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Treatment of luteal phase mares with the aromatase inhibitor letrozole does not change characteristics of the estrous cycle, ovarian function, and embryo characteristics

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ABSTRACT

Letrozole is a potent and highly selective third-generation aromatase inhibitor. In the present study, we aimed to investigate the effects of letrozole treatment of non-pregnant mares on estrous cycle characteristics. Cyclic, non-lactating mares ($n = 9$) were included. After estrus synchronization, mares were monitored to detect ovulation (d0:) of the treatment cycle. Each three mares were randomly assigned to one of three treatments: control, 0.5, and 1 mg/kg body weight letrozole in three consecutive cycles (d7 to d13 of the treatment cycle). Mares were inseminated in the estrus after treatment and the day of ovulation was recorded (dOv). Embryo collection was performed 8 days after ovulation (dflush) of the post treatment cycle. Blood samples for analysis of progesterone, estradiol, testosterone and anti-Mullerian hormone were collected repeatedly. Antral follicle counts (AFC) were determined on d0, d4, d7 and d13 of the treatment and dOv and dflush of the post-treatment cycle. There were no effects of letrozole treatment on estrous cycle characteristics, pregnancy rate, and embryo characteristics. There were no differences in AFC among treatments, but follicle counts in the size classes 15 – <20 mm, 20 - <25 mm, 25 - <30 mm and ≥ 30 mm changed over time ($p < 0.05$). Letrozole treatment did not affect any hormone concentrations in serum. The results of this study scrutinize the suitability of this aromatase inhibitor for assessment of estradiol function in non-pregnant horses.

1. Introduction

Estradiol-17β (estradiol) is the major ovarian estrogen in the mare. Its main source is the ovarian follicle. The theca interna cells of the equine follicle produce androgens that are converted to estrogens in the granulosa cells by aromatase (Sirois et al., 1991). Estrogen synthesis and secretion is stimulated by follicle stimulating hormone (FSH) pulses from the anterior pituitary. This gonadotrophin also stimulates the growth of antral follicles during the follicular phase. The emergence of a follicular wave is temporally associated with an FSH surge, but FSH concentration reaches a plateau when the largest follicle increases to a size of 13 mm in diameter (Gastal et al., 1999). This stage is known as follicle deviation and coincides with a decline in FSH to a concentration not enough to support further growth of subordinate follicles but sufficient for continuing growth of the dominant follicle. This FSH decline depends on follicular synthesis and release of estradiol and inhibin. Peripheral concentrations of estradiol in mares increase progressively from the beginning of estrus and peak two days before ovulation. They decrease again from ovulation and reach a nadir two days thereafter (reviewed by Aurich, 2011).

Aromatase is an enzyme of the cytochrome P-450 superfamily and product of the CYP19A1 gene. It converts androgenic substances to estrogens in many tissues, including granulosa cells of ovarian follicles. Letrozole is a potent and highly selective third-generation aromatase inhibitor that was developed in the early 1990s. In women, letrozole reduces peripheral estradiol concentration, which is associated with increasing FSH, luteinizing hormone (LH), inhibin B, and androgen concentrations during the follicular phase, and increasing androgen, inhibin A, and progesterone concentrations during the luteal phase (Poulsen et al., 2022). In human gynecology, letrozole is frequently used

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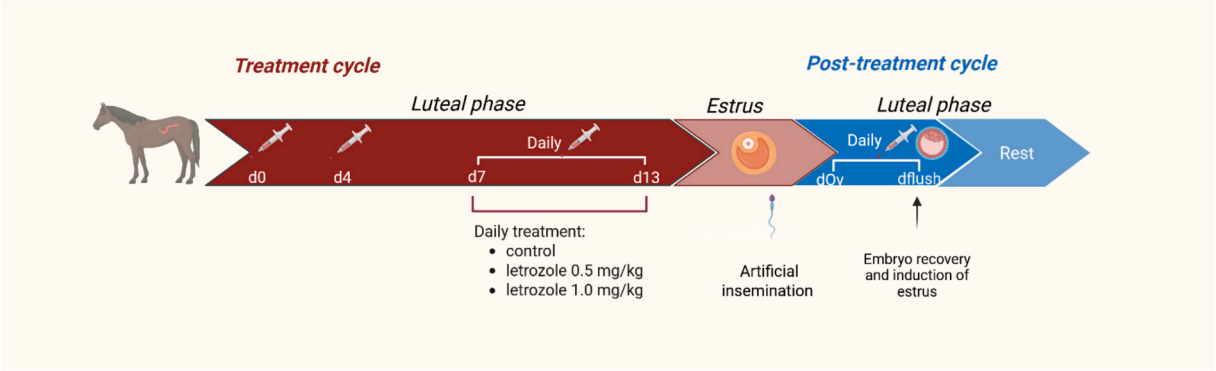

