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Evaluation of sperm head dimensions and chromatin integrity of epididymal sperm from domestic cats using the toluidine blue technique

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ABSTRACT

When using assisted reproductive technologies, there is seldom an evaluation of DNA integrity during sperm analysis, which is an important variable for proper embryo development. The toluidine blue staining technique allows the simultaneous evaluation of sperm chromatin and sperm head dimensions. The objectives of this study were to evaluate the applicability of the toluidine blue staining method for analyzing DNA abnormalities in epididymal sperm (from the caput, corpus, and cauda) of cats and to investigate the correlations among DNA condensation, morphology, and sperm head dimensions. The DNA alteration indexes were obtained using the toluidine blue and acridine orange techniques, and comparisons of these indexes indicated there was a 65.4% ($r = 0.654$; $P < 0.001$) correlation. The sperm from the cauda had greater chromatin stability (97.9%) than the sperm from the epididymal head (92.1%; $P = 0.0023$). There, however, was no difference in chromatin stability between sperm obtained from the corpus and cauda regions, indicating that these sperm were already mature. The sperm head dimension was correlated with chromatin decondensation, and the sperm head size decreased as the sperm were transported through the three epididymal regions ($P < 0.0001$). In addition, the percentage of sperm that were deficient in chromatin condensation decreased as the sperm were transported through the epididymal caput, corpus and cauda (26.4, 15.7, and 3.4%, respectively; $P < 0.0001$). Thus, the sperm head size predicts the quality of chromatin condensation in sperm cells.

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1. Introduction

The family Felidae includes 37 species, among which only the domestic cat (*Felis catus*) is not on the list of endangered species (IUCN, 2015). To preserve the genetic potential of these animals, several assisted reproductive technologies (ARTs) using spermatozoa from fresh or cryopreserved semen have been developed for domestic and non-domestic cats (Gomez et al., 2000; Pope et al., 2012). The ability to recover epididymal or even testicular spermatozoa increases the number of spermatozoa that can be obtained from the same individual for use in the event an animal is lost to sudden death (Bogliolo et al., 2001; Comizzoli et al., 2006; Buarpung et al., 2013).

The evaluation of sperm for ART routinely includes assessments of motility and morphology but does not give adequate importance to DNA integrity (Celik-Ozenci et al., 2004). Because sperm maturation and some chromatin compaction occur during epididymal transit (Hingst et al., 1995), the use of gametes from the testicles or epididymides makes the analysis of the DNA status even more important. Although spermatozoa with chromatin abnormalities are known to be fertile, embryonic development depends on the severity of DNA alteration (Ahmadi and Ng, 1999). Several methods have been developed to evaluate sperm DNA abnormalities, and these methods involve the terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay, sperm chromatin dispersion (SCD), the comet assay, chromomycin A3 (CMA3) and the sperm chromatin structure assay (SCSA), which uses acridine orange (AO) staining and flow cytometry (Ribas-Maynou et al., 2013; Vernocchi et al., 2014; Prochowska et al., 2014, 2016, 2017). The AO test, which is performed using fluorescence microscopy, consists of a simple, metachromatic technique in which green or red-orange fluorescence is emitted when the dye is bound to double- or single-stranded nucleic acids, respectively. The red-orange fluorescence intensity indicates the amount of DNA double-strand breakage, which indicates a loss of chromatin integrity (Evenson et al., 1980).

Toluidine blue (TB) staining is another method that is used to identify chromatin alterations and allows for the concomitant evaluation of sperm morphometry. Spermatozoa with less condensed chromatin have a greater number of phosphate groups available for binding to the metachromatic cationic dye, which results in a colour ranging from dark blue to violet. In normal spermatozoa, phosphate groups are blocked by protamines, resulting in only a few TB molecules being able to bind DNA and thus producing a light blue stain (Beletti and Costa, 2003). Because visual analyses are subjective, the TB staining technique also allows computerized evaluation, which increases the sensitivity, accuracy, rapidity and reproducibility of the assay (Beletti and Costa, 2003; Beletti and Mello, 2004; Beletti et al., 2005; Cruz et al., 2014).

