where bitches were treated as random effects while group (2 levels: control and treated), day from ovulation (repeated measure, 20 levels: from -10 to +10 days from day 0), and interaction represented fixed effects. Pairwise comparisons using Bonferroni correction were performed. Diagnostic graphics were used for testing assumptions and logarithmic transformations were used both for progesterone and estradiol-17β data. Results were expressed as estimated marginal means ± standard error (SE). Logarithms were back transformed but raw data are presented in the figures. The LH AUC (area under the curve), calculated by trapezoid method using GraphPad Prism version 5.01 software (Inc., San Diego, CA, USA) (Menchetti et al., 2018) was identified for each animal based upon LH values at each sampling time point from day -4 to 9 +1 from ovulation. Duration of the estrous phases and LH AUC between groups were compared using independent t-test checking for homogeneity of variance by the Levene's test. These results were expressed as means ± SE.

Statistical analyses were performed with SPSS Statistics version 23 (IBM, SPSS Inc., Chicago, IL, USA) with $p \leq 0.05$ considered as significant.

3. Results

There were no differences between groups in the number of days from the onset of proestrus to ovulation (9.8 ± 1.1 and 9.6 ± 0.5 days for control and treated groups, respectively; $p > 0.05$). However, compared to controls, treated animals showed a longer ovulation to cytological diestrus interval (9.2 ± 0.5 and 14.4 ± 2 for the control and treated groups respectively; $p < 0.01$) characterized by prolonged bleeding while progesterone was already increasing.

3.1. Progesterone and estradiol-17β concentrations during the periovulatory period

Mean progesterone increased from day -7 before ovulation ($P < 0.05$) when compared to day -10, but all values remained below 5.0 ng/mL until the day of ovulation (day 0). Later, progesterone concentrations continued to increase to reach the upper limit of the RIA (36.0 ng/mL) in early cytological diestrus for the control group, while treated animals were still in cytological estrus (Fig. 1). Progesterone concentrations were not affected by group ($P=0.136$) or interaction between group and day ($P=0.366$). Estradiol-17β concentrations were affected only by day ($P<0.001$) but not by treatments ($P=0.941$) or interaction between group and day ($P=0.919$). Mean concentrations progressively increased from day -10 peaking at day -6 ($P < 0.001$); then, it progressively decreased until 5 days post-ovulation where it returned to basal values ($P < 0.001$; Fig. 2) without significant differences between groups.

3.2. LH peak

No LH peaks were detected in the treated group (Fig.3 Panel 1-5 for each treated dog respectively), while long-lasting LH surges (ranging from 1 to 3 days with minimum of 3 to 5 consecutive over basal values) were clearly identified for all animals of the control group (Fig.4). In these control bitches, the LH peaks were observed on average 9 ± 1 days after the

beginning of proestrus or 0.8 to 2.4 days before ovulation. At the beginning of the LH surge (first significant positive value over basal values), the average progesterone serum concentration in the control group was 2.6 ± 0.7 ng/mL. The maximum serum LH concentrations were higher in control than treated group ($P<0.001$; Table 1SM). The LH AUC was significantly lower in the treated than in the control group (15.4±4.8 ng/mL x d and 3.3±2.9 ng/mL x d in Control and Treated groups, respectively; $P < 0.01$).

3.3. Ultrasound scanning

During proestrus, the ovaries had smooth regular margins and the follicles were clearly identified as anechoic spherical structures which grew progressively to reach an average size of 0.89 ± 0.06 cm (mean ± SE) the day before ovulation. The thickness of the follicle walls increased progressively to reach around 1 mm in width the day before ovulation without any differences between groups ($p > 0.05$). No differences in developing CL were noticed.

