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Domestic cat testicular aromatase activity as assessed by the tritiated water-release assay

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Abstract

Lower testicular tetosterone:17β-estradiol (T:E2) ratio was found in teratospermic domestic cats (<40% morphologically normal sperm). The aim of this study was to assess the reliability of the tritiated water-release assay (TWRA) to measure aromatase activity in domestic cat testes. Testicular T and E2 concentrations, measured by enzyme immunoassay, and sperm morphology were evaluated to verify the relationship between them. Aromatase activity was measured in microsomal fraction and in homogenates of cat testes. Rat ovaries and piglet testes were used for assay validation. Aromatase activity was not detected in cat testes microsomal fraction (n = 8), not even when the protein amount added to the assay was increased from 50 to 200 µg. In homogenates, however, it was detected (3.5 ± 0.5 pmol.g^{-1}.h^{-1}; n = 7), although in such low levels that no activity inhibition was detected when homogenates were incubated with increasing fadrazole concentrations. Although none of the cats in this study were classified as teratospermic, some sperm defects were correlated with testicular T:E2 ratio (abnormal acrosome, r = -0.76) and with E2 concentration (proximal cytoplasmic droplet, r = 0.77). However, we did not find any correlation between aromatase activity and hormonal or sperm morphology data. To our knowledge this is the first demonstration of testicular aromatase activity in domestic cats. Despite that, due to the low aromatase activity measured and the lack of correlation with other reproductive data, we could not infer that TWRA is a reliable method to detect differences in testicular aromatase activity in normospermic cats. Perhaps this method could be used in teratospermic individuals that probably have an increased aromatase activity. As an alternative, we suggest that more sensitive techniques should be used to compare aromatase activity between normospermic and teratospermic cats. This would allow a better understanding of the relationship between the level of aromatase activity, the testicular hormonal concentrations and the sperm abnormalities in domestic cats.

Keywords: androgen, estrogen balance, *Felis catus*, sperm quality, teratospermia, testicular aromatase.

Introduction

It has become increasingly apparent that the testicular androgen: estrogen balance is crucial for normal male sexual development and function in several species (Carreau and Ress, 2010). In the mammalian testis, this balance is under the control of several endocrine and paracrine factors, including the aromatase enzymatic complex. Aromatase is a microsomal enzymatic complex, which when complexes with NADPH-cytochrome P450 reductase is responsible for the irreversible conversion of androgens into estrogens (Simpson *et al*., 2002). Transcripts of this enzyme have been found in testicular cells of several species including humans (Carreau *et al*., 2010), rats (Carreau *et al*., 2002), mice (Nitta *et al*., 1993), bank vole (Bilinska *et al*., 2000), and Rhesus monkey (Pereyra-Martinez *et al*., 2001), and in the male reproductive tract of domestic and wild mammals, such as horse (Hejmej *et al*., 2005), deer (Schön and Blottner, 2008) and bison (Kopera *et al*., 2010), as well as in human ejaculated spermatozoa (Carreau *et al*., 2011). Leydig cells are the major site of testicular aromatase expression in adults, and its activity is controlled by various factors such as luteinizing hormone (LH), cyclic cAMP and testosterone (Carreau *et al*., 2003).

Apart from its effects on hypothalamic-pituitary-testicular axis, estrogens acts locally in the testis and the excurrent tubular system, regulating proliferation (gonocytes, spermatogonia, Leydig cells), apoptosis (pachytene spermatocytes, Sertoli cells), and differentiation (spermatides) of germ and somatic cells, as well as regulating spermiation, transport and motility of spermatozoa, epididymal sperm maturation, and scrotal testicular descent (reviewed by Meccarielo *et al*., 2014). Thus, testicular aromatase activity dysfunction may significantly compromise testis function through imbalances between testosterone (T) and estradiol (E2). Either the lack or excess of E2 may have a detrimental impact on testicular function. For example, spermatozoa recovered from the cauda epididymis of mice lacking estrogen receptor-α (α-ERKO) has reduced motility and failed to fertilize eggs *in vitro* (Eddy *et al*., 1996), in part due to changes in the epididymal environment, such as pH and osmolality (Joseph *et al*., 2010a, b). In addition, mice lacking aromatase activity (ArKO), with no source of estrogens, exhibited an arrest of spermatogenesis and higher apoptosis of round spermatids (Robertson *et al*., 1999; Murata *et al*., 2002). Corroborating with data from experimental models, all cases of male aromatase deficiency in humans were related to infertility due to different reasons: low sperm count and motility (Carani *et al*., 1997); altered

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hypothalamic-pituitary-gonadal axis (Deladoëy *et al*., 1999), oligoazoospermia and bilateral cryptorchidism (Maffei *et al*., 2004).

