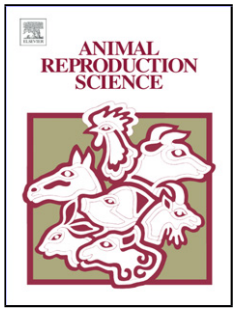

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Effect of the indenopyridine RTI-4587-073 (l) on feline testicle

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ABSTRACT

The aim of this study was to describe the seminal, histomorphological and hormonal effects of the oral indenopyridine RTI-4587-073(l) on the cat testicle. Side effects were also recorded. Sixty testicles of adult cats that had been treated (d 0) with RTI-4587-073(l) 12.5 mg/kg PO and randomly hemi-orchiectomized twice on: day -14 (n = 8), 6 h (n = 6), 12 h (n = 8), 24 h (n = 6), day 7 (n = 8), day 14 (n = 6), day 21 (n = 6), day 35 (n

= 6) or day 42 (n = 6) were studied. Before each hemi-orchiectomy, fecal samples for testosterone (T) measurement were collected and the testes were grossly and examined using ultrasonography. The indenopyridine treatment did not induce changes in testicular weight (P>0.1), volume (P>0.1), echostructure, gonadosomatic index (P>0.1), fecal T concentrations (P>0.1), nor clinical side effects. A severe disorganization of the cytoarchitecture of the seminiferous epithelium, sloughed cells and fluid, were observed in the 6 h samples up to a maximum at 24 h. Tubular diameter (P<0.01) increased twice, during the first 24 h and on d 35. Germinal epithelium was of minimal height on d 14 and rapidly recovered thereafter**.** After treatment, there was a significant decrease in the volume of all the seminiferous cell components, except spermatogonia. Values for all histotological variables were normal by the end of the study. It was concluded that treatment with RTI-4587-073(l) severely disrupted spermatogenesis during the first 24 h after treatment, however, there was a returning to normality in approximately one spermatic cycle without side effects.

Keywords: Felid; Contraception; Non-steroidal; Testis

1. Introduction

Domestic felids (*Felis catus*) are extremely prolific breeders mating from early puberty to the time of death and, if maintained indoors or at latitudes higher than 35º, throughout the year (Faya et al., 2011). Thus, contraception - reversible reproduction control - is often required for genetically valuable domestic or wild cats. As in most other

mammalian species, development of feline contraceptives is mainly focused on females and progestins are commonly used. Sometimes use of progestins lead to severe reproductive and general health side effects (Munson, 2006). Although steroidal and non-steroidal approaches have been assessed in males, controlling spermatogenesis is more complex than preventing ovulation in females. Furthermore, with use of most male contraceptive protocols there is a variable lag time to achieve azoospermia as well as an unpredictable return to fertility (Bowen, 2008). Safe, rapid and efficient pharmaceutical compounds are, therefore, still needed to temporally control the undesired reproduction in male felids. If tom cats were rendered temporarily sterile, female reproductive health could also be enhanced.

The group of indenopyridine derivatives, which were originally developed as antihistamine drugs, has anti-spermatogenic effects in rats (Hodel and Suter, 1978), stallions (Pozor et al., 2013) and dogs (Chang et al., 2002) without overt side effects, toxicity nor mutagenic potential (Fail et al., 2000; Mruk et al., 2008). Results from studies in laboratory animals indicate that the primary target of indenopyridines is the Sertoli cell, resulting in disruption of Sertoli - germ cell adhesion (Hild et al., 2001; 2007b; Koduri et al., 2008).

While in monkeys indenopyridines induced reversible severe oligoasthenozoospermia (Hild et al., 2007a), however, irreversible infertility occurred in rats as a result of this treatment (Hild et al., 2001). In horses and dogs, treatment with indenopyridines induced a rapid (few days) and transient inhibition of spermatogenesis with an increased number of immature germ cells in ejaculates (Hodel and Suter, 1978; Chang et al., 2002; Pozor et al., 2013, 2014). Histologically the seminiferous tubuli appeared devoid of spermatids and spermatocytes (Pozor et al., 2013). The common testicular effects with these four species indicate the indenopyridines could be promising

for non-steroidal, oral male contraception in felids, however, there is nothing known about effects, reversibility and tolerance of cats when there is administration of these compounds (Munson, 2006).

