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Feline testicular ultrasonogram differentiates pre vs. postpubertal and
normal vs. disrupted spermatogenesis

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Abstract

The aims of this study were: to ultrasonographically describe and compare testicular parenchyma echogenicity and heterogeneity using digital image analysis in: I) prepubertal (PREP), peripubertal (PERI) and mature (MAT) cats; II) Normal and abnormal mature felids. Secondary, the relationships between histomorphological and ultrasonographic attributes of the testes were also determined. I) Fourteen, PREP, PERI and MAT male cats were ultrasonographically examined and then castrated. II) Seven adult cats were ultrasonographically examined before and after a GnRH antagonist administration and then castrated. All the testes were grossly and histomorphometrically assessed. In the frozen digital images of the longitudinal ultrasound sections, 3 regions of interest (ROI, 1 mm2) were selected. Within each ROI the echogenicity and the heterogeneity of the testicular parenchyma were digitally analyzed. In experiment I, testicular volume (0.15±0.0 vs. 0.49±0.1 vs. 1.65±0.1; P<0.01) and gonadosomatic index (0.04±0.0 vs. 0.05±0.0 vs. 0.08±0.0; P< 0.01), echogenicity (56.54±0.75 vs. 81.87±5.88 vs.94.67±3.62; P < 0.01) and heterogeneity (10.2420±1.3740 vs.13.65±0.65 vs. 14.67±1.49; P< 0.01) augmented throughout PRE, PERI, and MAT. In experiment II, testicular volume (1.00 ± 0.09 vs. 0.85 ± 0.09; P< 0.05), echogenicity (87.74 ±1.53 vs. 83.32 ±1.54; P 0.01) but not heterogeneity (14.09 ± 0.26 vs. 14.19 ±0.29; P> 0.05) decreased in the post GnRH antagonist abnormal testes. For both experiments, testicular volume, seminiferous tubular diameter, percentage of spermatids as the most mature cell type, and luminal/intertubular ratio were highly correlated (P< 0.01) with their echotextural attributes. Computer-assisted image analysis of B mode ultrasonogram appears as a good indicator of pubertal development and mild alterations of spermatogenesis in felids.
Keywords: Puberty– Andrology – Ecography –Subfertility– Histology– Germinal epithelium

1. Introduction

Domestic cats have been increasing in popularity as pets over their canine counterparts. Several explanations for this shift have been postulated including that cats cost less to keep, and are more feasible in urban settings. Domestic felids have also been extensively used as models for human diseases, and for assisted reproductive technologies in wild endangered felids [1]. With the increasing interest in breeding cats the requests to determine puberty and to investigate cases of reproductive failure have become more frequent.

Sexual immaturity and testicular lesions, are causes of sub and infertility in domestic cats [2,3]. Both puberty and mild testicular lesions are difficult to clinically diagnose in this species. Although the most accurate test for diagnosis is testicular biopsy, this “gold standard” procedure is invasive and, therefore, rarely performed in practice. Thus, sensitive, non-invasive diagnostic methods are still required to contribute to the breeding soundness examination in this species.

Two-dimensional ultrasonography is a non invasive diagnostic technique that has been widely used in the andrological exam of most mammalian species. Although, testicular parenchyma is commonly described as a tissue of medium heterogeneity and homogeneous echotexture [4] these appreciations are subjective. The present availability of high-frequency and resolution equipments has permitted the accurate evaluation of the cellular composition of a tissue. Additionally, digital image analysis
supports ultrasound findings to be a quantifiable tool. Thus, echogenicity i.e. pixel intensity is described in terms of numerical values which range from 0 (absolute black) to 255 (absolute white) and heterogeneity as pixel standard deviation of echogenicity, which reflects the existence of interspersed hyper- and hypoechoic areas in a tissue [5]. These quantitative evaluations have been mainly carried out in farm animals testes [5-7] and there are also some reports in horses and dogs [8,9]. Although, fine tuning of this technique would contribute to the reproductive performance of this species, to date, no computer-assisted image analysis of testicular ultrasonograms appear to have been published in domestic cats. Thus the aims of this study were twofold: to ultrasonographically describe and compare testicular parenchyma echogenicity and heterogeneity using digital image analysis in: I) prepubertal, peripubertal and postpubertal cats and II) Normal and abnormal mature feline testes. Secondary, the relationships between histomorphological and ultrasonographic attributes of the gonads were also determined. In Experiment II, a pharmacological model of spermatogenic impairment was used. For this purpose, acyline, a potent gonadotrophin releasing hormone (GnRH) antagonist was selected as spermatogenic disruptor, based on previously described deleterious effects on feline spermatogenesis [10].