On the other hand, the excess of aromatase activity resulting in high intratesticular E2 and lower T:E2 ratio also causes male reproductive disorders. Transgenic male mice overexpressing human aromatase enzyme (AROM+) had structural and functional alterations in the reproductive organs such as cryptorchidism, Leydig cell (LC) hyperplasia, disrupted spermatogenesis and infertility (Li *et al*., 2001). LC hyperplasia and hypertrophy (Strauss *et al*., 2009; Yu *et al*., 2014) were accompanied by a simultaneous activation of testicular macrophages at 4-5 months of age leading to LC engulfment by macrophages (Yu *et al*., 2014), suggesting that E2/ER- α mediated inflammatory mechanisms maybe related to idiopathic chronic orchitis in a subset of infertile men. Furthermore, although in lower doses E2 has an antiapoptotic effect on germ cells of human seminiferous tubules cultured *in vitro* (Pentikainen *et al*., 2000), higher levels of E2 seem to have the opposite effect, leading to an unbalanced expression of the SCF/c-kit system, disrupting the survival and death communication between germ cells and Sertoli cells toward germ cell apoptosis (Correia *et al*., 2014).

The aforementioned studies demonstrate that a delicate balance between androgens and estrogens, controlled by the aromatase enzyme, is fundamental for normal testicular development and function. Recently, we found that randomly selected domestic cats, classified as with occasional teratospermic males based on epididymal sperm sample analysis (<40% morphologically normal sperm), presented higher testicular concentration of E2 together with a tendency to low testosterone T, resulting in decreased T:E2 testicular ratio when compared with occasional normospermic counterparts (>60% morphologically normal sperm; Müller *et al*., 2012). There are a few reports showing a lower T serum concentration in teratospermic wild (Wildt *et al*., 1988) and domestic cats (Howard *et al*., 1990), although no data on testicular or serum E2 are provided.

To our knowledge there are no data on testicular aromatase activity in felids. Therefore, the aim of this study was to assess the reliability of the tritiated water-release assay (TWRA) to measure aromatase activity in testis of randomly selected male cats. In addition, in order to search for any correlation between aromatase activity and testicular function in cats, we also evaluated the testicular hormonal concentrations as well as sperm morphology.

Materials and Methods

Chemical

Unless stated otherwise, all chemicals were of reagent grade and obtained from Sigma Chemical Co (St. Louis, Mo, USA).

Experimental design

All procedures were approved by the Ethics Committee on Animal Research of the Federal University of Paraná and performed after receiving formal consent from the animals' owners. Fifteen adult clinically healthy mixed-breed domestic cats, aging from 12 to 36 months, were subjected to bilateral orchiectomy under anesthesia with ketamine hydrochloride (Vetaset, Fort Dodge, São Paulo, SP, BR; 20 mg/kg, im) and xylazine (Rompun 2%, Bayer, São Paulo, SP, BR; 1.0 mg/kg, im). Cat testes were dissected and frozen until assayed for aromatase activity by the tritiated water-release assay (TWRA) and for T and E2 concentrations by enzyme immunoassay (EIA). Immediately after dissection, sperm was recovered from cauda epididymidis processed and analyzed for motility, progressive motility, sperm number and morphology.

To validate the TWRA in our lab, rat ovaries and piglet testes were used as reference tissues. Rat ovaries were obtained from six mature Wistar rats from the Federal University of Paraná breeding stock and testes from five piglets submitted to surgical orchiectomy in a local farm.

Aromatase activity was measured in microsomal fraction (n = 8) and in homogenates (n = 7) of domestic cat testes. The testes used in each assay were randomly selected. For rat ovaries, both microsomal (n = 3) and homogenate (n = 3) fractions were assayed, while for piglet testes only homogenate fractions (n = 5) were evaluated.