Considering the importance of paternal genome integrity on embryonic development (Seli and Sakkas, 2005), the analysis of DNA condensation assures the use of high-quality spermatozoa in ART. To the best of our knowledge, although the use of TB staining has been described for other animal species, including rabbits (Beletti and Mello, 2004), horses (Naves et al., 2006; Sardoy et al., 2008), humans (Rocha et al., 2002; Marchesi et al., 2010), and cattle (Kipper et al., 2017), its use in cats has not yet been reported.

The aim of this study was to assess the applicability of the TB staining technique for the spermatozoa of domestic felines by evaluating the chromatin compaction and head dimensions of spermatozoa from distinct epididymal regions.

2. Materials and methods

2.1. Selection of animals

Thirty mixed-breed male cats aged 17.1 ± 8.9 (mean ± SD) months were selected for this study. The animals weighed an average of 4.0 ± 0.6 (mean ± SD) kg and were considered healthy according to physical and clinical examinations, with no reports of sexual diseases. The serum testosterone concentrations of the 30 tomcats ranged from 0.2 to 3.8 ng/mL, indicating that all the animals were post-pubescent (Apparicio and Vicente, 2015). The animals were patients of the Veterinary Hospital (São Paulo State University, Araçatuba). The local Animal Ethics Committee approved all the procedures (protocol number 2015-00754).

Immediately prior to orchiectomy, a 1-mL blood sample was collected through a jugular vein puncture from each animal under general anesthesia. Each serum sample was separated by centrifugation, and the testosterone concentrations were measured using a chemiluminescent assay (Immulite® 1000, Siemens).

2.2. Epididymal sperm recovery

The surgically removed testicles and epididymides were immersed in PBS (Laborclin, Laboratório de Análises Clínicas e Anatomia Patológica, São José do Rio Preto, Brasil) at 37 °C and transported to the laboratory within 2 h. The epididymal regions (caput, corpus, and cauda) were macroscopically identified using the procedures of Kunkitti et al. (2015) by the same individual and separated using ligatures. Blood was removed by external washing with PBS at 37 °C. Each region was dissected, freed from visible blood vessels, transversally sectioned into small fragments, and stored separately in 2-mL Eppendorf tubes containing 200 µL of PBS at 37 °C to allow the outflow of sperm into the solution. Samples obtained from the epididymides (right and left) of the same animal were pooled.

After a 10-minute incubation at 37 °C, tissue segments were removed, and 50 µL from each region was diluted in 4% formalin-saline (1:1) for subsequent evaluation of sperm abnormalities. The remaining volume was used for making microscopic smears. The contents of the sperm smears were dried at room temperature and immersed in Carnoy's solution (three parts 70% ethanol to one-part acetic acid) (Beletti and Mello, 1996). Fixed contents on smears were stored in slide boxes at room temperature for further evaluation by staining with TB and AO.

