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Effect of coumestrol on the epididymis of adult dogs

Efecto del coumestrol sobre el epidídimo de perros adultos

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ABSTRACT

The concern of canine overpopulation is related to zoonoses such as rabies, which is responsible for 99% of human rabies cases, causing the death of approximately 59,000 people per year. Surgical sterilization is an effective, costly and invasive control method with limited impact. The effects of phytoestrogens on reproductive activity have been commonly studied in females and a limited number in males. Therefore, the objective of this study was to know the effect of subcutaneous administration of coumestrol on the gonadal activity of adult dogs as an alternative for their reproductive control. Basic seminal evaluation parameters, epididymal structure by ultrasonography, histological characteristics, as well as the presence of coumestrol by fluorescence and serum levels of testosterone and estrogens were determined. The administration of coumestrol for five weeks reduced sperm production, and evidenced changes in the echodensity and cellularity of the epididymis, associated with serum concentrations of estradiol and testosterone. Therefore, it is concluded that coumestrol administered subcutaneously has an estrogenic effect that can be used as a non-invasive method to help control fertility in adult dogs.

Keywords: epididymis, spermatozoa, estrogens, phytoestrogens, testis, dog, reproductive control.

RESUMEN

La preocupación de la sobrepoblación canina se relaciona con zoonosis como la rabia, la cual es responsable del 99% de los casos de rabia humana, causante de la muerte de aproximadamente 59,000 personas al año. La esterilización quirúrgica es un método de control eficaz, costoso e invasivo siendo su impacto limitado. Los efectos de los fitoestrógenos en la actividad reproductiva se han estudiado comúnmente en hembras, y un número limitado en machos. Por lo anterior, el objetivo de este estudio fue conocer el efecto de la administración subcutánea de coumestrol en la actividad gonadal de perros adultos como alternativa para su control reproductivo. Se determinaron los parámetros de evaluación seminal básica, la estructura del epidídimo mediante ecografía, características histológicas, así como la presencia del coumestrol mediante fluorescencia y los niveles séricos de testosterona y estrógenos. La administración de coumestrol durante cinco semanas redujo la producción espermática, y evidenció cambios en la eco densidad y celularidad del epidídimo, asociados a las concentraciones séricas de estradiol y testosterona. Por lo que, se concluye que el coumestrol administrado vía subcutánea tiene un efecto estrogénico que puede utilizarse como un método no invasivo para ayudar a controlar la fertilidad de perros adultos.

Palabras clave: epidídimo, espermatozoides, estrógenos, fitoestrógenos, testículo, perro, control reproductivo.

INTRODUCTION

Dog overpopulation is a worldwide problem which influences public and animal health (Rubel & Carbajo, 2019), because they are a source of health, political, socioeconomic and welfare problems, particularly in developing countries. These problems include roaming the streets causing traffic accidents, barking, aggression, and biting. The growing population of stray dogs in Latin American countries is alarming, several factors contribute to the increase in their population, the most common being abandonment by the responsible parties, which is the main cause of the number of dogs roaming streets or living in dog shelters (Mota-Rojas *et al.,* 2021).

The main concern of dog overpopulation is related to zoonotic diseases, especially canine rabies, an underreported and neglected disease in developing countries, which is responsible for 99% of dog-transmitted human rabies cases worldwide, killing approximately 59,000 [95% confidence interval: 25,000-159,200] people worldwide annually. Efforts are underway to reduce human deaths due to dog-mediated rabies to zero by 2030 (Hampson *et al.,* 2015; Hampson *et al.,* 2019).

Surgical sterilization is the best method to prevent reproduction and, consequently, dog overpopulation. However, the impact of sterilization programs has been limited, mainly due to high operational costs that make it difficult to reach as many dogs as possible (Evans *et al.,* 2022; Belsare & Vanak, 2020). Because of this, mass slaughter continues to be used as the main method of population control in much of the world, being little accepted by society, so the World Health Organization published guidelines in 1990 that discourage the use of slaughter and recommend alternative methods such as contraceptive methods (Smith *et al.,* 2019).