Fig. 1. Experimental protocol used in the study (Created in BioRender. Aurich, C. (2025) https://BioRender.com/jt4zgr2). All mares (n = 9) received one of the indicated treatments daily from d7 to d13 at random order in three estrous cycles with always one rest cycle between treatment cycles. Blood samples were collected on days indicated by the syringe icon.

for the improvement of fertility in anovulatory patients, and preparation of patients with diminished ovarian reserve for in vitro fertilization (IVF; Rose and Brown, 2020).

Letrozole and other aromatase inhibitors have facilitated research into the involvement of estrogens in the endocrine regulation of the estrous cycle for example in cattle (Yapura et al., 2011, 2012, 2018; Zwiefelhofer et al., 2022). In horses, letrozole has been used to investigate the role of pregnancy-related estrogens on placental function and angiogenesis as well as fetal development (Ball et al., 2016; Esteller-Vico et al., 2017; Haneda et al., 2021). To the best of our knowledge, there is only one study in non-pregnant mares (Akbarinejad et al., 2017). The authors suggested that letrozole treatment during the preovulatory period induced premature ovulation and impaired corpus luteum function although in this study with exception of progesterone concentrations no endocrine data were presented (Akbarinejad et al., 2017).

In the present study, we investigated the effects of letrozole treatment on estrous cycle characteristics in non-pregnant mares. It was the aim of the study to stimulate the simultaneous growth and ovulation of more than one follicle. Therefore, treatment was performed from the time of emergence of the future dominant follicle on d7 until its deviation on d13 of the cycle (Aurich, 2011). We hypothesized that inhibition of aromatase activity would increase the ratio of testosterone to estradiol concentration during letrozole treatment. This was expected to stimulate FSH concentration which in turn induces changes in the antral follicular count, follicular growth rate, and preovulatory size. Mares were also inseminated in the follicular phase following letrozole treatment to analyze embryo recovery rate and conceptus characteristics.

2. Materials and methods

2.1. Animals

This study included nine healthy, non-lactating Haflinger mares weighing 480 ± 22 kg and aged 5–12 years (8.7 ± 2.2 years, mean ± standard deviation). The mares shared a large outdoor paddock with a shed where they were housed as a single herd. They were fed hay ad libitum twice daily, but did not receive any concentrates. They had access to mineral supplements and fresh water. Body weight and body condition score were monitored at monthly intervals. Prior to the study, all mares passed a breeding soundness examination which included a transrectal ultrasonographic assessment, endometrial biopsy analysis (Ferris et al., 2015), and endometrial culture (Nielsen, 2005).

2.2. Experimental design

The study was approved by the Austrian Federal Ministry for Science and Research (experimentation license number 2023–0.122.306) and conducted in Vienna, Austria (longitude 16.4°, northern latitude 48.3°) from March to September, with ambient temperatures ranging from 1 °C to 27 °C, and average highs of 20–27 °C and lows of 1–16 °C and average relative humidity ranging from 55 % to 75 %.

This study was conducted during two consecutive breeding seasons. To synchronize estrus, all mares were given two intramuscular doses (250 μg each) of the $PGF_{2\alpha}$ analogue cloprostenol (Estrumate, MSD, Vienna, Austria) at a 14 day-interval. Each of the nine mares were randomly assigned to one of three treatments: controls (no treatment), letrozole 0.5 mg/kg (Letrozole 2.5 mg tablets, Actavis, Adelaide, Australia), or letrozole 1 mg/kg. The mares were weighed prior to starting letrozole administration to determine the number of 2.5 mg tablets to be added to each mare's feed and the study operator made sure that each mare consumed the entire prescribed amount of letrozole tablets by monitoring her until the food container was emptied.