The compound RTI-4587-073, formerly termed CDB-4022, is a new indenopyridine derivative, which is a mixture of L and D-isomers. The L-isomer has marked anti-spermatogenic activity (Pozor et al., 2013) and, therefore, was selected for the present feline trial. Thus, the aim of the present study was to describe the ultrasonographic, histomorphological and hormonal effects of oral indenopyridine RTI-4587-073(l) in the cat testicle. Secondly, some epididymal semen characteristics and clinical side effects of the drug were also recorded in the treated cats.

2. Materials and methods

2.1. Experimental protocol

Sixty testicles of 1.5 to 5 years old, domestic short- hair, fertile cats were included in this study. The toms had been born in our Institutional Colony and there was an imposed 14 h photoperiod in three 4 x 4 m enriched rooms, with animals being fed a commercial food and water being available *ad libitum.* The animals were treated (day 0) with RTI-4587-073(l) at a 12.5 mg/kg dose orally and randomly hemi-orchiectomized twice on: day -14 (n = 8), 6 hours (n = 6), 12 hours (n = 8), day 1 (n = 6), day 7 (n = 8), day 14 (n = 6), day 21 (n = 6), day 35 (n = 6) or day 42 (n = 6). The calculated dose was dissolved in 2 cc of saline and administered by a naso-esophageal tube after mild sedation. The dose and dosing regimen were selected based on studies with other mammals (Hild

et al., 2001; Pozor et al., 2013) and results from some preliminary studies before conducting the present study with cats.

Before each hemi-orchidectomy, fecal samples were collected and the testes were examined grossly and ultrasonographically in a way that each animal had two random evaluation time points on the days of surgeries. The eventual appearance of clinical side effects was also recorded in all the animals. 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 considering the guidelines established in The Guide for The Care and Use of Laboratory Animals, USA.

2.2. Ultrasonic evaluations

Ultrasonic examinations of the testes were undertaken by a single experienced evaluator using a real time B-mode ultrasonic machine (Toshiba Nemio XG, Japón) with a 14 MHz lineal transducer. All machine settings were established at the first examination based on the most distinct image quality and remained unaltered for all remaining examinations. 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 sagittal and transverse planes. Testicular dimensions were obtained from frozen images, using the ultrasonic calipers and the total testicular volume was calculated as described by Linn et al. (2009). Ecogenicity and homogeneity of the parenchyma were also assessed at each time point.

2.3. Fecal collection, extraction and hormone determinations

Fecal samples were collected at the time points of hemi-orchidectomies (subsequently described in this manuscript) and frozen for testosterone (T) quantification.

Fecal steroids were extracted using the methods described by Faya et al. (2013) and T (ng/ml) was quantified using a electrochemiluminescence immunoassay (Elecsys Testo II, Roche Diagnostics, Mannheim, Germany). Inter- and intra-assay coefficients of variation of the assays were < 10% and sensitivity was 0.025 ng/mL. All fecal data were expressed on a wet-weight basis

2.4. Hemiorchidectomies

All the cats were randomly hemi-orchidectomized twice using the procedures described by García Romero et al. (2012) in a way that a total of 60 testes were obtained. Briefly, the animals were pre-medicated with atropine sulfate, (Atropine Sulfate, John Martin; 0.04 mg/kg SC), acepromazine maleate (Acedan, Holliday; 0.03 mg/ kg SC), and butorphanol (Torbutol Plus, Fort Dodge; 0.2 mg/kg IM), anesthesia was induced with sodium thiopental (Pentovet TM, Richmond; 8 mg/kg IV) in all of the animals. After the cats were endotracheally intubated, anesthesia was maintained with isoflurane and oxygen in a closed system. Each testis was removed through a longitudinal midscrotal incision using sterile surgical procedures and a closed tunic technique, and subsequently the spermatic cord was ligated with 3.0 vicryl before transection. After surgery, ketoprofen (Ketofen®, Fort Dodge; 1 mg/kg) was administered SC (once) and then orally every 24 h for 4 additional days. All the cats were subsequently placed for adoption.