Measurement of aromatase activity

Tissue preparation

Microsomal fractions were prepared as described by Schatzman *et al*. (1988) with modifications. Briefly, 0.5 g of tissues were homogenized with 4 ml of phosphate buffer containing sucrose (sucrose 250 mM, EDTA 1 mM, dithiothreitol 1 mM, pH 7.4) and then centrifuged (800 x *g* for 10 min). The supernatant was subsequently centrifuged (9,000 x *g* for 20 min) and, again, the supernatant was recovered and centrifuged (120,000 x *g* for 1 h at 4ºC). The resulting pellet was suspended in 500 µl of phosphate buffer: glycerol (3:1; v:v) and frozen (-80ºC) until assayed. For homogenates, 0.5 g of tissue was homogenized with 1 ml of phosphate buffer with sucrose, centrifuged (800 x *g* for 10 min) and the supernatant recovered and assayed. Protein quantification was performed by the bicinchoninic acid protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Tritiated water-release assay (TWRA)

Aromatase activity was determined by the TWRA in which the release of ^{3}H as $^{3}H_2O$ from the

substrate 1β position during aromatization is quantified (Lephart and Simpson, 1991). Microsomes or homogenates triplicates were incubated with a NADPH generating system (glucose-6-hydrogenase 2 UI, glucose-6-phosphate 5 mM, $NADP^+$ 1 mM, NADPH 1 mM) and substrate 1β-^{3}H-Androstenedione 150 nm (^{3}H-A; 23,5 Ci/mMol, PerkinElmer, Boston, MA, USA) diluted in 50 mM phosphate buffer pH 7.4, in a final reaction volume of 200 µl. Reactions were started by substrate addition, carried out at 37ºC for 2 h, and stopped with 1 ml of cold trichloroacetic acid 30%. After extraction with 1 ml of chloroform and centrifugation (2,000 x *g* for 10 min), the aqueous phase (600 µl) was combined with 500 µl of charcoal 5% and dextran 0.5% and centrifuged (2,000 x *g* for 30 min). An aliquot of the supernatant (300 µl) was combined with 2 ml of scintillation liquid (Ecolume™ liquid scintillation - ICN Biomedicals, Irvine, CA, USA) and counted on a Beckman scintillation counter (LS380, Beckman Instruments, Schaumburg, IL, USA). For procedure validation pools of cat testes, rat ovaries and piglet testes homogenates were assayed with the aromatase inhibitor fadrazole hydrochloride to a final concentration ranging from 0.1 to 100 nm. The mean value for assay blanks (radioactivity in samples incubated in the absence of microsomes or homogenates) was 253 counts per minute (c.p.m.), which corresponds to approximately 0.05% of the substrate that had been added to the reaction (^{3}H-A 150 nm corresponds to approximately 490,000 c.p.m.). Background c.p.m. values were subtracted from each sample. Aromatase activity was expressed as the amount of substrate converted to tritiated water per gram of protein per hour ($pmol.g^{-1}.h^{-1}$). Different amounts of proteins (0.05 up to 3 mg) were used. The assay sensitivity was defined as the mean plus 2.0 standard deviations of blank tubes and calculated based on the mean amount of protein used in each assay and ranged from 0.19 to 0.46 $pmol.g^{-1}.h^{-1}$.

Testicular testosterone and 17β-estradiol measurements

To quantify testicular steroids, 0.2 g of testis were homogenized with 500 µl of PBS. The homogenate was extracted twice with a total volume of 2 ml of diethyl ether. The ether fraction was transferred to clean tubes and left to evaporate. The tube content was recovered in 400 µl of an ethanol solution (1:4, v:v in PBS) after vortexing and sonication (15 min). Testicular T and E2 concentrations were quantified via enzyme immunoassay (EIA; Munro *et al*., 1991) using polyclonal anti-testosterone (R156/7), anti-17β-estradiol (R0008), and specific horseradish peroxidase conjugates obtained from Dr. Coralie Munro (University of California, Davis, CA, USA). The assays were previously validated in our laboratory (Müller *et al*., 2012). The limits of detection were 46 pg/ml for T and 93 pg/ml for E2; intra- and interassay coefficients of variation were <10%. The results are expressed as ng/g of testis.

Collection and analysis of epididymal sperm

Procedures for sperm retrieval were described previously (Müller *et al*., 2012). Briefly, the cauda portion from each epididymis was isolated and incubated with supplemented Ham´s F-10 medium [Hepes, fetal calf serum 5% (Cultilab, Campinas, SP, BR), 100 UI penicillin/ml and 100 mg/ml streptomycin] in a water bath at 37ºC for 15 min. The obtained sperm suspension was centrifuged (600 x *g* for 10 min), the supernatant discarded and 30 µl of Ham´s F10 medium was added to the sperm pellet. Total sperm suspension volume, sperm concentration, motility and progressive motility were evaluated (Morais *et al*., 2002). For sperm morphology analysis, 5 µl of the sperm suspension was fixed in 100 µl of glutaraldehyde 0.3% (v:v in PBS) and 200 cells per sample were evaluated under phase-contrast microscopy (x 1,000 magnification). Sperm were classified as either normal or abnormal, if morphologic malformations were present, as described previously (Müller *et al*., 2012).