Once mares were in estrus, they were daily monitored by transrectal ultrasonography to detect antral follicles, endometrial edema, follicular growth, and eventually the day of disappearance of the preovulatory follicle (d0 = day of ovulation, i.e., start of the treatment cycle, Fig. 1). The treatment was then given orally once daily in the morning after blood sampling starting on d7 until d13 of the treatment cycle, i.e., during the luteal phase. During the subsequent follicular phase (estrus), mares were examined daily by ultrasound. Once the diameter of the largest follicle exceeded 35 mm, she was inseminated every 48 h until ovulation was detected (dOv). On dflush (i.e., 8d after ovulation), a uterine lavage was performed to retrieve embryos for macroscopic examination, morphological assessment, and cell counting. A subsequent rest cycle followed, where mares were only monitored with ultrasound for control of intrauterine fluid and ovulation in addition to uterine swabs for monitoring of inflammation and bacteriology. Each mare served as her own control and received all treatments in three estrous cycles with always one rest cycle between treatment cycles. Endometrial swabs for bacteriology were collected during the estrus phase of the rest cycles. Blood samples (2.7.) were collected from the external jugular vein once daily from the first ovulation until dflush.

2.3. Breeding management

The reproductive tract of the mares was examined by transrectal palpation and ultrasonography (DP-66Vet, Mindray, Shenzhen, China) following administration of the second dose of $PGF_{2\alpha}$. Upon detection of estrus, mares underwent daily ultrasound examinations to assess follicular growth, uterine edema, uterine fluid accumulation, and ovulation. The degree of endometrial edema was scored on a scale of 0 (no edema, diestrus-like structure) to 3 (maximum endometrial edema) as described previously (Mateu-Sanchez et al., 2016). When a preovulatory follicle (diameter > 35 mm) and uterine edema were

detected for the first time, mares were Inseminated with 500 million progressive motile spermatozoa of raw semen from a stallion of proven fertility at 48-h intervals until detection of ovulation (i.e., disappearance of the preovulatory follicle).

2.4. Antral follicle count

The antral follicle count (AFC) was evaluated on d0, d4, d7, d13 of the treatment cycle as well as dOv, and dflush of the post-treatment cycle following established protocols (Kaps et al., 2021). Both ovaries were scanned extensively from the ovarian pedicle to the ovarian ligament, with multiple passes conducted. Videos lasting 15 s were recorded in DICOM format. The video recordings were precisely analyzed frame by frame using the MicroDicom DICOM Viewer (Visus Health IT, Bochum, Germany) (Kaps et al., 2021). Follicles, along with their maximum diameter, were noted on an "ovarian map" generated from the analysis. In addition, the number of follicles within specific size ranges (0 - <5, 5 - <10, 10 - <15, 15 - <20, 20 - <25, 25 - <30 and ≥ 30 mm in diameter) was determined.

2.5. Ovarian cycle characteristics and follicular dynamics

The following reproductive parameters were evaluated: inter-ovulatory interval (days between consecutive ovulations), growth rate of the emerging and dominant follicle, diameter of the pre-ovulatory follicle at two days and one day before ovulation in the estrus of the treatment cycle (Claes et al., 2015, 2017). The dominant follicle growth rate was determined (mm/day) from deviation until two days before ovulation using the following formula: (diameter of the pre-ovulatory follicle two days before ovulation - diameter of the dominant follicle at deviation)/number of days from deviation until two days before (Ginther et al., 2004; Claes et al., 2017).

2.6. Embryo recovery and evaluation

Embryos were retrieved using an intrauterine silicone two-way Foley catheter (size CH 28 for mares, Minitube, Tiefenbach, Germany) via transcervical uterine lavage, and four sequential flushes of the uterus with 1 L of prewarmed (38 °C) Ringer's lactate solution (Fresenius Kabi, Graz, Austria), as described previously (Aurich et al., 2011).