2.5.Gross and histological examination of the testes

Immediately after surgical removal, the testes were measured (length, width and depth; cm) and weighed (g) and gonadosomatic index (%; Franca and Godinho, 2003) was also calculated. Sperm were recovered from the epididymal tail using a scalpel blade.

Spermatozoa morphology was evaluated using a Giemsa stain with 1000X magnification using bright field microscopy (Valiente et al., 2014).

The testes, were sectioned longitudinally, placed in Bouin's fixative for 12 h and the solution was subsequently changed to alcohol 70% and tissues were processed routinely with paraffin embedding. After processing, 5 µm serial sections were cut, mounted on slides, dyed, deparaffinized in xylene, rehydrated in graded ethanol solutions and stained with hematoxylin and eosin. Histological images were obtained using a microscope (Olympus BX50; 10X and 40X) with an attached video camera (Sony DXC 151) and digitalized in a 24 bit true color TIFF format. Twenty tubular profiles, which were round or nearly round, were chosen randomly and measured for each animal. The tubular diameter of seminiferous tubules (µm) and the germinal epithelium height (µm) were measured using planimetry (Image Pro Plus, Media Cybernetics, Silver Spring, MA, USA). The volume (cm^3) of the different testicular tissue components was determined by light microscopy using a 441-intersection grid placed on 940 magnification. For this, 15 fields were chosen randomly (6615 points) and scored for each animal. Points were classified as one of the following: spermatogonia, primary and secondary spermatocytes, round spermatids, elongated spermatids, spermatozoa, Sertoli and Leydig cells, intertubular compartment, and cellular debris.

2.6. Statistical analysis

Normality of distribution of results was tested using a Shapiro - Wilk normality test. All hormonal, gross and microscopic variables were compared among time points using the Kruskall-Wallis test followed by use of a Dunn's multiple comparisons test. In all the cases, descriptive data were expressed as mean ± SEM and P values <0.05 were considered significant.

3. Results

Neither gross testicular variables including weight (P>0.1), volume (P>0.1) and gonadosomatic index (P>0.1) nor body weight (P>0.1) were affected by RTI-4587-073(l) treatment at the different evaluation time points (Table 1). Although epididymal spermatozoa morphology was normal in all the cases, giant multinucleated cells appeared in the semen samples during the first 24 h after treatment (Fig 1).

Histological testicular structure was normal on day -14 (Fig 2;A) and a severe disorganization of the cy-architecture of the seminiferous epithelium was observed in the samples collected at the 6 h time point reaching a maximum impairment at 24 h, with the presence of vacuolization, immature germ and multinucleated cells as well as intra-tubular fluid (Fig 2; B). During the first 24 h after treatment, several seminiferous tubules were in the process of sloughing or had already sloughed material within the lumen occluding the tubules along with the fluid and cellular detritus (P<0.01; Fig 2; C and D). These abnormalities gradually disappeared by the end of the study (Fig 2, F, H, I) and there was no evidence of abnormalities through the echo-structure evaluations which was normal at all time points (Mattoon and Nyland, 1995).

Seminiferous tubular diameter (P<0.01; Fig 3, Inset) increased as the interstitium decreased (P<0.01) during the first 24 h with the greatest effects in these regards occurring on d 35. The size of the tubular diameter tended to return to the original size before treatments were administered by the end of the study.

Germinal epithelium height could not be measured during the first 24 h after treatment due to the major disorganization of its cyto-architecture (Fig 3). This epithelium was of a minimal height on day 14 and there was a rapid recovery with peak values on day 21 and,

thereafter, it maintained at the same height (Fig 3). Although the volume occupied by spermatogonia did not vary during the study ($P>0.1$) the volume occupied by primary spermatocytes decreased during this period ($P>0.01$). This decrease was even more marked in the case of secondary spermatocytes ($P<0.01$; Fig 4), round ($P>0.01$) and elongated *(*$P<0.01$; Fig 4 Inset) spermatids as well as luminal spermatozoa ($P<0.01$) which reappeared shortly before the end of the study on day 42. Conversely, the volume of tissue occupied by Sertoli cells increased 24 h after treatment and subsequently decreased up to day 14 when it began to return to a volume that existed pretreatment ($P<0.01$).