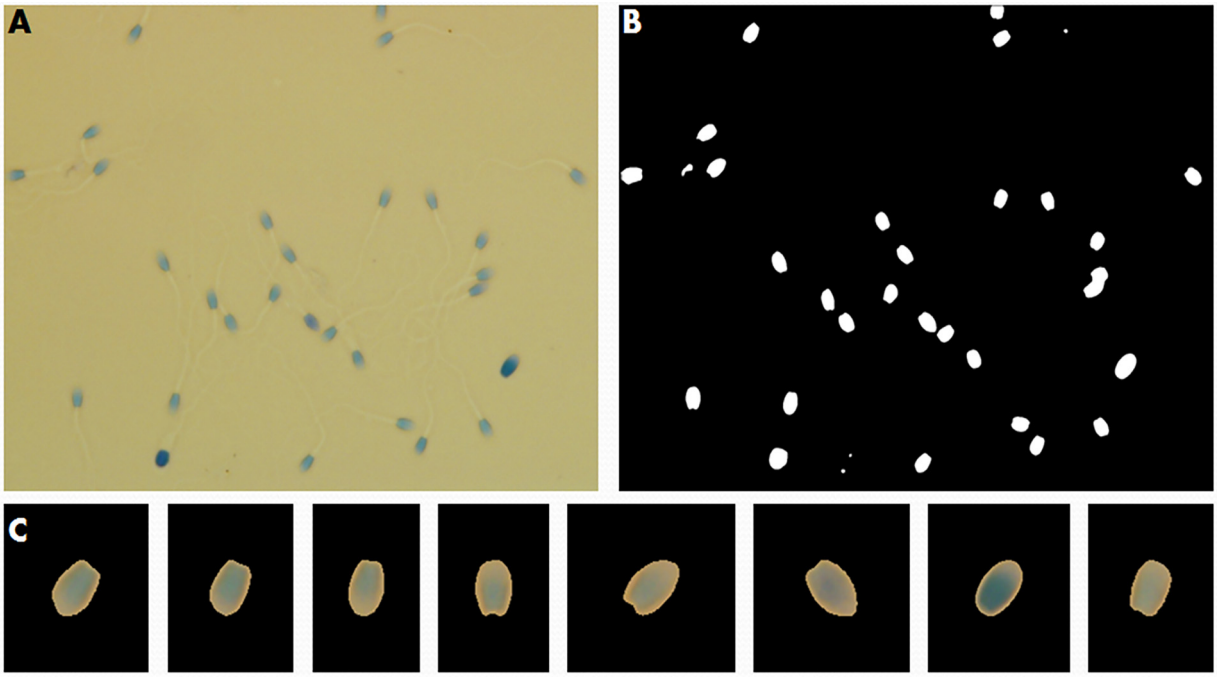

Fig. 1. (A) Spermatozoa obtained from the epididymal cauda of a feline (toluidine blue staining, x1000); (B) Identification and (C) segmentation of sperm heads using OCTAVE software.

2.3. Sperm chromatin condensation and fragmentation

In the present study, the condensation and fragmentation of DNA from 18,000 spermatozoa (3000 spermatozoa were obtained from each epididymal region) were evaluated: half of the spermatozoa were assayed via TB staining, and the other half was evaluated using AO staining.

2.3.1. Sperm head morphometry and chromatin condensation evaluation using toluidine blue

The computerized evaluation of sperm head dimensions and chromatin condensation was performed according to the method described by Beletti and Costa (2003). Images of 100 spermatozoa per smear were obtained using light microscopy with an Olympus BX 61 instrument coupled to a DP-71 Olympus camera (1000×) (Fig. 1). A software program was developed using MATLAB code and was utilized in the OCTAVE environment to obtain the total area of each sperm head and to minimize the area and region exhibiting

