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Effects of short-term oral letrozole on fresh semen parameters, endocrine balance, and prostate gland dimensions in domestic dogs

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Abstract

Background Aromatase inhibitors improve male fertility by modifying the hormonal control of spermatogenesis. The present study aimed to investigate the effects of oral administration of letrozole on testosterone and estradiol concentrations and their ratios in blood serum, seminal plasma, prostatic fluid, sperm quality in fresh semen, and prostate gland dimensions. Seven adult male intact mixed-breed dogs were selected. The animals received letrozole (72 µg/kg, PO) daily for four weeks. Blood samplings and semen collections were carried out on days 0 (control), 14 (treatment), 28 (treatment), and 42 (post-treatment).

Results Our results showed that letrozole administration resulted in a 4.3 fold significant increase in serum, seminal plasma, and prostatic fluid testosterone levels after 14 days. This remained high until the end of the study. Serum and prostatic fluid estradiol levels did not change significantly over the study period. However, the seminal plasma estradiol level showed a significant increase on day 14. The estradiol: testosterone ratio was significantly reduced on day 14 in serum, seminal plasma, and prostatic fluid samples. Letrozole significantly improved the ejaculated spermatozoa viability and concentration after 28 days of oral administration. However, the sperm plasma membrane functional integrity and kinematic parameters were not significantly affected by the treatment. Transabdominal ultrasound examination revealed a significant increase in the height, width, and volume of the prostate gland after 28 days of treatment.

Conclusions According to the present research, oral administration of letrozole for 28 days affects local and systemic sex hormone balance leading to an improvement of the ejaculated canine spermatozoa viability and concentration concurrent with an increase in the prostate gland dimensions.

Keywords Aromatase inhibitors, Hormone, Spermatogenesis, Prostate

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Background

Spermatogenesis is closely regulated through paracrine/endocrine mechanisms [1, 2]. Secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary stimulates the production of testosterone, dihydrotestosterone, and finally estradiol from Leydig and Sertoli cells. Both androgens and estradiol play crucial roles in maintaining normal spermatogenesis [3]. A concrete body of evidence shows that both absolute testosterone and estradiol concentrations, as well as the estradiol: testosterone ratio at either systemic or local levels are important determinants of normal spermatogenesis [4]. While estrogens play a role in regulating sperm maturation, motility, differentiation of somatic cells, and proliferation of spermatogonia and Sertoli cells [5], there are dose-dependent inhibitory and stimulatory effects of estradiol on testicular cell function [6]. An excess estrogen concentration may harm testicular function by decreasing the expression of androgen receptors [7]. Estrogen suppresses LH secretion using a negative feedback mechanism on the hypothalamus and pituitary. The suppression of estrogen increases testosterone levels by stimulating LH. Testosterone secretion mainly leads to germ cell differentiation by regulating Sertoli cell function, potentially improving reproductive performance [8].

Estrogen: testosterone ratios are influenced by various factors, especially the aromatase enzymatic complex, which is responsible for converting testosterone to estradiol in ovaries and testicular cells of different species [9]. However, increasing intratesticular estrogen and the resulting elevation of the estrogen: testosterone ratio has the potential to cause infertility in transgenic male mice [10]. Aromatase inhibitors (AIs) prevent the conversion of androgens into estrogens in the absence of high estrogen levels in oligozoospermic/azoospermic men [11]. The efficacy of AIs has been studied for their effects on spermatogenesis and semen quality in rats [12], fish [13], lizards [14], markhoz goats [15, 16], stallions [17], humans [18–20], and in dogs [21–23]. Letrozole, a reversible AI, is one of the options used to treat human oligospermia and azoospermia [24]. Rezaei et al. (2022) showed that subcutaneous injection of this drug in bucks increased seminiferous tubule diameter, Sertoli cell number, semen volume, and sperm concentration [15]. Consistent with the previous studies, Wang et al. (2018) found that this treatment could promote the proliferation rate of mouse spermatogenesis cell lines [25]. A study on stallion concluded that the early mention medication increases serum testosterone concentrations and decreases serum levels of estradiol and inhibin [17].