After uterine lavage, mares received the $PGF_{2\alpha}$ analog cloprostenol (250 μg i.m.) to induce luteolysis. The recovered uterine fluid was filtered (75 μm pores, EmCon embryo filter; Immunosystems, Spring Valley, WI, USA) and the fluid retained in the filter cup was examined under a stereomicroscope at 40 × magnification. Embryos underwent five washes in a holding medium (Minitube), were assessed for size at 90 × magnification and morphologically classified according to developmental stage and quality, using a scale ranging from 1 (excellent) to 4 (degenerated) as previously described (Thouas et al., 2001).

All retrieved embryos were stained as described previously (Thouas et al., 2001; Gastal et al., 2021) with minor modifications. Briefly, embryos were removed from the holding medium, washed in phosphate buffered saline (PBS), and fixed in ice-cold 4 % paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) for 25 min at room temperature (RT). Embryos were washed in PBS and kept for 25 min at RT in PBS with Tween (PBT; 0.05 % Tween-20 in PBS), permeabilized in 0.2 % triton X-100 solution for 40 min at RT, washed three times and stored in 100 μL of PBT at 4 °C. For analysis of the embryo cell count, embryos were washed five times in PBS and incubated in Hoechst 33258 solution (1 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 10 min at RT protected from light. Finally, embryos were washed in PBT, then blastocysts were mounted with a coverslip onto a glass slide in a drop of glycerol. The stained cell nuclei were visualized by a Spinning disk confocal laser microscope (Eclipse Ti2-E/Yokogawa CSU-W1 Spinning Disk, Nikon, The Netherlands) and a scientific CMOS camera (Prime BSI, Teledyne Photometrics, Birmingham, UK). The *Z*-stacks encompassing the whole embryo were acquired with a step size according to the appropriate Nyquist sampling rate, using 20×/NA 0.75 and 10×/NA 0.45 objective magnification, and analyzed using the open-source software Fiji (NIH, Bethesda, MD, USA), (Schindelin et al., 2012) using a customized analysis script. Maximum intensity projections of the image stacks were created to display all information. Nuclei were detected and analyzed with the AI-based Fiji plugin StarDist (Schmidt et al., 2018), which allows the detection of touching and overlapping objects. Results were controlled by overlay images of the raw image data with the segmented nuclei masks.

2.7. Hormonal analysis

Blood samples were collected by puncture of one jugular vein, alternating on the left and right side, into 9 mL Vacutainer tubes with CAT serum coagulation activator (Vacuette, Greiner bio-one, Kremsmünster, Austria). The tubes were kept at RT and centrifuged within 30 min after collection at 1200 ×*g* at 25 °C for 10 min. The serum was immediately frozen and stored at −20 °C until further analysis.

Progesterone concentration was analyzed by enzyme-linked immunosorbent assay (Enzo Progesterone ELISA, Cat. No.: ADI-901-011, Enzo Life Sciences, Farmingdale, NY, USA) as described (Beyer et al., 2019). The intra-assay coefficient of variation was 4.3 %, the inter-assay coefficient of variation was 28.5 % and the minimal detectable concentration was 9.6 pg/mL (recommended detection range 15.62 pg/mL to 500 pg/mL). Serum anti-Mullerian hormone (AMH) concentrations were determined with an enzyme-linked immunosorbent assay (AL-115, Ansh Labs, Webster, TX, USA) as described previously and validated for equine plasma in our laboratory (Scarlet et al., 2018). The intra- and inter-assay coefficients of variation were 4.7 % and 2.2 %, respectively, and the minimal detectable concentration was 0.02 ng/mL. Analysis of estradiol-17β concentration was performed by direct enzyme immunoassay (Estradiol ELISA DE2693; Demeditec Diagnostics, Kiel, Germany) as described previously and validated in our laboratory (Gautier et al., 2024). The intra- and inter-assay coefficients of variation were 6.2 % and 11.7 %, respectively, and the minimal detectable concentration was 11.9 pg/mL Testosterone concentration was analyzed by direct enzyme immunoassay (Testosterone ELISA, Demeditec Diagnostics) without extraction. The assay has been previously validated for equine plasma in the authors' laboratory (Schrammel et al., 2016). The intra- and inter-assay coefficients of variation were 10.3 and 15.3 %, respectively, and the minimal detectable concentration was 0.05 ng/mL.