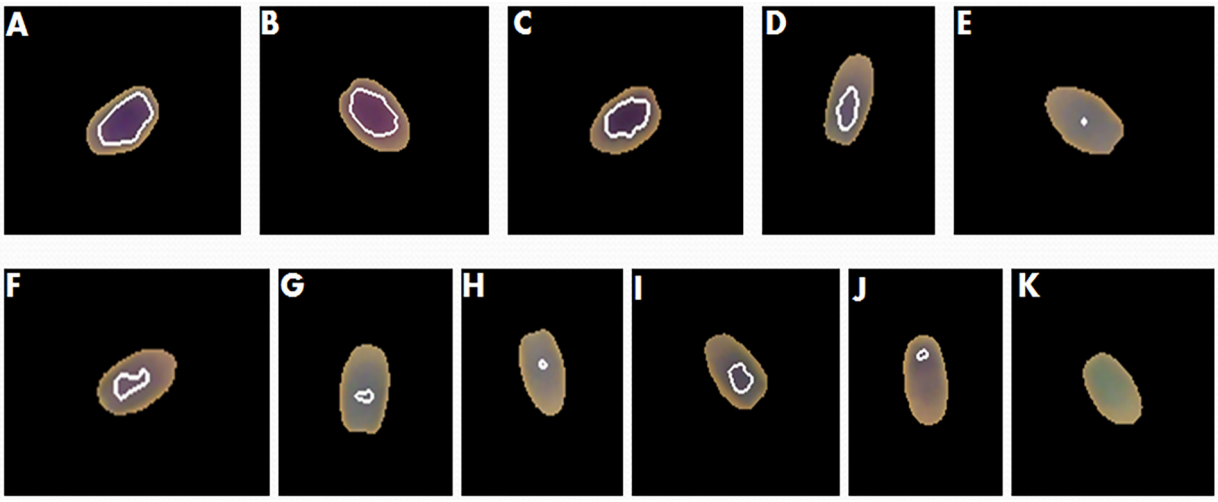

Fig. 2. Sperm heads obtained from the epididymal caput (A–E), corpus (F–H) and cauda (I – K) of a cat; areas with chromatin decompaction were identified through a computational analysis (MATLAB).

chromatin de-compaction (Fig. 2; Cruz et al., 2014).

2.3.2. Evaluation of sperm chromatin integrity using acridine orange

The chromatin integrity was assessed using the AO staining technique described by Tejada et al. (1984). Briefly, previously fixed contents of microscopic sperm smears were covered with 3 mL of staining solution for 3 min in a dark chamber and then washed with distilled water. Slides were examined using an epifluorescence microscope (Olympus BX61) equipped with a 460–570-nm excitation filter and a 460-610-nm emission filter (oil immersion, 1000×). For each smear, 100 cells were counted and classified according to Tejada et al. (1984). The cells that emitted green fluorescence were considered to have a normal chromatin integrity, whereas the emission of red, orange and yellow fluorescence, either regularly or irregularly distributed inside the sperm head, was considered to indicate complete or partial chromatin denaturation.

2.4. Sperm morphology

Sperm abnormalities were classified into major (i.e., acrosome defects, abnormal contour, abnormal loose heads, proximal droplets, abnormal midpiece, vacuoles, double forms, dag defect) and minor defects (i.e., micro and macrocephalic heads, normal loose heads, abaxial implantation, coiled tails, distal droplets) using the procedures of Blom (1973) and Barth and Oko (1989). A total of 200 cells with abnormalities (one per sperm) were examined, and the results are reported as percentages (DIC microscopy, oil immersion, 1000×, Olympus BX61). Spermatozoa in which cytoplasmic droplets were the only alteration were considered normal (Axnér et al., 2004; Müller et al., 2012) because such droplets move during epididymal transit and might be lost during ejaculation (Axnér et al., 1999).

2.5. Statistical analysis

The experimental data were tested for normality (Shapiro-Wilk). Data with a normal distribution were assessed using an analysis of variance (ANOVA), and the means were compared using the Tukey's test. Data without a normal distribution were evaluated using the Friedman's non-parametric test using Action® version 2.7 (Equipe Estatcamp, 2014). Pearson correlation analyses were performed to assess the correlations between the values for total sperm head area and chromatin decondensation area measurements, between the TB and AO staining methods, between morphological abnormalities and chromatin decondensation and between head abnormalities and chromatin decondensation. The results were considered significant if $P < 0.05$ (Zar, 1984).

3. Results and discussion

The mean percentages of major, minor and total defects observed in spermatozoa from each epididymal region are shown in Table 1. The percentages of abnormalities decreased as site of collection progressed from the caput to the cauda of the epididymis, indicating the existence of a mechanism that removes abnormal spermatozoa during epididymal transit (Axnér, 2006).