2. Materials and methods

2.1 Experiment I

2.1.1. Animals
Fourteen, 5 to 36 months of age, 2.5 to 4.7 kg body weight, prepubertal (PREP), peripubertal (PERI) and mature (MAT) healthy, crossbred male cats that were born in our institutional cat colony were included in this study. The animals were kept free in enriched 5 m$^2$ rooms with 14 hours of light/10 dark per day, fed *premium* commercial food and given water *ad libitum*. This study was reviewed and approved by the Animal Care and Use Committee of the Veterinary School of the National University of La Plata and all experiments were conducted under the guidelines established in The Guide for The Care and Use of Laboratory Animals, USA.

2.1.2. Ultrasonographic evaluations

Ultrasonographic evaluations were undertaken by a single experienced evaluator using a real time B-mode ultrasound machine (Toshiba Nemio XG, Japan) with a 14 MHz lineal transducer in both testicles of each animal. All machine settings were established at the first examination according to best image quality and remained unaltered for all remaining examinations (Gain: 100, focal depth: 2 cm). Acoustic gel was applied to the transducer and coupled directly to the clipped scrotum with minimum pressure to obtain the images. The testes were imaged in the longitudinal and transverse planes and dimensions were obtained from frozen images, using the ultrasound callipers. Total testicular volume was also calculated as described by Linn et al. (2009 [11]). In the frozen digital images (jpeg of 640 x 480 pixels) of the longitudinal sections, 3 regions of interest (ROI, 1 mm$^2$) were selected, between the central mediastinum and the testicular capsule (12 ROI/cat). Within each ROI the echogenicity
and the heterogeneity of the testicular parenchyma were analyzed using Image J software (National Institutes of Health, Bethesda, Maryland, USA).

2.1.3. Orchidectomies

A routine open castration was performed [12]. For the surgery, the animals were pre-medicated with atropine sulfate, (Atropine Sulfate, John Martin; 0.04 mg/kg, subcutaneously), acepromazine maleate (Acedan, Holiday; 0.03 mg/ kg subcutaneously), and butorphanol (Torbultol Plus, Fort Dodge; 0.2 mg/kg, intramuscularly). Anesthesia was induced with sodium thiopental (Pentovet TM, Richmond; 8 mg/kg, intravenous). After the males were endotracheally intubated, anesthesia was maintained with isoflurane and oxygen in a closed system. After surgery ketoprofen (Ketofen, Fort Dodge; 1 mg/kg) was injected subcutaneously (once) and then orally every 24 hours for 4 additional days. After orchidectomy all the cats were placed for adoption.

2.1.4. Gross, seminal and histological examination

Immediately after surgical removal, the testes were weighed (g). Gonadosomatic index (%; [13]) was also calculated. The testes were sectioned longitudinally, placed in Bouin’s fixative for 24 h and then changed to alcohol 70 and processed routinely with paraffin embedding. After processing, 5 µm serial sections were cut, mounted on slides, dried, deparaffinized in xylene, rehydrated in graded ethanol solutions and stained with hematoxylin and eosin. All histological determinations were made by a single operator without knowledge of the age at which the animals were castrated.
Histological images were obtained from a microscope (Olympus BX50, Tokyo, Japan); 10X or 40X through an attached digital RGB video camera (Omax A35180U3, China) and digitalized in a 24 bit true color TIFF format. Ten round tubular profiles per testis were evaluated for mean tubular diameter (µm), mean germinal epithelium height (µm), as well as the identification of the most developed germ cell found in the seminiferous tubules of each cat. The percentages of tubular cross sections containing different identified most mature cell were recorded. The proportion of the tubular/intertubular and luminal/intertubular compartments were also calculated. Images were analyzed by planimetry (Image Pro Plus v6.0-Media Cybernetics, Silver Spring, MA, USA).

Peripubertal state (PERI) was defined when tubular diameter was 100-150 µm and a multilayer germinal epithelium (up to the spermatide or spermatozoa stage) was present in more than 50% of the tubules. Lower and higher values were considered pre (PREP) and mature (MAT) states, respectively [14].

2.2. Experiment II

2.2.1. Animals and pharmacological protocol

Seven, 1 to 3 years old, 3.2 to 4.8 kg, cross-bred fertile cats from our institutional cat colony were administered acyline (Contraception & Reproductive Health Branch Center for Population Research, NIH, Bethesda, MD, USA) 330 µg/kg SC once a week for 4 consecutive weeks. Acyline [acetyl-D2Nal-D4CIPhe-D3PalSer-Aph(ac)-DAph(Ac)-Leu-Lys(lpr)-Pro-D-Ala-Nh2] was provided in a lyophilized powder which was suspended in sterile distilled water (concentration, 2 mg/mL). The antagonist dose and frequency of administration were selected according to our previous
studies [10,15]. The animals were kept under the experimental conditions described for Experiment I. This study was reviewed and approved by the Animal Care and Use Committee of the Veterinary School of the National University of La Plata and all experiments were conducted under the guidelines established in The Guide for The Care and Use of Laboratory Animals, USA.