4. Discussion

Our results indicate that when aglepristone is administered during the proestrus phase in bitches, it inhibits LH secretion from the pituitary. Ultrasound evidence of any abnormality in follicular development and ovulation, in accordance with other authors (Renton et al., 1992; Davidson and Baker 2009) and the increase in serum plasma progesterone concentration can only suggest that ovulation has occurred. Thereafter, as we have neither collected the oocytes nor carried out artificial insemination or natural mating, we do not have the certainty that aglepristone administered during the follicular phase in bitches likely does not interfere with the ovulation process. However, our results may be the reason for some reflections related to endocrinological control of the estrus cycle in bitches. In mammals, progesterone is an essential hormone during the whole estrous cycle and is involved in the maintenance of pregnancy, lactation, and sexual behavior (Reynaud et al., 2015). Progesterone is also involved in the

hypothalamic feedback regulating gonadotrophin secretion (Micevych et al., 2008). In bitches, plasma progesterone significantly increases as early as 2-3 days before the LH surge and reaches concentrations over 5 ng/mL at the time of ovulation (Manothaiudom et al., 1995). Concannon et al. (2009) suggested that this progesterone rise associated with the decrease in estradiol had a significant role in the induction of the LH surge and ovulation. In women and monkeys, based on similar experiments done in rodents, it was initially believed that the preovulatory LH surge is initiated by a similar rise in circulating progesterone (De Geyter et al., 2002). However, the very first descriptions of progesterone time changes in the human menstrual cycle made this notion difficult to accept as, opposite to dogs, progesterone is largely undetectable in blood until after the surge initiation (Rothchild, 1996). In those species, progesterone remains essentially intra-follicular and does not cause any significant (< 1 ng/ml) and early (3 12 hours before LH surge) changes in circulating concentrations (De Geyter et al., 2002; Rothchild, 1996; Abraham et al., 1974; Wu and Minassian, 1997; Dirnfeld et al., 1993; Sunderland et al., 1994). It is, essentially, the preovulatory rise in estradiol that acts on the hypothalamo-hypophysal system to initiate the LH surge under the permissive action of GnRH (Chappell and Levine, 2000; Chappell et al., 1999; Levine, 1997). Estrogens enhance neuroprogesterone synthesis in the hypothalamic astrocytes (Micevych et al., 2003) and this locally produced progesterone facilitates the switch of the estrogen action from negative to positive (Akison and Robker, 2012). It then mediates the hypothalamic-pituitary induced ovulation. The changes in estrogens are also responsible for the increased expression of progesterone receptors in the hypothalamus (Kazem et al., 1996). The hormone receptor antagonists are significant pharmacological tools used in therapy, biotechnology, and endocrine research to prevent reproductive hormonal effects. Once bound to the receptor before denaturation, they prevent progesterone receptor activation and consequently block the biological cascade that normally happens. After treatment of beagle dogs with aglepristone

during the follicular phase, Reynaud et al. (2015) did not observe any changes in progesterone profiles in treated versus untreated animals but observed delayed resumption of meiosis while *in vitro* progression and fertilization were prevented. Reynaud et al. (2015), who used a different experimental model (aglepristone administration at the end of the proestrus and 24 hours later), did not record significant changes in progesterone profiles between treated and control animals but observed, in vitro, a delayed resumption of oocyte meiosis and the inhibition of their progression and fertilization.

In a similar way, in our study, the aglepristone administration during follicular phase , did not alter the progesterone dynamic. However, we observed both an inhibition of the expected LH pre-ovulatory surges and a prolonged behavioral estrus. In none of the treated animals was a LH surge observed contrary to what was found in all control dogs where ovulation was always preceded by a LH surge of at least 36 hours (Onklin et al., 2002). Furthermore, the estrous phase was significantly prolonged as also reported by Bladowska et al (2018). In particular the prolongation of this phase could be due to a lack of action of progesterone, in the control of sexual behavior suggested by other authors (Concannon et al. 1979 a, b; Bladowska et al. 2018). The extended estrus that we observed could be due to the absence of progesterone effect on sexual behavior and/or absence of estrogen effect antagonism allowing the latter to continue their physiological and behavioral actions for an extended period.
Moreover the exact contribution of progesterone to the estrus signs in the bitch remains still to be clarified.