Although, the volume occupied by Leydig cells was less at all the post treatment observations than it was before treatments were administered ($P>0.01$), fecal T concentrations did not vary during the same period (data not shown; $P>0.1$).

4. Discussion

Widespread use of contraceptives has been limited in male felids as a result of safety concerns and the lack of effective options. The indenopyridines, however, have antispermatogenic effects in several mammals. There are, however, species differences in responses which indicates the need for the biological effects of this compound to be assessed in species such as cats if there is going to the use in those species. To the best of the authors´ knowledge, this is the first report that describes the effects of indenopyridines in cats.

Consistent with findings in rats (Hild et al., 2001; Koduri et al., 2008) and monkeys (Hild et al., 2007a) but different from those in horses (Pozor et al., 2014), T concentrations in cats did not appear to be affected as a result of treatment RTI-4587-073(l). While

suppression of T and, therefore, libido would have been desirable in domestic cats, T dependent changes in behavior and phenotype are a disadvantage for wild, endangered felids. Testicular volume occupied by Leydig cells was less as compared to volumes pre-treatment in all post-treatment observations in the present study. It should be borne in mind that in the present study, fecal determinations could have masked transient serum T variations that could have been present in blood samples. Consistent with results in horse studies with the same pharmacological protocol (Pozor et al., 2014), total testicular volume did not change during the period that the present study was conducted.

The rapidity of histological effects after treatment with RTI-4587-073(l) in the present study was one of the most marked findings when applying the antispermatogenic protocol. Similar rapid effects of treatment with RTI-4587-073(l) on testicular tissue were previously described in monkeys, horses, dogs and rats (Chang et al., 2002; Hild et al., 2001; 2007a; Pozor et al., 2014).

The seminiferous epithelium vacuolization and sloughing into the tubular lumen causing severe tubular disorganization is a common histopathological observation associated with Sertoli cell injury after exposure to testicular toxicants (Vidal and Whitney, 2014; Johnson, 2015). The multinucleated giant cells found both in the epithelium and semen are thought to arise from fusion of degenerate spermatids with abnormal intercellular bridges (Vidal and Whitney, 2014). This kind of cells was also previously observed in rats, horses and dogs treated with bisdiamines (Hild et al., 2001; Chang et al., 2002; Pozor et al., 2013).

Specifically, indenopyridines alter Sertoli cells leading to a massive germ cell loss (Koduri et al., 2008). As Sertoli cell junctions are an essential component of the blood-testis barrier required for normal spermatogenesis these sites have been considered a target for male contraceptive development (Lee et al., 2009). In the present study, the

injury in Sertoli cells could, not only, explain the early increase in the volume of these cells but also the augmentation of the whole tubular diameter associated with fluid production on Day 1 after RTI-4587-073(l) treatment (Vidal and Whitney, 2014).

As expected, in the presence of these severe histological findings almost all cellular components of the germinal epithelium were affected by the indenopyridine treatment. Importantly, the major decrease in numbers of germinal cells occurred because there were fewer spermatocytes and spermatids after treatment with RTI-4587-073(l).

Before the treatment on day -14, all characteristics for histomorphometric variables were similar to those previously described for the species (Franca and Godinho, 2003; Siemieniuch and Wocławek-Potocka, 2007). The early increase in the seminiferous tubule diameter in the present study has previously thought to be associated with the tubular fluid production after toxicant administration that affected Sertoli cell functions (Vidal and Whitney, 2014). The late augmentation of this diameter on day 35 could be explained as a rebound recovery of spermatogenesis at that time point (Meisami et al., 1994).