Because of this, different research has focused on finding and proposing canine fertility control alternatives. It includes the use of synthetic hormones (e.g., synthetic androgens), chemical sterilization (e.g., zinc gluconate neutralized with arginine, calcium chloride), immunocontraception (e.g., gonadotropin hormone inhibitor vaccine) (Sandam *et al*., 2021; Root Kustritz, 2018; Asa, 2018; Massei *et al*., 2013), as well as, the use of therapeutic ultrasound at different frequencies (Leoci *et al.,* 2015), among others. However, some methodologies may present different adverse effects and have not been shown to be fully effective (Sandam *et al*., 2021; Asa, 2018; Root Kustritz, 2018). For such reason, as a complementary method, the use of phytoestrogens is observed.

Phytoestrogens (PE) are plant-derived polyphenolic compounds, which are divided into several groups, including isoflavones (genistein), lignans (enterolactone), flavones (luteolin), stilbenes (resveratrol) and coumestanes (coumestrol) (Adler *et al.,* 2015; Pérez-Rivero *et al.,* 2009). They have similar structures to endogenous estrogens and can induce or inhibit the response in hormone receptors because they have the ability to bind to estrogen receptors (ER) alpha (ERα) and beta (ERβ) located in tissues in different organs including the reproductive. This binding is with higher affinity to ERβ (Rietjens *et al.,* 2017).

Some research indicates that PEs act as endocrine disruptors by competing with endogenous estrogens for binding to their receptors and can produce positive or negative biological effects on animal health (Beszterda & Frański, 2018). Similar to what happens with any drug, it depends on the dose or route of administration of PEs the intensity of the effect on the target tissue, because they are subjected to different processes of absorption, biotransformation, distribution and excretion. In addition, their effects may vary depending on the phytoestrogen, species exposed, sex, route of administration, dose and duration of exposure, as well as the time of exposure during reproductive development or adult life. Thus, EFs have the ability to act as estrogenic agonists producing classical estrogenic effects or as estrogenic antagonists (inhibiting the effect of estrogens) (Domínguez-López *et al.,* 2020; Mostrom & Evans, 2018).

Additionally, PEs can modulate the hypothalamus-pituitary axis, they also have the ability to inhibit aromatase (cytochrome P450) mRNA expression, producing the decrease of local estrogen biosynthesis, resulting in cellular inhibition, including spermatogenesis, participating as intracellular regulators of cell cycle and apoptosis (Domínguez-López *et al.,* 2020; Lephart, 2015; Cerendolo *et al.,* 2009; Kawakami *et al.,* 2004).
Coumestrol (COU) is present in alfalfa (*Medicago sativa*), white clover (*Trifolium repens*), spinach (*Spinacia oleracea*) and soybean (*Glycine max*) sprouts (Cerendolo *et al.,* 2009; Domínguez-López *et al.,* 2020). It has the ability to act as an endocrine disruptor of ERα and Erβ, which are widely distributed in the male reproductive tract during development and adulthood (Cooke *et al.,* 2021). In the dog, the presence of ER has been detected in interstitial Leydig cells, round spermatids, spermatogonia, and in the connective tissue of the epididymis (Serrano *et al.,* 2008).
A peculiarity of COU is that it can be naturally detected by fluorescence when bound to ER, it is excited at a wavelength of 390-400 nanometers (blue) and emits fluorescence with a wavelength of 532-559 nanometers (green color) (Wang *et al.,* 2014).

COU has been studied as a possible method of reproductive control in dogs. In females when administered orally, it induces a decrease in progesterone levels and an increase in serum estrogen values, without affecting the cellularity of the vaginal epithelium (Peña-Corona *et al*., 2020). In males, when administered orally, it affects the histoarchitecture of the germinal epithelium of the seminiferous tubes (Pérez-Rivero *et al.,* 2009). The same effect has also been reported in hematophagous bats (*Desmodus rotundus*) males (Pérez-Rivero *et al.,* 2014).

Spermatozoa that pass from the testes to the epididymis are non-functional gametes. It is during their transit through the epididymis where, through their interaction with proteins, they mature, acquire progressive motility and the ability to fertilize eggs (Ali Hassan *et al.,* 2021; Cornwall, 2009), so the epididymis can be a target for the reproductive control of species considered to be disease transmitters. Due to the above, the aim of this study was to know the effect of subcutaneous administration of coumestrol in the epididymis of adult dogs as an alternative for their reproductive control.