The LH assay used in this study was validated for canines (Abnova – Walnut, CA, USA Catalog Number KA2292; http://www.abnova.com/products/products_detail.asp?catalog_id=KA2292). While we cannot exclude that eventual low amplitude and/or short duration (< 6 hours) LH surges were not detected, we doubt this happened as the LH surges were identified in all control dogs. In the control group, maximum LH concentrations at peak values were significantly over

20 ng/ml and in all dogs at least 3 consecutive positive samples (significantly different from basal values) were always observed. In treated dogs, however, maximum values were significantly lower and 3 consecutive positive values, needed to define a peak, were observed only in one animal. In the other treated dogs, only scattered single high values were observed. The LH profile of treated dogs was clearly different and much lower than that of control dogs in this and previous studies. Concannon reported in a personal communication (2009) that, in some beagle dogs, ovulation occurred without a LH surge; however, it is possible that the LH peak was eventually not detected due to the poor assay specificity and sensitivity and to the reduced frequency of blood sampling (2 times a day vs. 4 times a day in the present work).

As shown in this study, aglepristone treatment during the periovulatory period did not affect progesterone secretion suggesting that the hormonal transition from the granulosa to theca cell phenotypes may be regulated independently from LH. In dogs, as in non-human primates, progesterone may enhance its own synthesis in the CL by promoting luteinization (Rothchild, 1996). However, some other mechanisms may also come into play as aglepristone blocks PR. Corpus luteum independency and autonomous secretion of progesterone before the actual LH surge and ovulation may play a role in the overall fertility mechanism and fertilization. These observations suggest that aglepristone administration during the follicular phase inhibits the LH surge possibly by blocking hypothalamic progesterone receptors and affecting GnRH pulses but without interfering with ovarian progesterone production.

If, in our study we may suppose, only based on clinical data, that ovulation can occur in the absence of the LH surge, it is possible to hypothesize the role of other local and/or systemic factors (i.e. EGF-like factors involved in prostaglandin synthase release associated with ovulation) but further studies would be needed to confirm our hypothesis.

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Declaration of interest

The Authors declare no conflict of interest.

Data Availability Statement

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

Figures and legends

Fig. 1. Serum progesterone concentrations (ng/ml) in treated (n =5, black line) and control (n= 5, gray line) bitches during the peri-ovulatory period, from days -10 before to 10 after the estimated ovulation (day 0). Individual progesterone curves are centered on day 0 based on sonographic evaluations and ovulation detection. Results are expressed as means ± standard error (SE).