The height of the germinal epithelium rapidly decreased to two layers of cells on day 14, however, there was recovery with values for germinal epithelial height being greater than the pretreatment values 1 week after treatment. With use of the pharmacological protocol in the present study, spermatogenesis recovery was possible because of the lack of an effect on spermatogonia throughout the study period. Treatment with indenopyridines have resulted in variable outcomes with there being either a reversible (Chang et al., 2002; Hild et al., 2007a; Pozor et al., 2013) or irreversible (Hild et al., 2001) effect on fertility, depending on the species. In the domestic cats of the present study, there was a reversible effect of this compound on the germinal epithelium during a time period that it would take for one feline spermatic cycle to be completed (Franca and

Godinho, 2003). Similarly, in indenopyridine treated horses and dogs, reversible spermatogenesis was noted 6 and 7 weeks after administration of this compound, respectively (Chang et al., 2002; Pozor et al., 2014).

Considering the rapid and severe histological changes as well as the reversibility of spermatogenesis it would be of interest in the future to test, *in vivo*, fertility during the time when there were the greatest indenopyridine effects after treatment and also after recovery so that there could be single- or serial-dose protocols developed for felids.

Consistent with findings in rodents (Hild et al., 2001) there were no observable adverse effects such as body weight loss or digestive problems in the treated animals of the present study, suggesting that this compound had no general toxicity.

In conclusion, treatment with RTI-4587-073(l) provides for a rapid, effective, reversible and safe non-steroid antispermatogenic oral protocol for male cat contraception because this treatment severely disrupts spermatogenesis during the first 24 h after treatment with there being initiation of spermatogenesis that results in the presence of normal sperm in the tail of the epididymis after a period that equates approximately to one spermatic cycle. Further refinement, including the study of effects of repeated doses and surveillance for long-term adverse effects, of these indenopyridine derivatives should occur before field implementations are widely recommended.

Conflict of interest

The authors do not have any financial nor personal relationships with other people or organizations that could inappropriately influence the study.

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Fig. 1. Giant multinucleated cell (Giemsa stain, 1000X) in epididymal sperm during the first 24 h after RTI-4587-073(l) administration

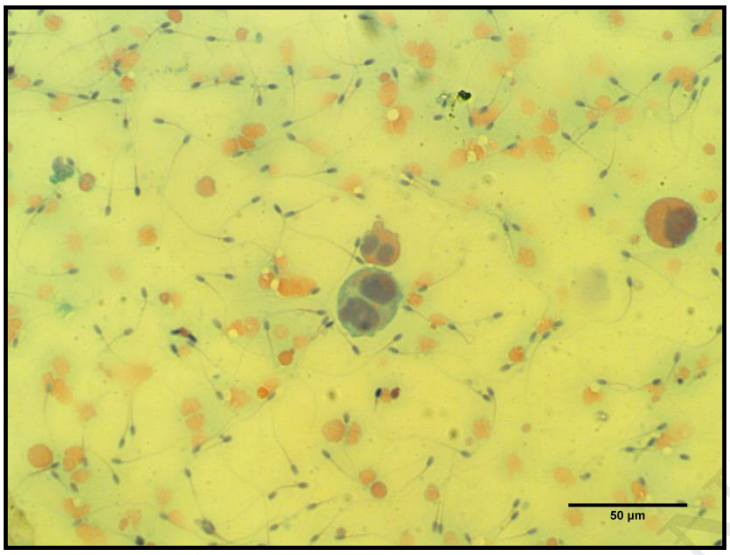

Fig. 2. Seminiferous tubule characteristics of cats at different time points subsequent to RTI-4587-073(l) treatment: -14 (A), 6 hours (B), 12 hours (C), day 1 (D), day 7 (E), day 14 (F), day 21 (G), day 35 (H) and day 42 (I); All sections were stained with hematoxylin and eosin (40X)

Notice normal germinal epithelium and spermatozoa at the luminal border (A), fluid in the lumen of a seminiferous tubule (B), seminiferous epithelium sloughing into the lumen with a giant multinucleated cell in the center (C), severe disorganization of the seminiferous epithelium with occluding detritus filling the lumen (D), epithelium sloughing is finishing and some vacuoles are appearing, luminal detritus is diminishing (E), thin germinal epithelium with frayed luminal border and some detritus in the lumen (F), quite recovered germinal epithelium with still some vacuoles and a clean lumen (G),

recovered germinal epithelium height with some vacuoles (H), fully recovered germinal epithelium with abundant elongated spermatids at the luminal border (I)