Some experimental studies support evidence that testosterone, dihydrotestosterone, and the balance of androgens and estrogen have prominent roles in prostate growth and maturation [26–28]. Prostatic hyperplasia and hypertrophy can occur in AI-treated mice. It may be attributed to long-term estrogen deficiency and high concentrations of androgens [29]. Similarly, another study found that letrozole causes an increase in the size of the prostate lobes in rats [30]. It needs to be noted that the etiopathology of prostatic hyperplasia and hypertrophy is different among various species [31].

Some dogs are referred to veterinary clinics due to low semen quality. AIs such as 4-androstene-4-ol-3,17-dione and anastrozole [22, 23] have been used to improve semen quality but the effect of letrozole on canine semen quality, reproductive hormones, and prostatic volume has not been studied. Animal studies have served as the basis for further experimental studies and applications in human subjects [32–34]. In this study, our objective was to assess the impact of orally administering of the third generaion type II AI on the semen quality of healthy adult dogs. We assessed the prostatic volume, size, and shape during this treatment by ultrasonography.

Results

Serum testosterone concentration

The testosterone concentration was increased from 11.61±18.54 ng/ml (day 0, control) to a maximum concentration of 61.8±2.11ng/ml on day 42. The serum testosterone concentration increased 4.32 times during the study (Table 1). Testosterone concentrations on days 14, 28, and 42 were significantly increased compared with the pre-treatment (control) testosterone concentration on day 0 ($P<0.01$), but no significant differences were found between testosterone concentrations on days 14, 28, and 42. The trend of standard deviation indicated a decrease from the beginning to the end of the study, so some differences in testosterone concentrations were detected between individual dogs at the start of the study (Table 1).

Serum estradiol concentration

The changes in estradiol concentrations were not significantly different between the sampling days (Table 1).

Serum estradiol: testosterone ratios

The serum estradiol: testosterone ratios were significantly decreased during and after treatment compared to before it ($P<0.03$; Table 1).

Seminal plasma testosterone concentration

The seminal plasma testosterone concentration was increased from day 0 to day 28 and 42 ($P<0.04$), and from day 14 to 42 of sampling ($P<0.001$). The concentration of seminal plasma testosterone increased gradually from day 0 (0.14±0.15 ng/ml) to day 42 (26.3±11.42 ng/ml) about 186.85 times (Table 1).

Table 1 The effect of letrozole administration (72 µg/kg, PO) on concentration (means ± SD) and changes of testosterone, estradiol, and estradiol: testosterone ratio in serum and semen samples studied before (day 0), during (day 14 and 28), and after (day 42) treatment of adult mixed breed dogs

Factors		Day				Changes (%)
		0	14	28	42	
Hormones						
Testosterone (ng/ml)	*Serum*	11.61 ± 18.53^{a}	44.55 ± 18.90^{b}	40.95 ± 19.48^{b}	61.80 ± 2.12^{b}	432.30
	Seminal Plasma	0.14 ± 0.15^{a}	5.89 ± 8.91^{ab}	13.95 ± 11.02^{b}	26.30 ± 11.42^{bc}	18685.71
	Prostatic fluid	0.12 ± 0.09^{a}	6.62 ± 12.26^{ab}	14.44 ± 16.18^{ab}	18.33 ± 13.80^{b}	15175.00
Estradiol (pg/ml)	*Serum*	116.39 ± 46.71	352.63 ± 279.14	396.76 ± 302.04	385.40 ± 424.83	231.13
	Seminal Plasma	75.09 ± 32.89^{a}	111.70 ± 11.56^{b}	106.27 ± 11.50^{ab}	104.96 ± 24.54^{ab}	39.78
	Prostatic fluid	90.49 ± 28.78	110.23 ± 20.27	117.17 ± 14.27	94.59 ± 33.24	4.53
Estradiol: Testosterone Ratios	*Serum*	0.11 ± 0.12^{a}	0.01 ± 0.01^{b}	0.01 ± 0.01^{b}	0.006 ± 0.006^{b}	-94.55
	Seminal Plasma	0.82 ± 0.62^{a}	0.11 ± 0.10^{b}	0.05 ± 0.08^{b}	0.005 ± 0.003^{b}	-99.39
	Prostatic fluid	1.15 ± 0.81^{a}	0.17 ± 0.13^{b}	0.07 ± 0.09^{b}	0.01 ± 0.02^{b}	-99.13

[abc] Different superscript letters indicate significant different ($P < 0.05$) in each row

Seminal plasma estradiol concentration

The seminal plasma estradiol concentration significantly increased from day 0 compared to day 14 ($P=0.02$). There were no significant differences in estradiol seminal plasma concentrations among other days of sampling (Table 1).