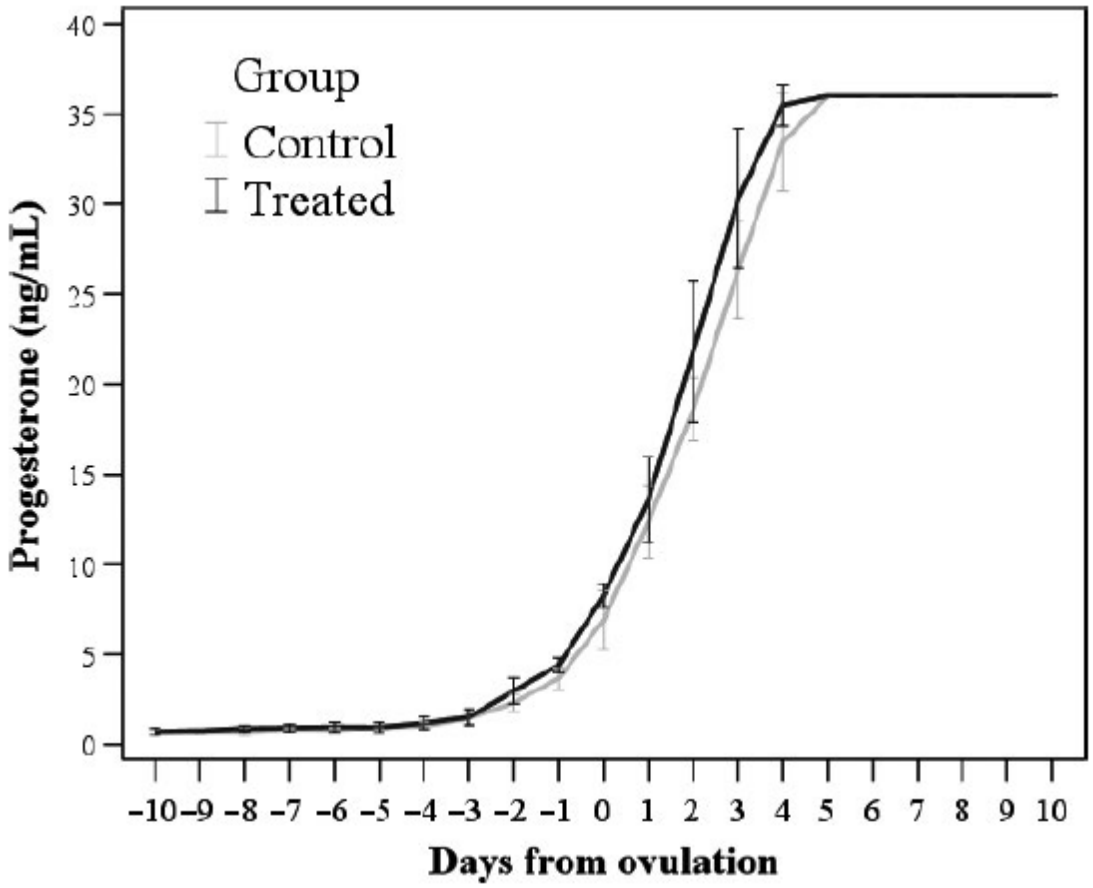

Fig. 2. Estradiol-17 serum concentrations (pg/ml) in treated (n = 5, black line) and control (n = 5, gray line) bitches during the peri-ovulatory period, from days -10 before to day 10 after the estimated ovulation (day 0). Individual curves are centered on day 0 based on sonographic evaluation and ovulation detection. Results are expressed as means ± standard error (SE).