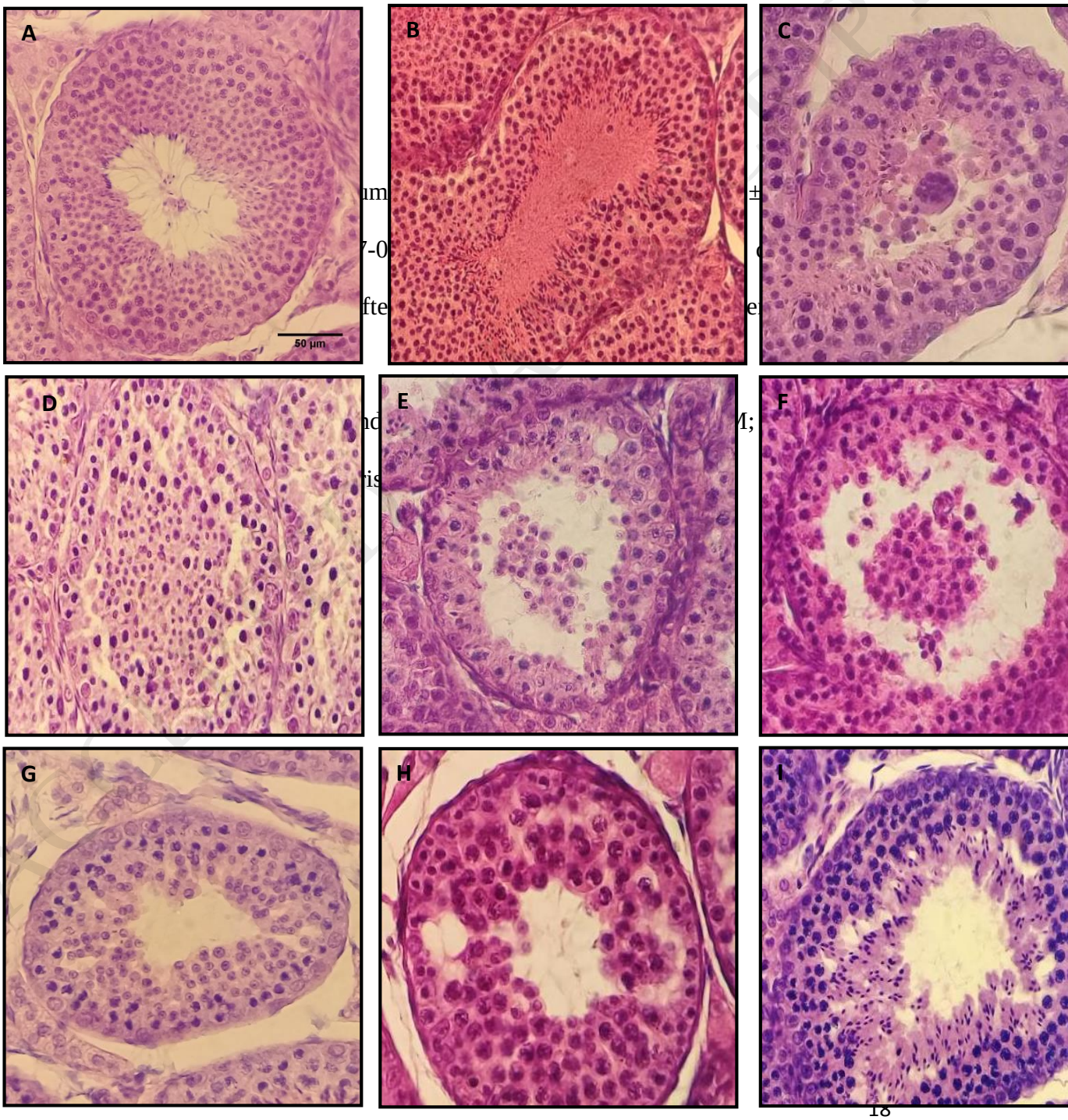

Table 1

Morphometric data of the 30 male cats administered (d 0) a single PO dose of 12.5 mg/kg RTI-4587-073(l) and randomly hemi-orchiectomized twice on: day -14 (*n* = 8), 6 hours (*n* = 6), 12 hours (*n* = 8), day 1 (*n* = 6), day 7 (*n* = 8), day 14 (*n* = 6), day 21 (*n* = 6), day 35 (*n* = 6) or day 42 (*n* = 6)