MATERIAL AND METHODS

Animal Welfare

The study was approved with protocol number DC-2017/2-4, by the Internal Committee for the Care and Use of Animals from Veterinary Medicine and Zootechnics Faculty of the National Autonomous University of Mexico. It was performed with adherence to the principles of the 3 R's of research with animals (NCRRAR).

Animals

Ten male dogs between 2 and 3 years old, of different breeds, sexually mature, with an average weight of 14±2.9 kg, were included. Clinically healthy with both testes scrotalized.

Experiment

They were randomly divided into two groups with five dogs each: Control (C) and treatment with coumestrol (TCOU), and followed for 5 continuous weeks. In the first week, the TCOU group received a single dose of 1.2 mg/kg subcutaneously of coumestrol (COU, Sigma Aldrich® Lot# BCBH0742V Pcode: 101171142 and Lot# BCBN3969V Pcode: 101652427), dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co®) to have a COU concentration: 100 µg/µl, which was mixed with 300 µl chitosan vehicle. Group C only received the DMSO and chitosan vehicle in the same amount and route of administration (Miranda-Castro & Lizarraga-Paulin, 2012).

Clinical studies

Each dog in both groups: C and TCOU, prior to treatment administration, at the first week of treatment administration and subsequently every week for 4 occasions, underwent four evaluations: evaluation of seminal production and quality, ultrasound to evaluate testes and epididymis, and blood collection for determination of hormonal indicators.

Sperm concentration, morphology and motility

After obtaining the ejaculate, progressive motility was evaluated at 4X and 10X, sperm concentration and morphology were determined according to the methodology proposed by Zuvela & Matson (2020) and Johnston (1991).

Ultrasonography

Dogs were placed in left lateral decubitus without sedation, to perform ultrasound evaluation of both testes and epididymis in a transscrotal manner. A K10-Vet ultrasound equipment (KontroLab®) and a 7.5 MHz microconvex transducer were used to quantitatively determine the echogenicity of the testicles, mediastinum and the head of the epididymis. From each anatomical region, digital images were obtained and evaluated in the sagittal plane, twenty areas were randomly selected on each image to determine the gray scale pixels of each one. A value of "0" totally black and "255" totally white was considered. ImageJ software (NIH; Morales *et al.,* 2021) was used.

Gonadal morphology

In the fifth week, all dogs in both groups underwent bilateral orchiectomy under inhalation anesthesia (isoflurane-oxygen) and epididymal dissection of both testes was performed. Sections of epididymal head and body were fixed in Bouin's solution for processing by kerosene embedding and 3-micrometer thick sections were obtained and stained with hematoxylin and eosin (Aziz & Zeman-Pocrnich, 2022; Kurowicka *et al.,* 2015).
Sections of the epididymal head were evaluated by light microscopy (Optisum® MIC 900T) microphotographs were taken at 20X and 40X. Subsequently, tubule diameter, area and thickness of the tubular epithelium, as well as, the area occupied by the sperm packet in the lumen of each tubule were determined with the LSM 5® (Carl Zeiss) computer program.

Identification of estrogen receptors

Two unstained histological sections of each testis from both groups (C and TCOU) were evaluated by Leica TCS SP2 confocal microscopy with a wavelength of 400 nanometers (blue light) for excitation and 550 nanometers to detect green fluorescence emission associated with COU bound to the estrogenic receptors present (Serrano *et al.,* 2008; Pérez-Rivero *et al.,* 2009). Digital images were also obtained to determine the intensity of

the emitted fluorescence expressed in pixels with the ZEN Blue 3.5 Carl Zeiss Microscopy GmbH program.

Serum parameters of testosterone and estradiol

Blood samples were obtained by jugular venipuncture during daylight hours with at least 8 h of fasting, dispensed in tubes without anticoagulant. Serum testosterone and estradiol concentration was determined by enzyme-linked immunoadsorption assay (ELISA) using commercial kits (DRG Testosterone ELISA EIA-1559 and DRG Estradiol Sensitive ELISA EIA-4399, DRG Instruments GmbH®, Germany) and following the manufacturer's protocols. The analytical sensitivity of tests for testosterone and estradiol was 0.083 ng/ml and <1.399 pg/ml respectively. The intra-assay and inter-ensayo coefficients of variation of the tests were 3.59%, and 7.12% for testosterone, and of 6.36%, and 7.60% for estradiol. If the serum concentration was below the limit of quantification, that sample was assigned the lower limit value.