Table 1
Data on estrous cycle characteristics, follicular size and growth rate, pregnancy outcome and embryo characteristics in Haflinger mares (n = 9) with control, letrozole 0.5 mg/kg, and letrozole 1 mg/kg treatment during the luteal phase. Data are means ± SEM (Abbreviations: IOI: Inter-ovulatory interval, DF: dominant follicle; n.s.: not statistically significant).

	Control	Letrozole (0.5 mg/kg)	Letrozole (1 mg/kg)	Statistical analysis
IOI (days)	23.0 ± 0.8	21.1 ± 0.8	21.7 ± 1.0	n.s.
Size of preovulatory follicle (mm)	46.4 ± 2.0	41.9 ± 1.7	44.1 ± 0.9	n.s.
Size DF d13 (mm)	18.2 ± 3.0	18.7 ± 2.2	22.2 ± 2.5	n.s.
Size DF two days before ovulation (mm)	42.5 ± 3.2	39.1 ± 0.9	42.5 ± 1.2	n.s.
DF growth rate (mm/day)	3.2 ± 0.4	2.9 ± 0.3	3.0 ± 0.4	n.s.
Pregnancies (n)	6	8	5	n.s.
Embryo size (μm)	590 ± 126	616 ± 30	622 ± 53	n.s.
Cell count of the embryo inner cell mass (n)	4286 ± 1273	3907 ± 588	3562 ± 268	n.s.