Seminal plasma estradiol: testosterone ratios

The estradiol: testosterone ratios were significantly decreased between day 0 and day 14, 28, and 42 ($P<0.001$). This ratio decreased from day 0 to day 42 about 99.39% (Table 1).

Prostatic fluid testosterone concentration

The prostatic fluid testosterone concentration was increased about 151.75 times from day 0 compared with day 42 ($P=0.04$; Table 1). There was no significant increase in prostatic fluid testosterone concentration on other days.

Prostatic fluid estradiol concentration

There were no significant differences in prostatic fluid estradiol concentration between different sampling days (Table 1).

Prostatic fluid estradiol: testosterone ratios

The decrease in prostatic fluid estradiol: testosterone ratios was significant between day 0 and days 14 and 28 ($P<0.001$). The prostatic fluid estradiol: testosterone ratios decreased significantly decreased by approximately 99.13% between day 0 and day 42 ($P<0.001$; see Table 1).

Sperm analysis

Oral letrozole administration for four weeks improved significantly ($P<0.01$) the percent of live sperm using eosin-nigrosine staining in the fresh semen. In contrast, the sperm membrane functional integrity (HOST-positive) and abnormal morphology were not affected by the treatment. The sperm concentration increased significantly after 28 days of this treatment in compared to days 0 and 14 ($P<0.02$). However, it did not change until the end of treatment period (Table 1). This treatment did not affect the fresh sperm kinematic factors during the current study ($P>0.05$; Table 2).

Prostate gland dimensions

The transabdominal ultrasound examination of the prostate gland indicated a significant increase in the prostatic gland height, width, and volume after 28 days of letrozole treatment in compared with days 0 and 14 ($P \leq 0.05$, Table 3).

Discussion

Oral letrozole administration (72 µg/kg) for 30 days increased testosterone concentration and decreased estradiol: testosterone ratios in serum, seminal plasma, and prostatic fluid. Furthermore, the viability and concentration of sperm improved following this treatment and the prostate gland volume increased in treated dogs. Testosterone, FSH, and LH concentrations are mainly affected by the circulating estradiol level, as estradiol acts a negative feedback on GnRH. In men, low estradiol levels cause a rapid increase in testosterone, FSH, and LH levels [35–37]. The treatment of two azoospermic dogs with high plasma estradiol-17 beta concentration, using AI 4-androstene-4-ol-3,17-dione for 4 weeks, decreased the plasma estradiol concentration. Consequently, plasma testosterone concentration increased, and a small number of sperm were detected in the semen 3–6 weeks after the start of treatment [23].

It has been described that type I estrogen antagonists, such as tamoxifen, inhibit the function of estrogen. These drugs may induce some estrogenic responses [38]. A reduction in prostatic volume, testosterone

Table 2 The effect of letrozole administration (72 µg/kg, PO) on semen quality parameters and sperm kinematics (means ± SD) studied before (day 0), during (day 14 and 28), and after (day 42) treatment of adult mixed breed dogs