Statistical analysis

Statistical analysis was performed with PAST 3.01® software (Hammer, 2001). The medians ± standard error (SE) and the range between quartiles (Q1-Q3) were obtained for the cellularity parameters determined in gonads, as well as for the intensity of the ultrasound pixels and fluorescence images. Differences between groups were analyzed with the Mann-Whitney test. The significance level was set at $p < 0.05$.

RESULTS

Sperm production

The initial sperm concentration of group C was 1.79±0.001 X108 spermatozoa/ml, which in the following weeks did not show variations. On the contrary, in the TCOU group (Table 1), the sperm concentration in the ejaculates showed an evident numerical decrease from week one to four, the concentration determined in the fifth week which was $0.420±0.002X10^8$ spermatozoa/ml was lower ($p<0.05$) than the concentrations determined in the previous four weeks.

Sperm motility decreased significantly ($p<0.05$) from week 3 onwards. Regarding sperm morphology, an increase was observed at week 3 of alterations in the tail and proximal and distal cytoplasmic droplets associated with maturation in the epididymis (Table 1).

Sonographic indicators and gonadal morphology

Gonadal echogenicity (Table 2), showed that the gonadal parenchyma of the TCOU group (69.9±2.9) was increased ($p>0.05$) with respect to the echogenicity determined in the C group (63.9±2.6).

In testicular ultrasonography (Figure 1), pixel intensity showed a decrease in echogenicity in the mediastinum region (testicular network) and in the epididymal head region ($p<0.05$). The evaluation of cellularity in the epididymal head of both groups C and TCOU (Table 3) showed no differences between them; however, ranges of the values determined in each group observed in the quartiles were evident.

In the histological observations of epididymal cellularity (Figure 2), it is highlighted that the TCOU group presented a decrease in sperm mass and luminal microvilli.

Table 1. Weekly sperm concentration, morphology and motility of dogs treated with coumestrol (TCOU group)

Week	1*	2	3	4	5	Mann-Whitney *P*
	Sperm concentration X10^8 spermatozoa/ml Median±SE (Q1-Q3)					
Treated	1.79±0.001 (1.78-1.79)	1.58±0.05 (1.48-1.68)	1.17±0.09 (1.01-1.32)	0.952±0.03 (0.41-1.49)	**0.420±0.002^a (0.41-0.42)**	<0.05
Control	1.79±0.001 (1.78-1.79)	1.68±0.03 (1.68-1.78)	1.68±0.04 (1.58-1.70)	1.60±06 (1.55-1.79)	1.78±0.02 (1.70-1.79)	>0.05
	Sperm motility %					
	Median (Q1-Q3)					
Treated	>90 (89-92)	>90 (88-91)	**50^a** (50-50)	**50^a** (45-60)	**60^a** (50-60)	<0.05
Control	>90 (89-92)	>90 (89-92)	>90 (89-92)	>90 (89-92)	>90(89-92)	>0.05
	Sperm morphology % ± SE					
Treated Normal	86±1.5	88±1.1	**65±7.0^a**	**56±5.5^a**	**57±2.9^a**	<0.05
Control Normal	86±1.5	87±2.0	88±1.0	86±2.0	89±1.5	>0.05
Treated Tail rolled up	1±0.80	4±1.5	**10±4.5^a**	**9±4.5^a**	5±2.5	<0.05
Control Tail rolled up	1±0.80	1±0.5	-	-	-	>0.05

Treated Proximal drop	-	-	-	2±2	7±4	>0.05
Control Proximal drop	-	-	-	-	-	-
Treated Distal drop	-	1±0.5	1±0	**7±1[a]**	-	<0.05
Control Distal drop	-	-	-	**-**	-	-
Treated Tail rolled up	4±2	2±0.25	18±10	**34±13[a]**	**32±6.9[a]**	<0.05
Control Tail rolled up	4±2	2±1	-	-	2±1	>0.05

*Start of treatment. [a]Indicates the parameter where there is a significant difference in the Mann-Whitney test