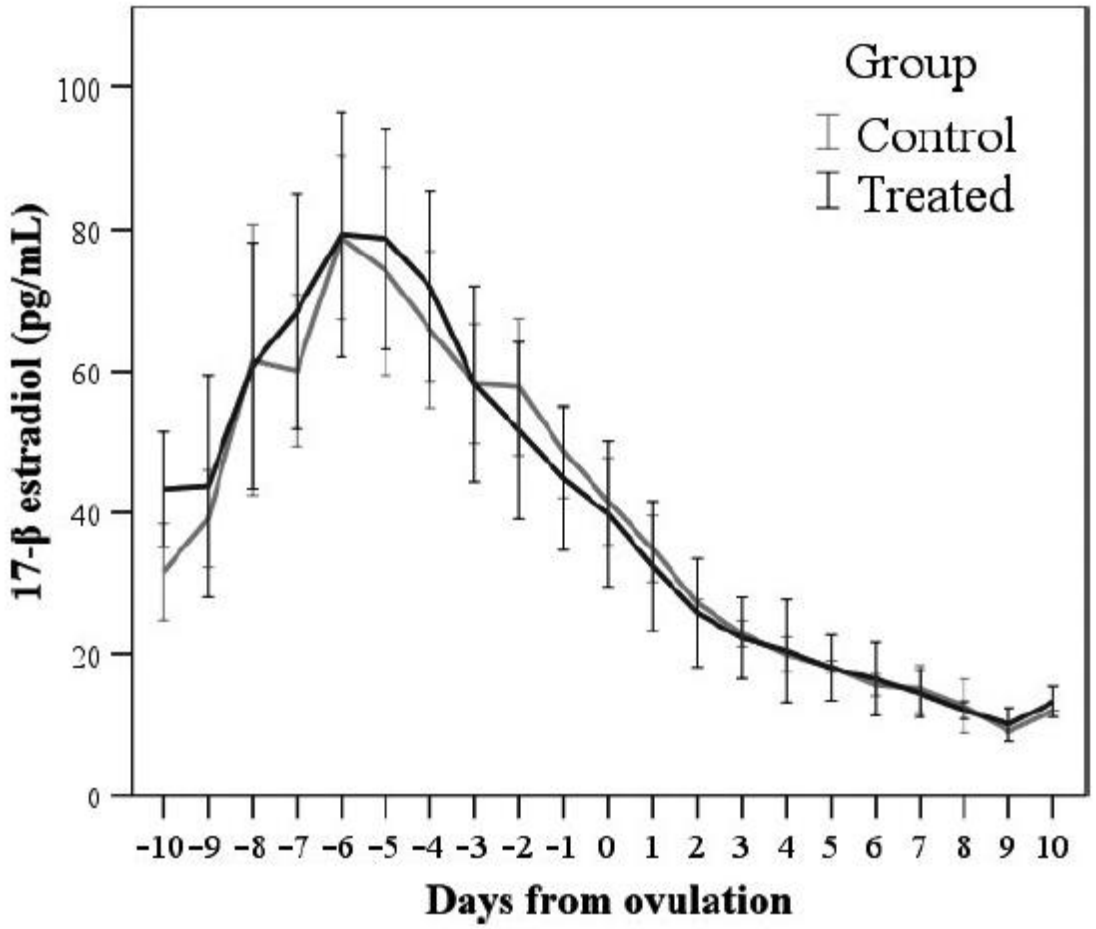

Fig. 3. Serum LH profiles (ng/ml) in treated bitches (panel 1-5). Arrows indicate the days of treatment; the dashed gray line shows the estimated day of ovulation as identified by sonography in individual bitch.