Factors	Day				Changes (%)
	0	14	28	42	
Sperm parameters					
Live Sperm (%)	88.43 ± 1.57^{ac}	87.43 ± 1.78^{a}	93.00 ± 1.00^{bc}	94.00 ± 0.69^{b}	6.30
Abnormal Morphology (%)	2.29 ± 0.18	3.29 ± 0.52	3.14 ± 0.74	4.86 ± 0.91	112.23
HOST Positive (%)	93.86 ± 2.14	95.00 ± 1.15	91.43 ± 2.84	95.71 ± 0.84	1.97
Sperm Concentration (million/ml)	184.79 ± 45.89^{a}	202.60 ± 27.05^{a}	335.29 ± 30.68^{b}	324.43 ± 37.40^{b}	75.57
Sperm kinematic parameters					
TM	95.51 ± 3.34	93.48 ± 2.94	91.70 ± 3.05	96.03 ± 1.23	0.54
PM	47.58 ± 4.19	44.38 ± 2.15	49.34 ± 2.29	50.58 ± 2.87	6.31
VCL	75.74 ± 8.59	77.03 ± 5.68	92.29 ± 5.45	79.66 ± 5.59	5.18
VAP	39.36 ± 3.88	38.35 ± 2.68	48.24 ± 2.49	41.71 ± 3.08	5.97
VSL	26.74 ± 2.72	25.5 ± 2.02	34.45 ± 1.80	28.81 ± 2.55	7.74
STR	67.95 ± 1.75	66.25 ± 1.23	71.43 ± 0.94	68.52 ± 1.55	0.84
LIN	36.26 ± 1.97	33.07 ± 0.64	37.62 ± 1.83	35.93 ± 1.64	-0.91
WOB	53.21 ± 2.05	49.92 ± 0.51	52.56 ± 1.96	52.31 ± 1.41	-1.69
ALH	3.82 ± 0.37	4.02 ± 0.22	4.37 ± 0.21	4.09 ± 0.18	7.07
BCF	4.12 ± 0.55	4.13 ± 0.28	3.11 ± 0.21	4.25 ± 0.25	3.16
MAD	68.80 ± 7.44	70.82 ± 4.77	69.35 ± 6.00	72.31 ± 3.46	5.10

^{abc} Different superscript letters indicate significant different ($P < 0.05$) in each row

ALH = Amplitude of Lateral Head Displacement (µm), BCF = Beat Cross Frequency (Hz), HOS = Hypo Osmotic Swelling, LIN = Linearity (%), MAD = Mean Angular Displacement (°), STR = Straightness (%), VAP = Velocity Average Path (µm/s), VCL = Velocity Curvilinear (µm/s), VSL = Velocity Straight Path (µm/s), and WOB = Wobble (%)

Table 3 The effect of letrozole administration (72 µg/kg, PO) on prostate dimensions and volume (means ± SD) studied before (day 0), during (day 14 and 28), and after (day 42) treatment of adult mixed breed dogs

Factors	Day				Changes (%)
	0	14	28	42	
Prostate measures					
Height (cm)	3.06 ± 0.14^{a}	3.17 ± 0.07^{a}	3.73 ± 0.08^{b}	3.72 ± 0.09^{b}	21.57
Length (cm)	3.15 ± 0.22	3.17 ± 0.19	3.16 ± 0.17	3.19 ± 0.14	1.27
Width (cm)	3.68 ± 0.19^{a}	3.68 ± 0.18^{a}	4.18 ± 0.10^{b}	4.38 ± 0.10^{b}	19.02
Volume (cm^3)	18.94 ± 2.56^{a}	19.62 ± 2.20^{a}	25.94 ± 2.00^{b}	26.98 ± 1.99^{b}	42.45

^{abc} Different superscript letters indicate significant differences ($P < 0.05$) in each row

concentration, and quality of semen was observed when this treatment was used in Beagle dogs [21]. Corrada et al. (2004) also showed the estrogenic impact of this medication on hypothalamic-pituitary levels, resulting in lower plasma FSH and LH levels and, consequently, lower plasma testosterone concentrations [21]. Although several researchers have challenged the effect of this drug, the results of some studies support the idea that AIs can increase spermatogenesis in humans, dogs, and rats [12, 39].

Letrozole has been found to cause parallel estradiol reduction, thereby increasing testosterone and stimulating spermatogenesis [40]. Several studies investigating the effects of this treatment on the quality of semen have demonstrated a positive effect on sperm concentration and motility [20, 40]. Ribeiro et al. (2016) hypothesized that AI therapy has a progressive impact on spermatogenesis and LH concentrations [11]. Other studies have focused on the effects of letrozole and anastrozole on the quality of semen and estradiol: testosterone ratios in infertile men, with the results indicating improved sperm concentration, motility, semen volume, estradiol: testosterone ratios, and serum testosterone [40]. Similarly, Saylam et al. (2011) prescribed this treatment in infertile men, noting significant decreases in estradiol: testosterone ratios and increases in sperm motility and concentration after treatment [41]. Party et al. (2009) reported that, after using letrozole for up to four months in a man with primary infertility, normal spermatogenesis was detected in the testicular biopsy [42]. A study on the effects of this medication in healthy adult male horses further supports the effect of AIs, with researchers concluding that the drug increases serum testosterone concentration and decreases serum levels of estradiol and inhibin in stallions. However, this study showed that the treatment had no effects on sperm production [17].