Table 2. Gonadal echogenicity in control group and coumestrol-treated dogs

Group/Quartile	Control Median±SE	Q1-Q3 Median	Coumestrol Median±SE	Q1-Q3 Median	Mann Whitney *P*
Parenchyma	63.9±2.6	45.5-77.5	69.9±2.9	51.5-83.5	>0.05
Mediastinum	67.3±2.0	55.5-77.0	60.0±2.4	46.5-73	<0.01
Epididymal head	53±2.9	35.5-64	44±1.8	39-47	<0.05

Gonadal ultrasound/Gray pixels

Table 3. Cellularity parameters of the epididymis of dogs treated with coumestrol

Epididymal head	Control Median±SE	Q1-Q3 Median	Coumestrol Median±SE	Q1-Q3 Median	Mann Whitney *P*
Morphometry					
Diameter µm	273±5.7	235-306	314±5.3	279-340	<0.01
Area µm^2	60882±2593	43665-73608	79688±2716	61362-90872	<0.01
Epithelium thickness µm	59.7±1.0	53.4-64.4	75.9±1.0	67.0-83.6	<0.01
Length of microvilli in µm	23.6±0.4	20.8-25.6	13.0±0.6	8.6-17.9	<0.01
Spermatozoa (Area of the sperm packet in µm^2)	12703.9±2026.7	7991.1-18201.5	8371.8±2605.5	781.0-14181.5	<0.05

Presence of estrogen receptors

The presence of estrogenic receptors (Figure 3 and Table 4), was evidenced by the numerical increase in the mean of the pixels, in the connective tissue in the epididymal structures of the TCOU group of 21.5±0.9 compared to the C group which presented 6±0.5 ($p>0.05$).

Table 4. Fluorescence intensity associated with the presence of estrogen receptors

Group/Quartile	Control Median±SE	Q1-Q3 Median	Coumestrol Median±SE	Q1-Q3 Median	Mann Whitney *P*
Epithelium	1.4±0.1	0-3	3.1±0.4	0-5.7	<0.01
Connective tissue	6.0±0.5	3-8	20.9±0.9	14.2-26	>0.05