Statistical analysis

Descriptive analyses of the data were performed, and the results are presented as mean ± SEM. Pearson correlations were calculated between aromatase activity, hormonal concentrations and sperm morphology (Statistica 7.0; Stat Software Inc., Tulsa, OK, USA). The level of significance was defined as 5%.

Results

Aromatase activity

Aromatase activity was successfully measured in rat ovary microsomal proteins or tissue homogenates, and in piglet testicular homogenates (Table 1). Besides that, the aromatase activity of rat ovaries and piglet testes homogenates was inhibited in a dose-response manner when incubated with fadrazole, resulting in an IC50 value of 1.95 nm and 3.97 nm, respectively (Fig. 1).

Aromatase activity was not detected in any microsomal fraction of domestic cat testis, either with 50 (n = 4) or 200 µg (n = 4) of microsomal protein added to the reaction. The c.p.m. values for all samples did not differ from blank tubes. Nevertheless, when using testis homogenates, a mean value of 3.5 ± 0.5 $pmol.g^{-1}.h^{-1}$ of aromatase activity was detected (Table 1). However, no inhibition was observed in the assay with fadrazole using a pool of cat testes homogenates (Fig. 1).

Table 1. Aromatase activity measured in microsomes (M) or homogenates (H) of domestic cat testes, piglet testes and rat ovaries. Incubations were carried out with 150 nm of 1β-^{3}H-androstenedione (^{3}H-A) and different amounts of protein. Values are Mean ± SEM of triplicate incubations.

Tissue	n	Protein (mg)	Aromatase activity (pmol/g/h)
Domestic cat testes M	8	0.05 to 0.2	same as blank
Domestic cat testes H	7	3.3 ± 0.3	3.5 ± 0.5
Rat ovaries M	3	0.05	14,960.7 ± 808.2
Rat ovaries H	3	2.3 ± 0.1	1,460.1 ± 73.6
Piglet testes H	5	3.1 ± 0.2	1,422.3 ± 47.1

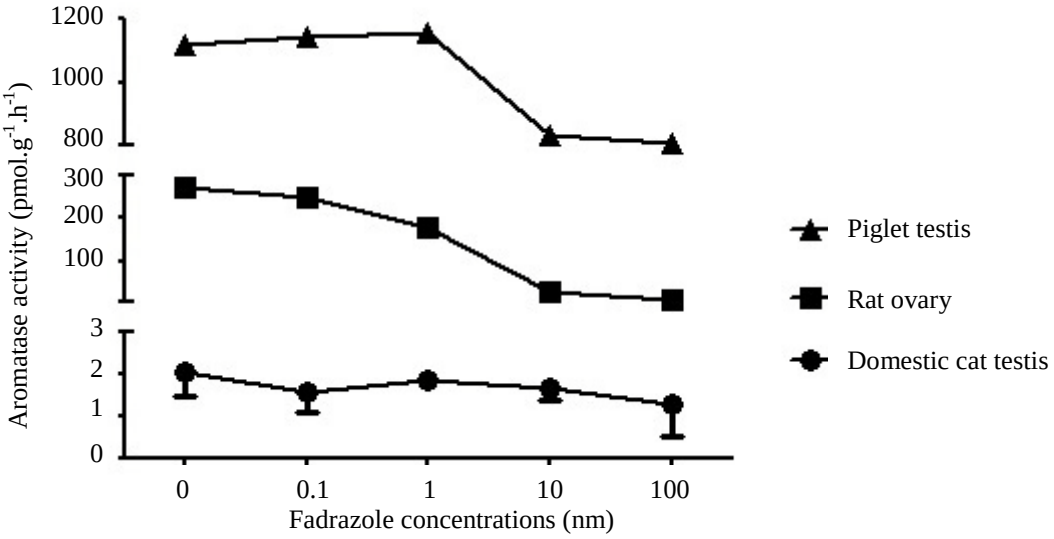

Figure 1. Aromatase activity inhibition curve in homogenates of rat ovary, piglet and domestic cat testes in the presence of increasing concentrations of the aromatase inhibitor fadrazole. Assay sensitivity was 0.19 pmol.g^{-1}.h^{-1}. Values are mean ± SEM of triplicate incubations.