The reduction in estradiol levels caused by AIs was linked to higher levels of serum FSH and LH, as well as an increase in circulating testosterone levels in elderly men [43]. The main sites where the aromatase substrate, essential for estrogen synthesis, is produced are predominantly Leydig, Sertoli, and germinal cells [43]. Therefore, it is likely that letrozole enhances the process of spermatogenesis and boosts sperm concentration through a potential increase in FSH levels [43]. Rezaei et al., (2020) demonstrated an elevation in the Sertoli cell count within the testes of bucklings injected with letrozole. The stimulation of Sertoli cells by this medication is attributed to various mechanisms including: (i) augmentation of LH-induced testosterone secretion, (ii) direct enhancement of FSH levels, and (iii) suppression of estrogens and/or estrogen receptor blockade [16]. Sertoli cells play a crucial role as the primary recipient cell for FSH and testosterone, creating a conducive setting that supports the development, maturation, and proliferation of germ cells into spermatozoa through physical interaction [44]. While it is widely documented that anastrozole and letrozole effectively inhibit aromatase activity by nearly 100%, the use of these inhibitors in men does not lead to complete suppression of plasma estradiol levels [36, 37, 45].

Gonzalez et al. (2009) investigated the effect of 60-day administration of the AI anastrozole and the antiestrogenic receptor blocker tamoxifen on normal and hyperplastic prostate glands in dogs. Anastrozole decreased prostate volume to a greater exent than tamoxifen in dogs with hyperplastic prostate glands. In normal dogs, libido, testicular consistency and scrotal diameter, semen volume, sperm count, motility, and morphological abnormalities remained unaltered in the anastrozole group throughout the study [22]. AIs are effective in counteracting stromal changes associated with estrogens due to the build-up of androgens, the aromatase substrate. Some developed AIs have been found to influence the epithelial and stromal compartments of canine benign prostatic hyperplasia, decreasing intraprostatic aromatase activity, estradiol levels, and androgenic receptors [45]. The prostate is an androgen-dependent organ, but both animal [46] and human [47] studies suggest that estradiol has an independent effect on the prostate. This is because testosterone is aromatized to estradiol, meaning that some of the trophic effects of testosterone on the prostate might be mediated via its aromatization to estradiol [43]. In a study in older men treated with transdermal testosterone gel, prostate volume significantly increased (despite on-treatment serum testosterone levels being similar in the AI group), suggesting that the trophic effects of testosterone on prostate volume are mediated via its aromatization to estradiol. In contrast, serum prostate specific antigen (PSA) increased significantly (although within the normal range) in both the testosterone and AI groups, suggesting that the increase in PSA is primarily an androgen-driven process [43].

In our study, prostate width, height and volume increased significantly after 28 days treatment of dogs with letrozole. The focal prostatic hyperplasia, as well as the enlargement of the anterior and dorsal prostate lobes, were observed in peripubertal rats following both short-term and long-term treatment with AIs [30]. The equilibrium between androgens and oestrogen plays a crucial role in both the normal function and disorders of the prostate [28]. The manifestation of prostatic hyperplasia resulting from temporary suppression of aromatase in peripubertal animals aligns perfectly with the finding that prolonged lack of estrogen leads to prostatic hyperplasia and enlargement in mature aromatase knockout mice [48].

The present study is the first investigation into the impact of letrozole on canine semen quality and reproductive hormone (testosterone and estradiol) concentrations in serum, semen, and prostate. The results and conclusions of this study are limited by the small number of dogs, the short period devoted to monitoring, the lack of measurement of testicular size and volume, and the lack of FSH and LH assay.

Conclusions

In conclusion, this study has demonstrated the efficacy of letrozole in changing serum, semen, and prostatic fluid testosterone concentrations and estradiol: testosterone ratios. Increasing sperm viability and concentration may have been attributed to the reduction in estradiol: testosterone ratio. Therefore, the potential clinical effects of this treatment in low fertility or infertile dogs should be considered. Further studies are suggested to improve our understanding of the mechanism behind this drug's efficacy and to evaluate the clinical efficacy of this medication on subfertile and infertile dogs.