2.2.2. Follow up

Testicular ultrasound evaluations were carried out one week before treatment (PRETR) and one week after the last antagonist administration (POSTR). Then all the cats were submitted to orchidectomies. Immediately after surgical removal, the testes were grossly and histologically assessed. All ultrasonographic and histological procedures were carried out as explained for Experiment I. The castrated cats were also placed for adoption.

2.3. Statistical analysis

Normality of obtained data of both experiments was confirmed by Shapiro-Wilk test. Gross testicular parameters and ROIs echogenicity and heterogeneity values of the 3 groups (PREP vs. PERI vs. MAT) of Experiment I and the 2 groups (PRETR vs. POSTR) of Experiment II were compared by one way ANOVA followed by Tukey test and paired Student’s t test, respectively. For both experiments, echotextural parameters were correlated with gross and microscopic attributes of the testes by Pearson correlation test. Descriptive statistic was expressed as mean±SEM and the level of significance was set at P < 0.05 (SPSS, Inc., Chicago, IL, USA).
3. Results

In Experiment I, 6, 2 and 6 cats were histomorphometrically classified as PREP, PERI, and MAT, respectively. Testicular volume (0.15±0.0 vs. 0.49±0.1 vs. 1.65±0.1; \(P<0.01\)) and gonadosomatic index (0.04±0.0 vs. 0.05±0.0 vs. 0.08±0.0; \(P<0.01\)) increased with reproductive development, showing differences among the 3 groups. Both echogenicity (\(P<0.01\)) and heterogeneity (\(P<0.01\)) also augmented throughout PREP, PERI, and MAT (Fig. 1).

In Experiment II, acyline caused mild histological spermatogenic impairment with a diminution of 34% and 18% of normal germinal height and tubular diameter, respectively when compared to what has been reported for normal testes in this species [13]. In this experiment, testicular volume (cc; 1.00±0.09 vs. 0.85±0.1; \(P<0.05\)) and parenchyma echogenicity (\(P=0.01\)) but not heterogeneity (\(P>0.05\); Fig. 2) decreased in POSTR. Correlations between gross (also Fig. 3) and histomorphological testicular parameters with echotextural attributes of both experiments are shown in Table 1.

4. Discussion

Male cats are particularly difficult to andrologically examine not only because of their comparatively reduced body size but also for their innate tendency to experience stress requiring sedation or even anesthesia for minor maneuvers. Thus, in this study, computer-assisted image analysis of B mode ultrasonogram was evaluated as a non-invasive technique to contribute to the male cat breeding soundness examination.
As expected, in the growing cats of Experiment I, testicular volume and gonadosomatic index increased up to what has been previously described as adult values [13]. In line with reports in humans [16], ruminants [5,17-19] and horses [9], in these cats, computer-assisted analysis of testicular ultrasonograms could differentiate pre from postpubertal state.

In domestic felids, the same as in other mammals, the onset of spermatogenesis during peripubertal development is accompanied by changes in testicular microstructure [14]. An increase of seminiferous tubule diameter and lumen as well as of the epithelial height, due to the appearance of more mature spermatogenic cell types, occur [19]. Thus, the increase in echogenicity is associated with these testicular changes leading to the onset of pubertal spermatogenesis [5,17,20]. Furthermore in agreement with a similar study in ram lambs, the seminiferous tubular diameter of the present study had the strongest correlation with testicular echotexture [5].

To investigate the echotextural characteristics of adult cats with impaired spermatogenesis, a suitable pharmacological model was used for Experiment II. As previously reported [15], in these cats, the GnRH antagonist treatment reduced testicular volume causing mild seminiferous tubular deterioration which could mimic subfertility cases. In spite of these mild histomorphometric testicular changes, computer-assisted image analysis of B mode ultrasonogram could differentiate abnormal from normal spermatogenesis by a significant decrease in pixel intensity. Although it can be assumed than more severe lesions, e.g. testicular degeneration or atrophy, could be quantitatively more evident, the reproductive importance to these end stage lesions is much less clinically relevant. Similarly, in bulls echogenicity decreased in the first 2–3 weeks after scrotal insulation, coincidently with the diminution in sperm motility and normal morphology [21]. In stallions, retained abdominal testes have also lower and less
heterogeneous echogenicity than scrotal testes [9]. In domestic dogs, poor seminal quality has been associated with decreased parenchyma echogenicity [22] and future sperm motility was positively related to gonadal echogenicity [23].