Fluorescence intensity/Green Pixels

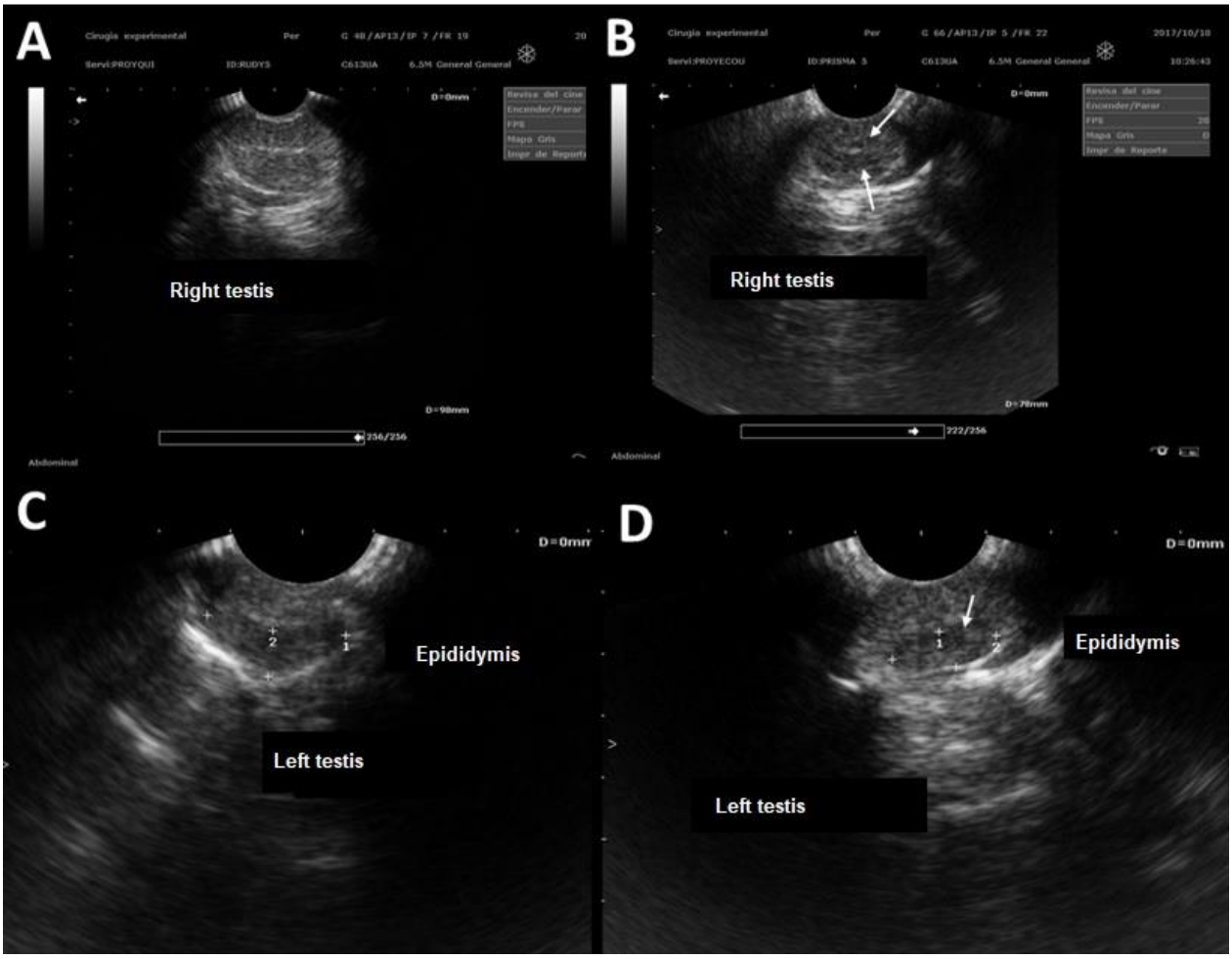

Figure 1. Ultrasound of the testis and epididymis. A. Control group. **B.** OCT group, note the decrease in echogenicity at the height of the testicular hilum (arrows). **C.** Evident echogenicity in epididymis of dogs in the control group. **D.** Lower echogenicity in epididymis of dogs in the TCOU group

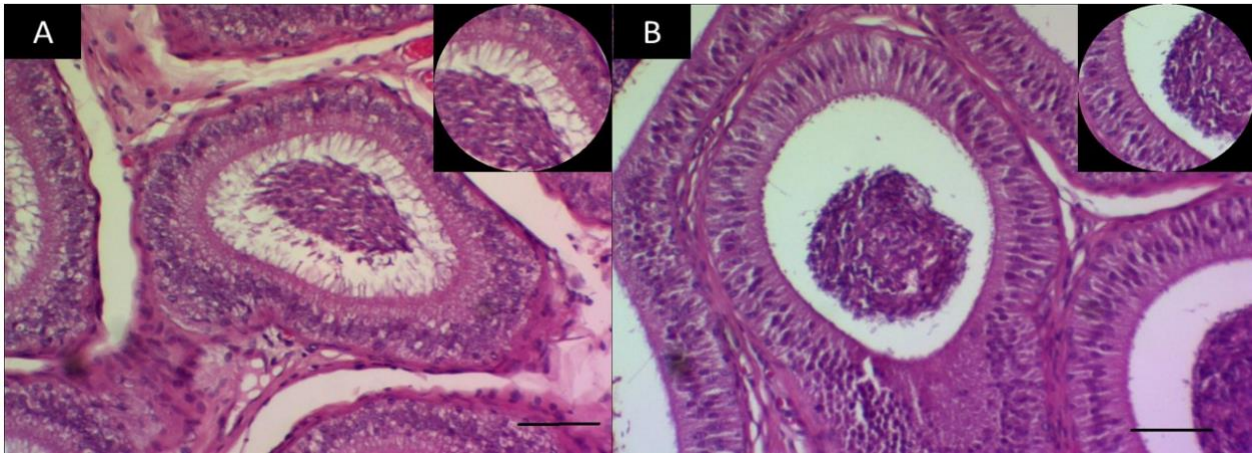

Figure 2. Histological section of a segment of the epididymal head. A. Head of epididymal head control group (40X), inset detail of microvilli **B**. Head of epididymis coumestrol treated group (40X), inset detail magnification showing absence of the microvilli. Bar 100µm