Methods

The Iranian animal ethics framework under the supervision of the Iranian Society for the Prevention of Cruelty to Animals and Shiraz University Research Council approved experimental protocols in this study (IACUC no: 4687/63). The European Council Directive (2010/63/EU) recommendations of September 22, 2010, regarding the standards in the protection of animals used for experimental purposes, were also followed.

Animals

Seven adult male intact mixed-breed dogs, aged 3 ± 1 years old and weighting 20 ± 2 kg, were selected and enrolled for this study. General and reproductive examinations were performed using palpation and ultrassonography and healthy animals with fertile

reproductive histories were included in the study. The dogs were trained for semen collection and produced normal ejaculates [49]. They were owned and kept by the School of Veterinary Medicine of Shiraz University. All dogs received 300 g/dog/day of commercial dog food (Nutri® dry dog food; Behintash Co. Iran), and they had free access to water. The dogs were adapted to the new condition for two weeks. Anti-parasitic treatment was performed using Panvermic® tablets (praziquantel 50 mg; mebendazole 220 mg; pirantel Pamoate 144 mg; Drag Pharma, Chile). Praziquantel and mebendazole were administered at doses of 5 mg/kg and 22 mg/kg, respectively.

Experimental design

This research was designed as a longitudinal cohort study for 42 days, with the sampling on day 0 considered the control group compared with other days of sampling. Blood and semen samples were collected on days 0 (control), 14, 28 (treatment), and 42 (post-treatment). The dogs received 72 µg/kg of oral letrozole tab (Letrofem 2.5 mg, Iran Hormone, Iran) daily for four weeks from day 1 of the study [50]. The canine dose of this drug was estimated based on the human dose of this drug and the following equation: [Canine allometric coefficient (1.8)×human dose (mg/kg)] [51]. The blood samples were collected from the jugular vein into simple glass tubes at 9 a.m., centrifuged for 10 min at 750 ×g, and the serum samples stored at -20 °C. The semen samples were collected by one operator using manual masturbation into pre-warmed (37 °C) plastic tubes (Falcon, USA). Prostate fluid was collected at the end of the semen collection. The fresh semen centrifuged for 10 min at 600×g and the seminal plasma preserved at -20 °C for further analysis.

Prostate ultrasound

Ultrasound examination of the prostate was performed using an ultrasound machine equipped with a transabdominal micro convex probe 3.5–10 MHz (SIUI 900 V, China) before semen collection on days 0, 14, 28, and 42. Prostate volume was calculated by measuring length in the longitudinal section and width and depth in the transverse section of preserved ultrasound images. The volume of the prostate was calculated using the following function: prostate volume (cm3)=[(L×W×D)/2.26]+1.8 [52]. The expected normal prostate volume of dogs was estimated by their weight and age: Prostate volume=(0.867×BW)+(1.885×age)+15.88 [53] and compared with the calculated prostate volume using ultrasound exam to confirm that there was no prostatic hyperplasia at the beginning of the study.

Hormone assays

The concentration of estradiol (intra-assay C.V. <9%; sensitivity 8.68 pg/ml; Diametra, Italy) and testosterone (intra-assay C.V. 4.8%; sensitivity 57 pg/ml; Monobind, USA) in serum, seminal plasma, and prostatic fluid were measured using solid-phase sandwich ELISA commercial kits. The ELISA kits were validated for measuring estradiol and testosterone in dog`s serum, seminal plasma, and prostatic fluid samples in the Laboratory of Clinical Pathology, School of Veterinary Medicine, Shiraz University [54].

Fresh semen primary preparation

Upon receipt, all semen samples including three fractions of ejaculation were centrifuged for 10 min at 600 ×g in a pre-warmed (37 °C) laboratory centrifuge equipped with a swing-out rotor [55]. Next, a part of the seminal plasma was removed for hormone measurements so that 2 ml remained above the sperm pellet. The sperm pellet was then gently resuspended in remaining seminal plasma, and the resulting sperm suspension was used for the downstream procedures as follows:

Kinematic parameters

Sperm motion parameters were objectively evaluated using Computer-Assisted Sperm Analysis (CASA) software (Houshmand Fanavar, Tehran, Iran). A 10 µL sample of sperm was loaded onto a pre-warmed (37 °C) sperm chamber (Sperm meter™, India) with a chamber depth of 10 micrometers. Immediately, the sperm motion parameters were analyzed under a bright-field light microscope (Pro-Way, China). A minimum of 400 cells in 4 random microscopic fields (×100 magnification) were included in the analysis. A sperm cell was classified as immotile when the Velocity Average Path (VAP) value was less than 10 mm/second, and the CASA frame rate was set to 50 Hz.