Testicular spermatogenic cells are known to affect the grey-scale appearance of scrotal ultrasonograms [5] and changes in the composition of tubular cell population affect echotexture. Thus, in both experiments, the cytological and echotextural correlations shifted from negative with the less mature cell types -i.e. spermatogonias- to positive with the more mature cell types -i.e. elongated spermatids- in spermatogenesis evidencing a marked increase in echogenicity.

It was concluded that, in domestic cats, testicular parenchyma variations were accompanied by changes in their echotexture, thus, computer-assisted image analysis of B mode ultrasonogram appears as a good indicator of pubertal development and mild alterations of spermatogenesis. Further research in a larger number of animals needs to be carried out before computerized image analysis of ultrasonograms may be widely used in clinical settings.

Author statement


Declaration of competing interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgements

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Changes in circulating hormone concentrations, testes histology and testes during sexual maturation in beef bulls. Theriogenology 1996;46(2):345e57.


Figure legends

Fig. 1: Testicular (mean ± SEM) echogenicity (solid; P< 0.01) and heterogeneity (scattered; P< 0.01) of prepubertal (PREP; n=6), peripubertal (n=2; PERI), and mature (MAT; n=6) male cats. Different letters above the columns show differences of P< 0.01.

Fig. 2: Testicular (mean ± SEM) echogenicity (solid; P< 0.01) and heterogeneity (scattered; P> 0.05) of 7 adult male cats before (PRE) and after (POSTR) 4 weekly administrations of sc acyline (330 μg/kg) SC. Different letters above the columns show differences of P = 0.01.

Fig. 3: Correlations between gonadosomatic index and echogenicity (A; P < 0.01) and heterogeneity (B; P < 0.01) of cats of Fig 1 and 2. See also Table 1.

Table 1: Correlations between testicular gross and histomorphological with echotextural attributes of cats of Fig 1 and 2.
<table>
<thead>
<tr>
<th>Gross &amp; microscopic parameters</th>
<th>Echogenicity</th>
<th>$p$</th>
<th>Heterogeneity</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular volume (cc$^3$)</td>
<td>0.58</td>
<td>$&lt; 0.01$</td>
<td>0.69</td>
<td>$&lt; 0.01$</td>
</tr>
<tr>
<td>Gonadosomatic index</td>
<td>0.63</td>
<td>$&lt; 0.01$</td>
<td>0.46</td>
<td>$&lt; 0.01$</td>
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<tr>
<td>Tubular diameter (µm)</td>
<td>0.69</td>
<td>$&lt; 0.01$</td>
<td>0.71</td>
<td>$&lt; 0.01$</td>
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<tr>
<td>Epithelium height (µm)</td>
<td>0.08</td>
<td>$&gt; 0.1$</td>
<td>0.22</td>
<td>$&gt; 0.1$</td>
</tr>
<tr>
<td>Spermatogonia (%)</td>
<td>-0.75</td>
<td>$&lt; 0.01$</td>
<td>-0.78</td>
<td>$&lt; 0.01$</td>
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<tr>
<td>Primary spermatocytes (%)</td>
<td>0.14</td>
<td>$&gt; 0.1$</td>
<td>0.12</td>
<td>$&gt; 0.1$</td>
</tr>
<tr>
<td>Round spermatids (%)</td>
<td>0.41</td>
<td>0.01</td>
<td>0.43</td>
<td>0.01</td>
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<tr>
<td>Elongated spermatids (%)</td>
<td>0.62</td>
<td>$&lt; 0.01$</td>
<td>0.69</td>
<td>$&lt; 0.01$</td>
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<tr>
<td>Spermatozoa (%)</td>
<td>0.39</td>
<td>$&lt; 0.05$</td>
<td>0.23</td>
<td>$&gt; 0.1$</td>
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<tr>
<td>Tubular/intertubular ratio</td>
<td>0.38</td>
<td>$&lt; 0.05$</td>
<td>0.23</td>
<td>$&gt; 0.1$</td>
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<tr>
<td>Luminal/intertubular ratio</td>
<td>0.54</td>
<td>$&lt; 0.01$</td>
<td>0.42</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Echogenecity

Heterogeneity

PRETR                POSTR

a
b

PRETR    POSTR

c    c

c    c

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**Highlights**

In domestic felids, increasing testicular echogenicity and heterogeneity characterize pubertal attainment.

In mature cats, decreasing testicular echogenicity indicates spermatogenic impairment.

Testicular volume and gonadosomatic index correlate with echogenicity and heterogeneity.

Testicular echogenicity and heterogeneity correlate with tubular diameter, percentage of elongated spermatids and luminal/intertubular ratio.

Testicular echogenicity correlates with tubular/intertubular ratio.
Theriogenology
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