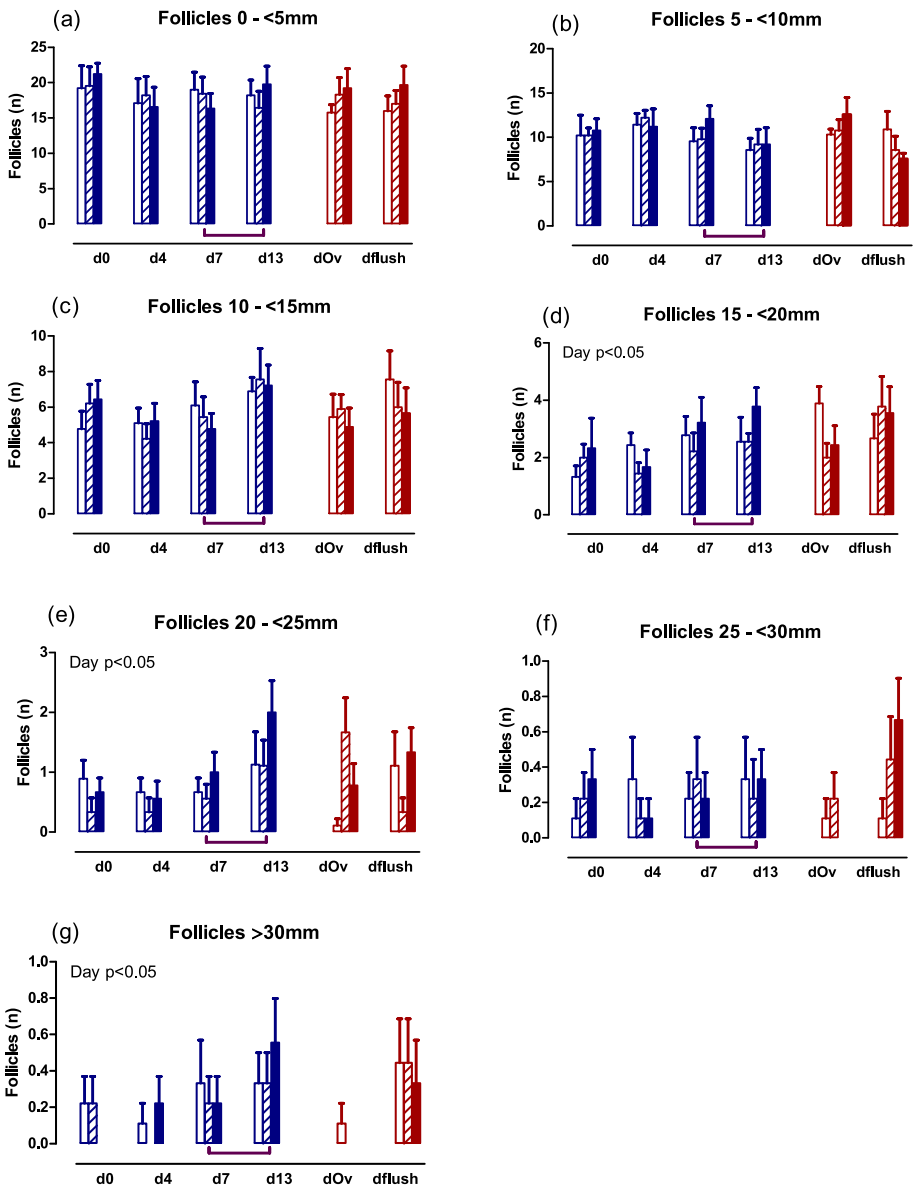

Fig. 2. Antral follicle counts (AFC) in mares after treatment with letrozole 0.5 mg/kg (hatched columns), letrozole 1 mg/kg (solid columns) and control (open columns) during the treatment cycle (blue colors) and the subsequent cycle (red colors). The treatment duration is indicated with the purple bracket. The follicle counts were grouped according to follicle size categories: (a) <5 mm, (b) 5 - <10 mm, (c) 10 - <15 mm, (d) 15 - <20 mm, (e) 20 - <25 mm, (f) 25 - <30 mm, and (g) ≥30 mm. Data are means ± SEM. The results of statistical analysis are given in the figures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.8. Statistical analysis

Statistical analysis was done with the IBM SPSS statistics software (version 29.0, IBM-SPSS, Armonk, NY, USA). Data were analyzed for normal distribution by the *Shapiro-Wilk* test and homogeneity of variance by *Levene* test. If data were not normally distributed, log transformation was performed. Differences between treatments over time were compared by the general linear model for repeated measures with time and treatment (control, letrozole 0.5, letrozole 1) as between subject factors. Differences in ovarian cycle characteristics, follicular dynamics, pregnancy rate, and embryo characteristics among treatments were compared by Friedman test. All values are given as mean ± standard error of mean (SEM) if not stated differently. A *P*-value <0.05 was considered significant.

3. Results

3.1. Estrous cycle and embryo characteristics

There were no effects of letrozole treatment irrespective of the dose on any of the estrous cycle characteristics determined in this study (Table 1). No significant differences in pregnancy rate, embryo quality, embryo size, and cell count of the inner cell mass of the embryo among the treatments were detected.

3.2. Ovarian follicular dynamics

The AFC before and after dOv did not differ among treatments, data are summarized in Fig. 2. There were no significant differences in follicle counts in the different size classes irrespective of treatment with letrozole. Follicle counts in the size classes 15 - <20 mm, 20 - <25 mm, and ≥ 30 mm changed over time ($p < 0.05$).