Sperm concentration

The sperm concentration was calculated using the standard hematology hemocytometer method. A sample of sperm suspension was diluted at 1:100 (dilution factor, df) in double distilled water, loaded into a hemocytometer (Neubauer, Germany) chamber, and subjected to sperm counting for the final cell concentration calculations in each mL of the sample. The total number of sperm (n) was counted in five squares, with at least 200 sperm for normal semen samples. Then, the number of sperm in each milliliter was calculated using the following formula: n×df×50,000.

Sperm viability and morphology

The eosin-nigrosine supravital staining procedure was used to determine the viability status of sperm cells.

The stain and microscopic slide need to be prewarmed (37 °C). Equal volumes (200 µl) of the sperm suspension and stains were mixed on a microscopic slide, smeared after 30 s, and air-dried immediately. At least 200 cells were observed under a bright-field light microscope (Olympus, Japan) with ×1000 magnification in immersion oil. Sperm cells, excluding the eosin stain in the head region, were considered to be membrane-intact and live spermatozoa and sperm cells with a stained head were considered to be membrane disrupted and dead spermatozoa. Also, the sperm with abnormal morphologies in the head, mid-piece, or tail regions were classified as abnormal [56].

Sperm plasma membrane functional integrity

The functional integrity of the sperm was evaluated using a hypo-osmotic swelling test in a hypotonic sucrose solution. A 10 µL sample of sperm was mixed with 90 µL of pre-warmed (37 °C) hypotonic solution and incubated for 30 min at 37 °C. Next, a wet smear was prepared and evaluated under a bright-field light microscope (Olympus, Germany) with ×400 magnification. At least 200 cells were evaluated, and sperm cells with swelling/coiling of the tail were considered to have intact plasma membranes [56].

Statistical analysis

At first, the normal distribution of data was assessed and confirmed using the Kolmogorov–Smirnov test. The treatment effects were then analyzed by a one-way repeated-measures analysis of variance (ANOVA) test. Tukey's multiple comparison tests were used to compare differences between the sampling days. The analysis was performed using Graphed Prism Version 6 software. To calculate estradiol: testosterone ratios, the raw data of estradiol concentration for each dog was converted from pg/ml to ng/ml, and then the ratios were calculated for each dog on every sampling day. The percentage of changes was calculated using the following function: ((new value - original value) / original value) × 100. P values less than 0.05 were considered significant. Data were expressed as the mean and standard deviation (SD).

Abbreviations

AIs	Aromatase Inhibitors
CASA	Computer-Assisted Sperm Analysis
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
LH	Luteinizing Hormone
MAD	Mean Angular Displacement
PSA	Prostate Specific Antigen
SD	Standard Deviation

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Not applicable.

Author contributions

All authors contributed to all parts of the study from designing a study to writing and preparing of manuscript. A.M., N.D., and M.R.D. contributed to the study design, performing study, sampling, data collection and analysis, and preparing manuscript. S.N. and I.A. aimed in designing and performing study and laboratory analyses.

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Data availability

The data-sets analyzed during the current study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate

Ethical approval was provided by the ethics committee of the Iranian Society for the Prevention of Cruelty to Animals and the Shiraz University Research Council (IACUC no: 4687/63). The recommendations of European Council Directive (2010/63/EU) of September 22, 2010, regarding the standards in the protection of animals used for experimental purposes, were also followed. As the corresponding author, I hereby affirm, on behalf of all researchers engaged in this study, that we have collectively granted our informed consent to participate. Additionally, before the commencement of data collection, a comprehensive overview of the study's aims, methodologies, potential risks, and benefits was presented to the owner, and informed consent was duly acquired from all owners participating in the research.

Consent to publish

Not applicable.

Competing interests

The authors declare no competing interests.

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