Sperm chromatin condensation begins in the testicle and continues throughout the passage of spermatozoa through the epididymis, and as a result, the nuclei of sperm become increasingly more compact due to the substitution of histones by protamines through a process termed protamination (Hingst et al., 1995; Balhorn, 2011; Sringam et al., 2011). Chromatin reorganization decreases the size of spermatic cells (Balhorn, 2011), consistent with the data obtained in conducting the present study. There was a significant reduction in the values for both the total sperm head area and the chromatin decondensation area from the head to the tail of the epididymis, as shown in Table 2.

Because the sperm head is almost entirely composed of DNA, slight differences in the sperm nuclear shape are expected to be related to the chromatin content and organization (Ostermeier et al., 2001; Lange-Consiglio et al., 2010; Enciso et al., 2011; Mangiarini et al., 2013). Although results from a previous study indicated there was a correlation between the percentage of spermatozoa having chromatin decondensation and the percentage of spermatozoa with morphologically abnormal heads (Hingst et al., 1995), there was no correlation between these variables in the current study ($r = 0.23$; $P > 0.05$). Results from the present study are consistent with the findings reported by Vernocchi et al. (2014), where sperm morphology was not correlated with chromatin alterations, even when only head defects were analyzed.

There was a significant correlation in values between the total sperm head area and the chromatin decondensation area, i.e., larger heads with an increased chromatin de-condensation ($r = 0.68$; $P = 0.0001$). Thus, the computerized analysis was more

Table 1
Percentages (mean ± SD) of major, minor and total defects in spermatozoa from the caput, corpus and cauda of the epididymis of domestic cats ($n = 30$).

Epididymal region	Major defects	Minor defects	Total defects
Caput	14.4 ± 5.1[a]	17.5 ± 6.4[a]	31.9 ± 8.9[a]
Corpus	13.3 ± 5.3[b]	12.6 ± 5.6[b]	25.9 ± 9.3[b]
Cauda	9.2 ± 5.0[c]	7.5 ± 6.3[c]	16.7 ± 9.9[c]

[a, b]Different letters in columns indicate differences ($P < 0.05$).

Table 2
Total head area and area with altered chromatin condensation (mean ± SD) in spermatozoa from the caput, corpus and cauda of the epididymis of domestic cats (n = 30).

Epididymal region	Toluidine blue staining		
	Total area (μm²)	Altered area (μm²)	Altered area (%)
Caput	10.22 ± 1.55[a]	0.47 ± 0.38[a]	3.38 ± 2.72[a]
Corpus	9.40 ± 2.71[ab]	0.20 ± 0.24[b]	1.58 ± 1.68[b]
Cauda	8.55 ± 2.18[b]	0.04 ± 0.05[b]	0.39 ± 0.49[c]

[a, b]Different letters in columns indicate differences (P < 0.05).

sensitive and accurate for detecting the relationship between chromatin compaction and head size in cat sperm.

The use of the TB staining technique for the analysis of cat sperm has not been previously evaluated. Thus, there were attempts in the present study to compare the detection of DNA abnormalities in sperm from cats using this technique with that observed using the AO staining technique, as was previously performed for humans (Rocha et al., 2002), horses (Naves et al., 2006), roosters (Rocha and Beletti, 2008), sheep and goats (Kamimura et al., 2010).