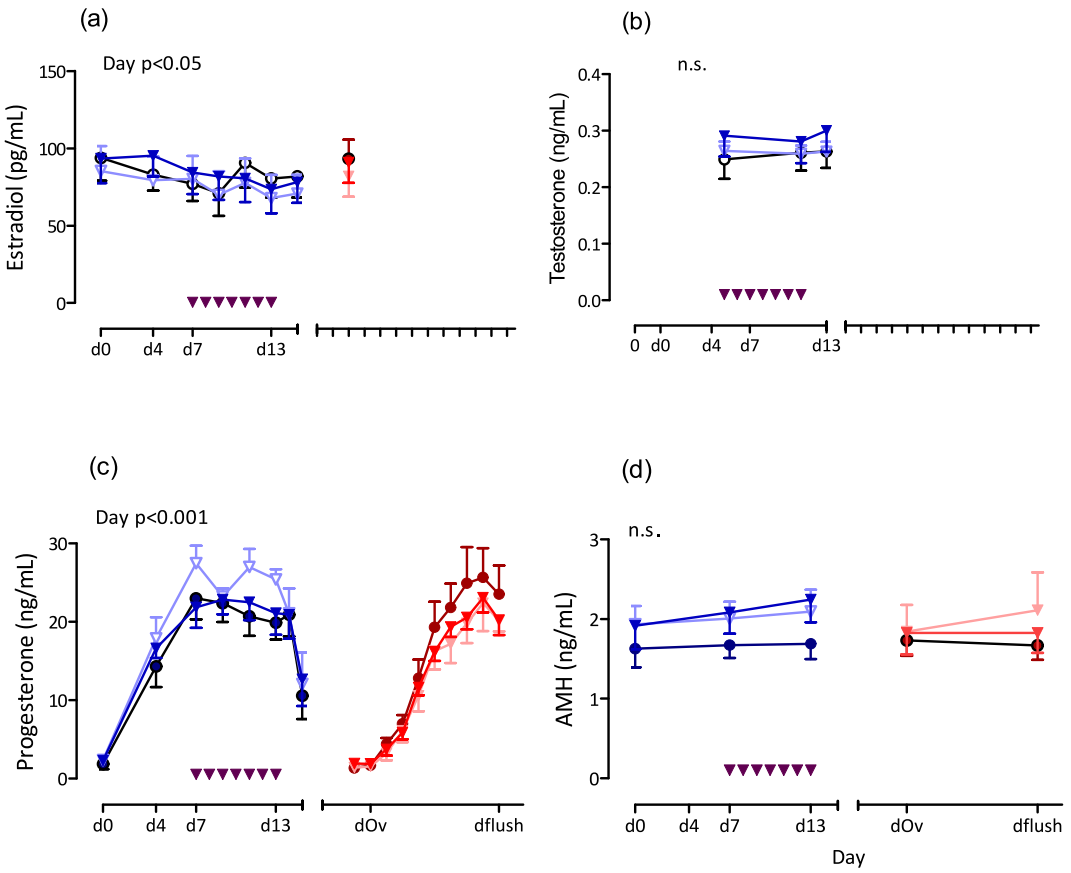

Fig. 3. Concentrations of (a) estradiol, (b) testosterone, (c) progesterone and (d) anti-Mullerian hormone (AMH) in mares during the experimental period, starting with the day of ovulation of the treatment cycle. Days with treatment are represented by purple arrowheads. Data from the treatment cycle are in blue color, from the post-treatment cycle in red color. Dark color circles: control, light color triangles: letrozole 0.5 mg/kg; medium color triangles: letrozole 1.0 mg/kg). Data are means ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Hormone concentrations

Serum estradiol concentrations showed some variations over time ($p < 0.05$) but there were no differences in estradiol concentration among treatments (Fig. 3). Similarly, testosterone concentration in serum before, during, and after letrozole application was not different among treatments. Serum progesterone concentrations increased after ovulation in both cycles and peaked around day 8 without any differences between letrozole and control treatments. There were no changes in AMH concentrations, irrespective of treatment.

4. Discussion

In this study, we analyzed the effects of the aromatase inhibitor letrozole on estrous cycle characteristics and fertility in non-pregnant mares during the breeding season. The letrozole treatment protocol used in this study was based on protocols reported to be successful in horses in previous studies (Akbarinejad et al., 2017; Esteller-Vico et al., 2017; Haneda et al., 2021). Nevertheless, in the present study, the results were largely negative and none of the animals showed any detectable changes of the characteristics investigated. Therefore, the question arises if the letrozole doses that we used for treatment were not efficient. In a previous study in non-pregnant cyclic mares, letrozole was given at a dosage of 0.5 mg/kg for three consecutive days starting at luteolysis (Akbarinejad et al., 2017). In pregnant mares, a dosage of approximately 1 mg/kg every four days for several weeks was chosen (Esteller-Vico et al., 2017; Haneda et al., 2021). The letrozole dosages used in this study are therefore comparable and should have been effective.

The AFC is the only endpoint of the present study where some changes during the treatment period between d7 and d13 could be determined, but notably no differences were seen regarding the three different treatments including untreated controls. This strongly suggests that letrozole treatment during the luteal phase did not influence subsequent follicular dynamics in mares. Temporal fluctuations in follicle numbers within specific size classes (15 – <20 mm, 20 – <25 mm, and ≥ 30 mm; $p < 0.05$) detected in this study most likely reflect physiological follicular dynamics independent of treatment.