Testicular T and E2 and sperm traits

Mean values for testicular T and E2 concentrations were 1,318.7 ± 378 and 6.9 ± 1 ng/g of testis, respectively, and the resulting T:E2 ratio was 171.6 ± 39.5 (n = 15). All males included in this study were classified as normospermic once they presented more than 60% of morphologically normal sperm. The mean percentage of sperm with normal morphology was 60.9 ± 2.2% and the most prevalent sperm defect was distal cytoplasmic droplet (12.2 ± 2.6%), a defect normally found in high proportions in epididymal sperm samples. Other sperm traits, such as sperm number, motility and percentages of sperm abnormalities were also considered normal for cats (Table 2).

Correlations

Significant correlations were found between hormonal data and sperm defects. Testicular E2 concentration correlated with the percentage of sperm presenting proximal cytoplasmic droplet (r = 0.77) and T:E2 ratio correlate negatively with the percentage of sperm with abnormal acrosome (r = -0.76). No correlation was found between aromatase activity and hormonal or sperm morphology data.

Table 2. Sperm traits and incidence of morphologically normal and abnormal sperm recovered from the cauda epididymis of domestic cats (n = 15). Distal cytoplasmic droplet was the main morphological sperm defect found that is normal for sperm collected from the cauda epididymis.

Parameters	Mean ± SEM
Total sperm number (x 10^6)	44.8 ± 10.5
Sperm motility (%)	61.8 ± 5.3
Sperm progressive motility (0 to 5)	3.6 ± 0.2
Sperm motility index †	66.6 ± 4.7
Normal sperm (%)	60.9 ± 2.2
Primary sperm defects (%)	7.0 ± 1.2
Head defects	1.9 ± 0.3
Abnormal acrosome	0.7 ± 0.2
Abnormal midpiece	0.6 ± 0.2
Tightly coiled flagellum	3.6 ± 1.3
Secondary sperm defects (%)	32.1 ± 2.1
Bent midpiece with droplet	5.4 ± 1.2
Bent midpiece without droplet	3.4 ± 0.7
Bent flagellum with droplet	3.2 ± 0.8
Bent flagellum without droplet	4.6 ± 0.7
Proximal cytoplasmic droplet	3.2 ± 0.5
Distal cytoplasmic droplet	12.2 ± 2.6

†Sperm motility index = (% sperm motility + [20 x sperm progressive motility])/2.

Discussion

To our knowledge, this is the first study to demonstrate aromatase activity in domestic cat testis. We used both homogenates and microsomal protein, but we were able to measure aromatase activity only in homogenates. Unlike data described for males known for their exceptionally high testicular aromatase activity such as the pig (Moran, 2002) and the stallion (Gaillard and Silberzahn, 1987), aromatase activity was undetectable in the microsomal fraction of the domestic cat testis, even when the amount of protein was increased from 0.05 to 0.2 mg. As a quality control for the assay, we also ran homogenates from rat ovary and piglet testis and, as expected, we found significantly higher levels of aromatase activity than that of the domestic cat testis. We could infer that the assay sensitivity does not allow the detection of aromatase activity in small amounts of microsomal protein for tissues displaying low activity as the domestic cat testis. Furthermore, the aromatase activity levels measured on homogenates of the domestic cat testis were very low and too close to the limit of the detection of the assay. Perhaps this was the reason why we did not find a significant inhibition of the aromatase activity in a pool of homogenates from domestic cats testis incubated with fadrozole, as we did for rat ovary and piglet testis homogenates. The dose-response inhibition curves we found for rat ovaries and piglet testis are in accordance with previously published data (Moran, 2002; Satoh *et al*., 2008).