	d -14	h 6	h 12	h 24	d 7	d 14	d 21	d 35	d 42
Body weight (kg)	4.83± 0.28	5.35± 0.35	4.90± 0.49	4.40± 0.20	4.74± 0.28	4.48± 0.16	4.65± 0.35	4.93± 0.19	4.47± 0.32
Testis weight (g)	1.95± 0.12	1.76± 0.26	1.85± 0.24	1.93± 0.04	1.87± 0.35	1.87± 0.34	1.77± 0.21	1.89± 0.47	1.92± 0.55
Testicular volumen (cm^3)	2.69± 1.43	2.54± 0.40	2.10± 0.29	2.76± 0.29	2.67± 0.97	1.85± 0.92	2.28± 0.62	2.43± 0.66	2.54± 0.68
Gonadosomatic index (%)	0.08± 0.01	0.08± 0.08	0.08± 0.01	0.09± 0.00	0.08± 0.01	0.08± 0.01	0.08± 0.01	0.08± 0.01	0.08± 0.01

Table 2

Component (cm^3)	d -14	h 6	h 12	h 24	d 7	d 14	d 21	d 35	d 42
Sertoli cells	0.15±0.11[a]	0.22±0.04[b]	0.20±0.07[b]	0.23±0.05[b]	0.12±0.0[a]	0.11±0.07[a]	0.12±0.02[a]	0.13±0.09[a]	0.15±0.05[a]
Spermatogonia	0.11±0.05	0.08±0.03	0.07±0.02	0.10±0.03	0.08±0.05	0.06±0.02	0.07±0.04	0.10±0.06	0.07±0.05
Primary spermatocytes	0.76±0.20[a]	0.47±0.20[b]	0.43±0.12[b]	0.51±0.16[b]	0.45±0.20[b]	0.31±0.11[b]	0.29±0.15[b]	0.51±0.15[b]	0.46±0.09[b]
Secondary spermatocytes	0.00±0.01[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.01±0.00[c]
Round spernatids	0.55±0.30[a]	0.39±0.15[b]	0.25±0.05[b]	0.39±0.19[b]	0.33±0.15[b]	0.24±0.06[b]	0.18±0.06[b]	0.25±0.07[b]	0.34±0.09[a]
Elongated spermatids	0.45±0.28[a]	0.14±0.08[b]	0.13±0.06[b]	0.14±0.07[b]	0.17±0.09[b]	0.12±0.09[b]	0.16±0.08[b]	0.18±0.11[b]	0.36±0.12[a]
Spernatozoa	0.02±0.01[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.03±0.00[b]	0.01±0.01[b]	0.00±0.00[b]	0.00±0.00[b]	0.01±0.01[a]
Leydig cells	0.21±0.06[a]	0.04±0.03[b]	0.05±0.04[b]	0.11±0.04[b]	0.09±0.06[b]	0.07±0.02[b]	0.07±0.01[b]	0.12±0.02[a]	0.12±0.08[a]

Intertubular compartment	0.39 ±0.24[a]	0.21±0.06[b]	0.19±0.05[b]	0.22±0.07[b]	0.25±0.07[b]	0.21±0.09[b]	0.22±0.08[b]	0.29±0.05[a]	0.30±0.11[a]
Celular debris	0.10 ±0.07[a]	0.26±0.06[b]	0.317± 0.14[b]	0.33±0.16[b]	0.20±0.08[c]	0.18[c]± 0.06	0.09±0.01[a]	0.09±0.02[a]	0.05±0.03[a]