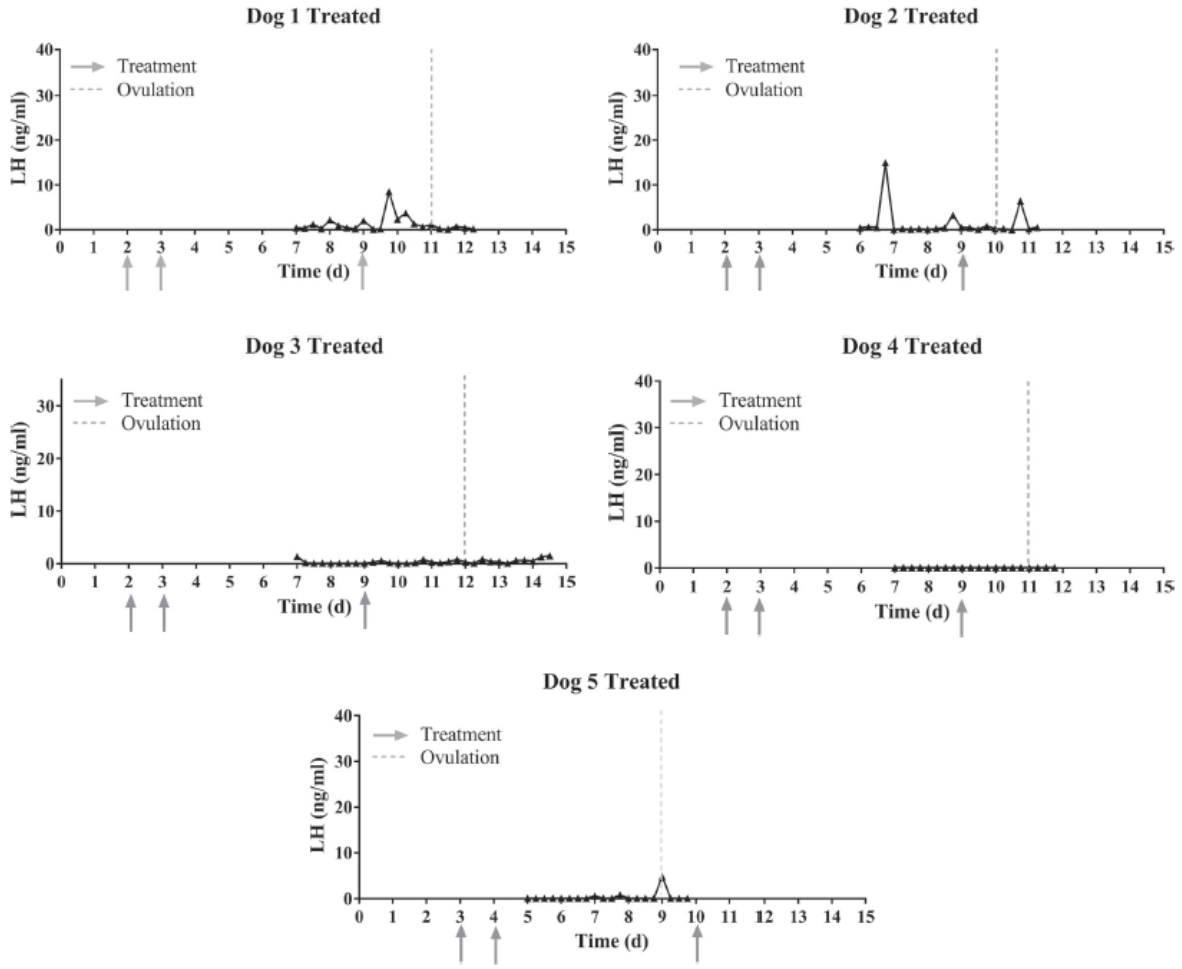

Fig. 4. Means ± SEM of serum LH concentrations (ng/ml) in control bitches (n = 5). Individual curves are centered on day 0 based on LH evaluation.

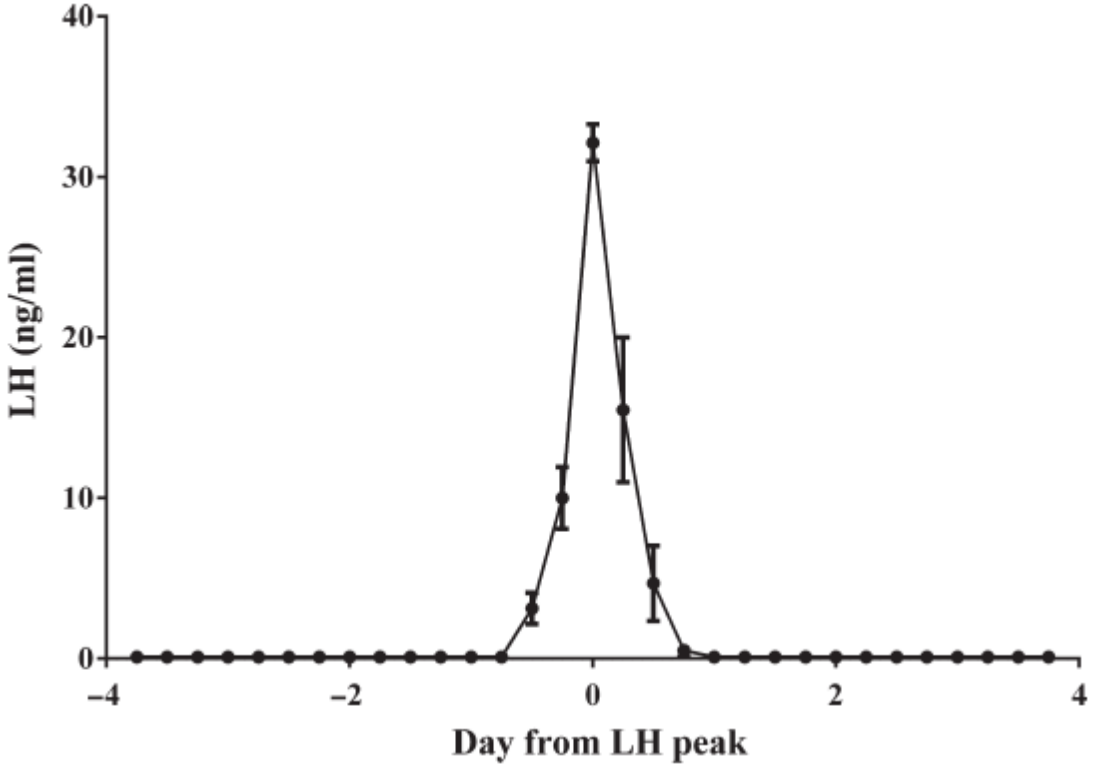

Table 1 SM. Maximum serum LH concentrations (ng/mL) in control and treated bitches

Bitch ID	Group	
	Control	Treated
1	30.0	8.5
2	34.2	15.0
3	34.5	0.9
4	33.1	0.1
5	28.8	4.8
Mean ±SEM	32.1±1.15	5.9±2.73

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