The TB staining can be used to indirectly assess the presence or absence of protamines because sperm heads with a greater number of free phosphate groups, which are not blocked by protamines, are stained with a greater intensity (Mello, 1982). Because with use of AO staining there is an evaluation of DNA double-strand breaks (Evenson et al., 1980), the TB and AO staining techniques can be used to evaluate different types of DNA abnormalities. The chromatin compaction process, however, requires the breaking of DNA double strands to allow for DNA reorganization. Breaks caused by topoisomerase II beta result in unwinding of DNA strands that are organized by histones, allowing the substitution for protamines. At the end of this protamination process, which occurs during epididymal transit (Andrabi, 2007), these breaks must be repaired because after re-compaction, the sperm lose the repair capacity and become quiescent (Balhorn, 2011). The comparison of these techniques resulting in greater TB staining resulted in a greater proportion of spermatozoa with chromatin alterations compared with that obtained with AO staining (Table 3), regardless of the epididymal region from which the sperm were collected. The TB induces metachromasia even when only a few TB molecules are bound to the DNA-protein complex, allowing the detection of minor changes in chromatin. In a previous study, there was detection of chromatin alterations in 71.9% of human spermatozoa using TB, whereas only 24.2% were observed to have such alterations using AO (Rocha et al., 2002). The TB staining technique is more sensitive than AO staining for assaying sperm from buffalo (Machado and Beletti, 2002), horses (Naves et al., 2006), sheep and goats (Kamimura et al., 2010). Furthermore, a comparison between these techniques indicated correlations of 55% in stallion spermatozoa (Naves et al., 2006), 35% in goat spermatozoa and 89% in ram spermatozoa (Kamimura et al., 2010), whereas in cat sperm, the correlation between the two techniques was 65.4% (r = 0.6538; P = 0.0002).

The results indicated there was an increase in the percentage of spermatozoa with DNA compaction and integrity during epididymal transit (Table 3). This finding indicates the occurrence of a continuous substitution of histones for protamines, which compacts the genetic material to ensure paternal genome integrity (Oliva, 2006; Sharma and Agarwal, 2011).

The analysis of the percentage of spermatozoa having DNA integrity (AO staining; Table 3) indicated there was no difference between spermatozoa from the corpus and cauda, which is consistent with the results from other studies where that spermatozoa from the epididymal corpus had reproductive maturity (Hingst et al., 1995; Sringam et al., 2011). Similar results were obtained in studies comparing spermatozoa collected with urethral catheterization and epididymal slicing (Prochowska et al., 2016, 2017).

Spermatozoa from the caput, however, must be considered for use in association with those from the corpus and cauda because large quantities of these spermatozoa have chromatin condensation (73.6%) and there is relatively greater DNA integrity (92.1%). Further studies are needed to evaluate the quality of epididymal caput spermatozoa and thus confirm the possibility of preserving these cells and widen the range of options for obtaining a greater number of gametes from the same individual animal.

Table 3
Percentages (median, range) of DNA compaction, decompaction obtained by toluidine blue staining, and integrity or fragmentation obtained by acridine orange staining in spermatozoa from the caput, corpus and cauda of the epididymis of domestic cats (n = 30).

Epididymal region	Toluidine blue		Acridine orange	
	DNA compaction	DNA decompaction	DNA integrity	DNA fragmentation
Caput	75.3[c] 36.0–100.0	24.7[a] 0.0–64.0	96.0[b] 54.0–100.0	4.0[a] 0.0–46.0
Corpus	90.6[b] 44.0–100.0	9.4[b] 0.0–56.0	98.5[a] 66.0–100.0	1.5[b] 0.0–34.0
Cauda	97.7[a] 87.4–100.0	2.3[c] 0.0–12.6	99.0[a] 81.0–100.0	1.0[b] 0.0–19.0

[a, b, c]Different letters in columns indicate differences (P < 0.05).

4. Conclusion

In the present study, there was a correlation between the cat sperm head area and chromatin condensation. The sperm head size decreased due to increases in DNA compaction during epididymal transit. In the present study, the TB staining technique was applicable for cat sperm and that this technique allows for an efficient analysis of sperm DNA condensation and has greater sensitivity than AO staining.

Conflict of interest

None.

Author contributions

I.P. Alves collected and analyzed the samples and wrote the manuscript; C.H.B Cancelli conducted the anesthetic procedures; T.L.M. Grassi performed the statistical analyses; P.R.H Oliveira and D.A. Franciscato participated in the laboratory analyses; J.T. Carreira helped elaborate the methodologies; and M.B. Koivisto is responsible for the design, supervision and coordination of the project. All authors read and approved the final manuscript.

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