Our primary goal was to validate the assay for the measurement of testicular aromatase activity in domestic cats as a tool to search for biological significance between potentially increased levels of testicular aromatization and teratospermic cats. To accomplish that we also evaluated other testicular traits including testicular testosterone and estradiol concentrations and the quality of sperm obtained from epididymis. As testicular aromatase activity levels may define the testicular androgen:estrogen balance by regulating the rate of conversion of androgens into estrogens, its level of activity can be implicated in testicular endocrine imbalances and low spermatogenic efficiency. In domestic cats we have recently found randomly selected teratospermic males had higher testicular concentration of E2 together with a tendency for low T, giving a decreased T:E2 ratio when compared with normospermic counterparts (Müller *et al*., 2012). Besides of that, testicular E2 correlated with the percentages of normal sperm (r = -0.55), primary defects (r = 0.58), and abnormal acrosomes (r = 0.64), suggesting that increases in testicular E2 may be related to the development of sperm abnormalities in cats. In this study we also found that testicular E2 concentration correlated positively with the percentage of sperm presenting proximal cytoplasmic droplet and T:E2 ratio correlated negatively with the percentage of sperm with abnormal acrosome. These findings together with the knowledge that permanent teratospermia in felids is accompanied by a high sperm production with remarkable increase of Sertoli cell efficiency (Jewgenow *et al*., 2013), and reduced germ cell apoptosis (Jewgenow *et al*., 2009) led us to hypothesize that unbalances in the T:E2 ratio in cats may be related to the pathophysiology of teratospermia in felids, and consequently it may also be related to the level of testicular aromatase activity. This hypothesis seemed to be confirmed previously for other species. For example, men presenting idiopathic infertility presented a high

testicular aromatase activity, which was highly correlated to testicular E2 concentration (r = 0.88) and T:E2 ratio (r = 0.85), suggesting that this increased aromatase activity might be related to impaired spermatogenesis (Ichikawa, 1995). Many infertile men with severe idiopathic oligozoospermia (<5 x 10^6 sperm cells/ml) and nonobstructive azoospermia can exhibit a decreased serum T:E2 ratio without alteration in FSH and LH levels. When treated with the aromatase inhibitor letrozole or anastrazole, serum T and E2 values were normalized and sperm motility, sperm number and ejaculate volume were improved (Saylam *et al*., 2011; Gregoriou *et al*., 2012; Schlegel, 2012). Pavlovich *et al*. (2001) characterized men with normal spermatogenesis as having a serum T:E2 ratio of 14.5, whereas men with severe infertility had a mean serum T:E2 ratio of 6.9, and based on these observations, a cutoff of 10 was set as the lower limit of normal serum T:E2 ratio in men (calculated using T in ng/dl, and E2 as pg/ml). These studies demonstrate that defective sperm production is commonly accompanied by an excess of aromatase activity that can be reverted with the use of aromatase inhibitors, improving semen characteristics. Similarly, aromatase inhibitor treatment in oligo and azoospermic dogs with high serum E2 concentration and low T and LH has been reported to increase their ejaculate volume and sperm number, motility and viability (Kawakami *et al*., 2004).

Unfortunately, we were not able to demonstrate any correlation between the level of aromatase activity and the reproductive data in this study and this hypothesis for cats still remains to be confirmed. Limitations of this study that might have played an important role include the method of animal selection and the reduced sample size. As part of a population control program, cats in this study, as well as in previous studies in our lab were randomly selected. Based on historical data, we were expecting to get some teratospermic males. However, none of them had a percentage of morphologically abnormal sperm below 40%. On the contrary, from sperm traits, hormonal parameters as well as spermatogenic activity (data not shown), all the cats in this study were classified as normospermic males (Howard *et al*., 1990; Neubauer *et al*., 2004; Jewgenow *et al*., 2009). When we searched for a correlation between the aromatase activity and percentage of sperm morphological abnormalities, testicular concentrations of E2 and T or testicular T:E2 ratio no significant correlation was found. Although unexpected, this lack of correlation may be related to the reduced sample size and the good quality of the samples evaluated. Another possibility is that the values detected for testicular aromatase activity for the domestic cats in our study were too close to the assay detection limit, not allowing us to discriminate among individuals when searching for biological significance of these measured levels of aromatase activity. To better clarify this, we could suggest using another technique such as HPLC for the measurement of the estrogens produced during the aromatization instead of measuring the tritiated water. Furthermore, to evaluate if TWRA can be useful to search for differences in the testicular aromatase activity between normospermic and teratospermic cats it would be helpful to select testis from permanent teratospermic male cats, based on Sertoli cell efficiency (ratio of round spermatids to Sertoli cells), as suggested by Jewgenow *et al*. (2013).

In conclusion, we were able to detect very low levels of aromatase activity in domestic cat testis. However, due to the lack of correlation with any of the other reproductive traits evaluated, including testicular E2 concentration, we suggest that more sensitive techniques should also be used to evaluate testicular estrogen synthesis in domestic cats and its relation to spermatogenic activity.

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