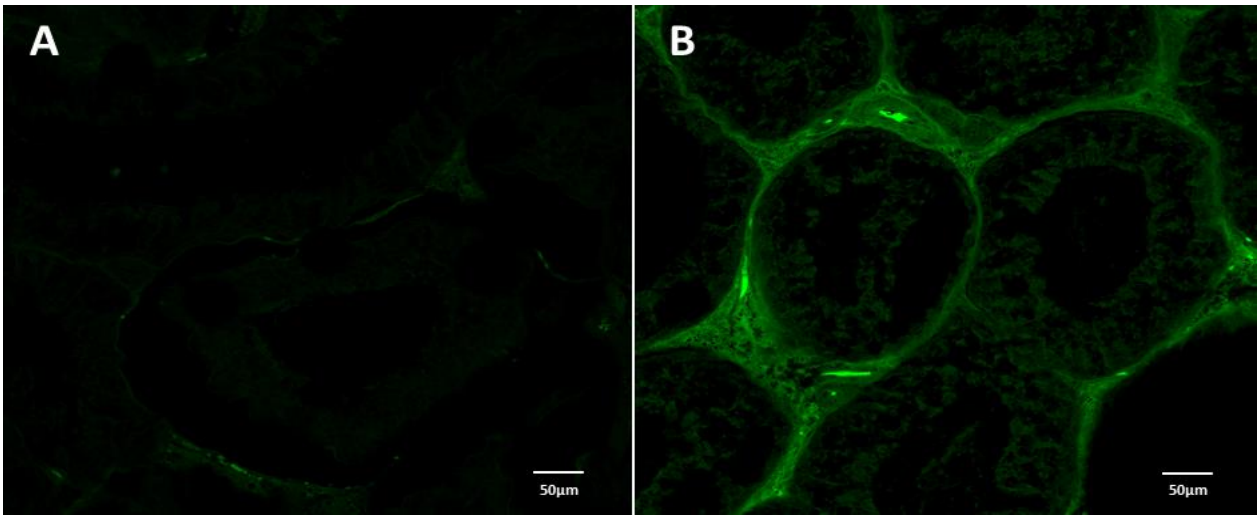

Figure 3. Fluorescence in epididymis showing the presence of estrogen receptors (coumestrol). A. Scanty fluorescence in the epididymis of dogs of the control group, in which the decrease of epithelium stands out. **B.** Intense fluorescence in the epididymis of dogs treated with coumestrol in which there is evidence of increased connective tissue

Hormonal parameters

The hormone levels determined (Figure 4) showed differences between group C and the TCOU group. In-group C, testosterone levels were constant; on the other hand, there was a variation in estradiol levels. In the TCOU group, a decrease in testosterone levels, associated with an increase in estradiol levels, was evident from week four until week five.

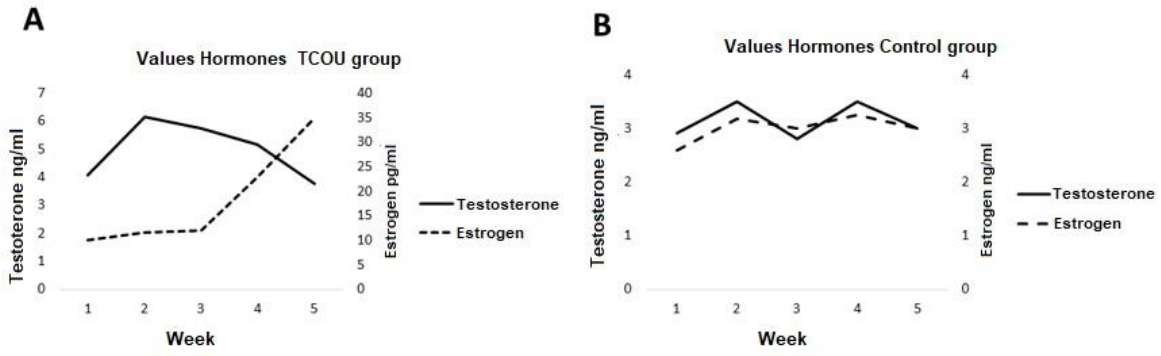

Figure 4. Serum testosterone and estrogen values. A. In dogs treated with coumestrol, the decrease of testosterone associated with the increase of estradiol was evident, **B.** In dogs of the control group, there was no evident relationship between hormones due to the effect of coumestrol

DISCUSSION

Normal canine ejaculates should have at least 80% morphologically normal and viable spermatozoa (Chłopik & Wysokińska, 2020). In this study, normal sperm morphology of 60% or lower was found from the third week of treatment, which is similar to that reported in dogs considered infertile (Barbosa de Souza *et al.,* 2015). The decrease in sperm concentration and the increase in spermatozoa with cytoplasmic droplet are evidence of the inhibitory effect of coumestrol on gonadal activity.