Letrozole is an aromatase inhibitor, i.e., it reduces the aromatization of androgens into estrogens which will in turn increase the androgen/estrogen ratio in both, the circulation and body tissues (Bhatnagar, 2007). In the present investigation, there were no changes in the concentrations of estradiol and testosterone in response to letrozole treatment. This result contrasts with previous findings in pregnant mares where the blood estradiol concentration was clearly reduced by letrozole. The effect in pregnant mares was inhibition of placental and not of follicular estrogen synthesis (Esteller-Vico et al., 2017; Haneda et al., 2021). In agreement with the present findings, letrozole treatment did also not change estrogen concentrations in the circulation of mares before day 100 of pregnancy (Haneda et al., 2021), i.e., before the onset of estrogen biosynthesis by the feto-placental unit (Conley and Ball, 2019) but only thereafter (Haneda et al., 2021) and during the last trimester of gestation (Esteller-Vico et al., 2017). It is also important to note that in these previous investigations, letrozole-treatment never resulted in complete ablation of estrogen synthesis in pregnant mares, i.e. estrogen concentrations during the phase of placental estrogen synthesis were still increased in comparison to estrus or early pregnancy (Esteller-Vico et al., 2017). Why letrozole did not inhibit follicular

estradiol synthesis to an extent that resulted in changes of estrous cycle characteristics in the present study is unclear. In horses, there is a very active aromatase enzyme system responsible for the conversion of androgens of thecal origin into estradiol by granulosa cells (Sirois et al., 1991; Christensen, 2011). To the best of our knowledge, there is no evidence that the follicular aromatase system of horses should not be affected by letrozole - at least to a certain extent.

In letrozole-treated women, aromatase inhibition is apparently also not complete, and estrogens are still detectable in the circulation (Poulsen et al., 2022). Nevertheless, letrozole treatment stimulates ovarian function, i.e., induces follicular development and ovulation in anovulatory women via enhancement of FSH release (Holzer et al., 2006). Increasing the letrozole dose from 2.5 and 5 mg daily to doses as high as 12.5 mg per day was beneficial regarding the ovarian response of treated women and without apparent negative side effects (Pritts et al., 2011). In the present study, we treated mares with two dosages of letrozole that were already reported to be successful in female horses (Esteller-Vico et al., 2017; Haneda et al., 2021; Akbarinejad et al., 2017). In our study, the treatment was started not at the beginning of estrus (Akbarinejad et al., 2017), but shortly before follicle deviation during the luteal phase (Aurich et al., 2011). Follicle deviation involves important changes in the future dominant follicle which include an increased sensitivity to circulating concentrations of FSH (Beg and Ginther, 2006). On the day before follicle deviation, estradiol production in the future dominant follicle increases (Gastal et al., 1999), resulting in an inverse relationship between circulating concentrations of FSH and inhibin (Bergfelt et al., 1991). When we designed the experimental design of the present study, we therefore expected that starting letrozole treatment at this time would reduce the inhibitory actions of the dominant follicle and thus allow for simultaneous growth of more than one follicle to preovulatory size. This expectation was unfortunately not met in this study.

Interestingly, in stallions, letrozole has been reported to boost the testosterone/estradiol ratio and enhance overall reproductive performance (Stein et al., 2002), but the fact that this information is available in abstract form only may question the reliability of these data. Nevertheless, the effects of letrozole treatment on reproductive function were also reported in male pigs (At-Taras et al., 2006, 2008), goat bucks (Rezaei et al., 2020), and roosters (Ali et al., 2017). Differences among species, sexes, and treatment regimens may account for unpredictability in the effectiveness of the aromatase inhibitor letrozole in altering reproductive functions.

5. Conclusion

Treatment with the aromatase inhibitor letrozole from the moment of follicle deviation did not result in any changes in estrous cycle characteristics and ovarian function in mares. Previous studies in mares suggest a stronger effect of letrozole on placental than on ovarian estradiol synthesis in the horse. The results of this study scrutinize the suitability of this aromatase inhibitor for assessment of follicular estradiol function in non-pregnant mares. Future studies should aim at investigating letrozole treatment at higher doses, longer duration and during other phases of the estrous cycle of mares.

CRediT authorship contribution statement

Younis Khan: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Camille Gautier:** Validation, Methodology, Data curation, Conceptualization. **Christine Aurich:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Amr El-Shalofy:** Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors do not have any interest to declare.

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