The sperm motility reported in fertile dogs should be located above 70% (Johnston,1991), in the present study in the dogs of the TCOU group from week 3 was located at 60% or lower, a value similar to that reported in infertile dogs (Barbosa de Souza *et al.,* 2015). In the present study, secondary alterations of sperm morphology were found, which are associated with their maturation in the epididymis or during the preparation of semen samples. Among the main alterations were strongly coiled tail from week 3 and distal cytoplasmic droplet from week 4 post-treatment. A point to consider when interpreting these results is that the average time of passage through the epididymis of spermatozoa in domestic dogs is 15 days (3 weeks) (Chłopik & Wysokińska, 2020), the same time interval at which alterations began to be detected in the spermiogram.

Because the structure of phytoestrogens is similar to that of endogenous estrogens and they have the ability to bind to ER, it is possible to observe physiological effects in tissues expressing the receptor (Rietjens *et al*., 2017; Beszterda & Frański, 2018). Once the COU molecule binds to any of the estrogen receptors (ERα and ERβ) it can emit a fluorescent signal (Serrano *et al.,* 2008; Wang *et al.*, 2014). In the present study, such signal was detected with greater intensity in the connective tissue of the epididymis of dogs in the TCOU group, confirming that COU is absorbed and probably binds to estrogenic receptors present in this tissue. Pérez-Rivero *et al*. (2014) previously reported it in adult hematophagous bat (*Desmodus rotundus*) testes and Serrano *et al.,* (2008) in male dogs, in both cases when COU was administered orally.

With respect to the echogenicity of the testicular parenchyma, it is homogeneous throughout the periphery, an area with decreased echogenicity is observed in the mediastinal region, which corresponds to the location of the vas deferens and the testicular network. It is consistent with the changes found at histological level related to the dilation of the tubules of the epididymis, loss of microvilli and decreased sperm content in its lumen (Mantziaras, 2020; Lubinus *et al.,* 2006).

The process of estrogen biosynthesis depends on the enzyme aromatase activity, a member of the cytochrome P450 superfamily (Hess & Cooke, 2018). Aromatase is responsible for converting testosterone and androstenedione to aromatic estrogens, 17β-

estradiol and estrone, respectively (Hess & Cooke, 2018). It has been described that COU can bind to the enzyme aromatase, resulting in inhibition of estrogen biosynthesis, so, it is possible to consider that the increase in serum testosterone concentration is due to this effect (Wyse *et al*., 2021; Lephart, 2015).

Estrogens are found in high concentrations in epithelial cells lining efferent ducts, their main function being to reabsorb the luminal fluid and thereby increase the concentration of spermatozoa. The biological effect absence of estrogens induces the accumulation of liquid in the efferent ducts, which finally generates dilatation of the same and of the rete testis. Subsequently there is a decrease in the epithelial height, in the number and height of the microvilli in the epididymis and subsequently atrophy of the seminiferous epithelium, all these changes favor semen dilution (Hess & Cooke, 2018; Pérez-Rivero *et al.,* 2009).

CONCLUSION

The studies performed showed that subcutaneous administration of coumestrol could be a non-invasive alternative to decrease the fertilizing capacity of dogs, since it induced alterations in gonadal activity and cellularity associated with a decrease in sperm production and sperm quality. However, studies are still required to evaluate its possible reversible effect and sperm fertilizing capacity.

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Conflict of interest
The authors declare that they do not have any conflict of interest

Authors' contribution

A.J.R: Designed and conducted the study, collected the data and biological samples, performed the laboratory work, analyzed and interpreted the results, drafted and approved the final manuscript.
J.J.P.R: Conceived the original idea and designed the study, supervised the study, analyzed and interpreted the data, performed critical revision of the manuscript, and approved the final manuscript.
J.A.H.B: Performed critical revision of the manuscript and approved the final manuscript.
A.A.S: Performed critical revision of the manuscript and approved the final manuscript.
M.P.M: Performed critical revision of the manuscript and approved the final manuscript**.**

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Errata Erratum

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