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Choice of staining technique affects the morphological assessment of epididymal feline sperm

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ABSTRACT: Morphological examination of spermatozoa remains a valuable tool for determining the quality of feline sperm for cryopreservation and/or for use in artificial insemination. Numerous staining techniques have been employed for feline sperm assessment, and several variations have also been reported. Forty-eight domestic cats were castrated and epididymal sperm samples were obtained. Sperm morphology was assessed using two staining methods, an air-dried stain (Giemsa-Wright) and a wet stain (eosin-nigrosin), to determine their relative abilities for estimation of the proportion of normal and abnormal spermatozoa. The effects of centrifugation and the cooling process on sperm morphology were also analysed. The mean percentage of morphologically normal spermatozoa was significantly lower in eosin-nigrosin-stained samples than in Giemsa-Wright-stained preparations (46.5% vs 53.4%; $P < 0.01$), both examined by conventional light microscopy. Results concerning head, midpiece and tail sperm abnormalities suggest that these methods differ in their capacity to identify sperm anomalies in cat. Although no significant differences were observed for secondary abnormalities, cytoplasmic droplets were significantly lower in Giemsa-Wright-stained samples ($P < 0.01$). Centrifugation or refrigeration of sperm samples did not affect the epididymal sperm morphology in evaluated samples. This study highlights the necessity of minimising morphological assessment variations in order to eliminate subsequent errors and to improve the reproducibility of sperm morphology assessment in different laboratories.

Keywords: *Felis catus*; cat; spermatozoa; morphology; eosin-nigrosin stain; Giemsa-Wright stain

The recovery of spermatozoa is an important step in the use of assisted reproductive technologies in felines, and is frequently complicated, not only in wild felines, but also in domestic cats. Nowadays, different techniques, such as electroejaculation or epididymal and urethral collection, are used to obtain spermatozoa in cats. Epididymal sperm can be obtained after castration or post-mortem, recovering valuable genetic material that otherwise would be lost. However, epididymal sperm show a high percentage of abnormalities, ranging from 36 to 54% (Goodrowe and Hay 1993; Lengwinat and Blottner 1994). The morphological evaluation of epididymal sperm samples might be important for predicting the performance of frozen-thawed sperm in artificial insemination or *in vitro* fertilization programs. Studies on factors affecting the motility or functionality of feline sperm have been reported (Pukazhenthi et al. 2002; Villaverde et al. 2006; Chatdarong et al. 2009). However, few studies have been conducted to evaluate the morphological sperm variations in toms. There is not a standard method to assess the morphology in feline sperm, and reported variations could be attributed to the methods employed. Different staining methods have been reported to assess the feline sperm morphology, such as 0.3% glutaraldehyde (Howard et al. 1990; Penfold et al. 2003), carbolfuchsin (Axner et al. 1998; Axner et al. 1999; Axner et al. 2004), eosin-nigrosin (Glover and Watson 1987; Tittarelli et al. 2006), Giemsa-Wright (Mota and Ramalho-Santos 2006), Spermac® (Baran et al. 2004), Pope's staining (Tebet et al. 2006) and fast green FCF Bengal pink (Villaverde et al. 2006), or fixation in formol-saline (Axner 1999). Different percentages of morphologically normal spermatozoa have been reported depending on the staining technique (preparation, type of dye, osmolality) in species such as dog (Root

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Kustritz et al. 1998), human (Meschede et al. 1993; Bjorndahl et al. 2004), fowl (Bilgili et al. 1985) or bull (Sprecher and Coe 1996). The aim of this study was to analyse if staining methods (Giemsa-Wright vs eosin-nigrosin), as well as centrifugation or refrigeration could influence the morphology of epididymal cat sperm, with the aim of reducing variation between laboratories.

MATERIAL AND METHODS

Animals. Forty-eight domestic cats were orchidectomised for this study. Their mean age ranged from 12 to 24 months and their mean weight was 3.2 ± 0.6 kg. After that, deferent ducts were tied and the testes and epididymides were transported to the laboratory within 1 h after collection in a sterile 0.9% sodium chloride solution at room temperature for processing. The study was carried out in Cordoba (Spain), and private veterinary clinics supplied the specimens with previous authorisation by owners. The experimental procedures were performed in accordance with Spanish Animal Protection Law RD 1201/2005, based on Directive 86/609 of the European Union concerning the protection of animals used in scientific experimentation.

Blood vessels were removed from epididymides and proximal deferent ducts, and spermatozoa were collected by mincing the caudae epididymides in 3 ml Dulbecco's phosphate buffered saline (DPBS) (Oxoid, Hampshire, England) (osmolality, 260–290 mOsm) at 38 °C. After 10 min, the washed tissue was filtered to obtain the spermatozoa, and 3 ml DPBS were used to again wash the tissue retained in the filter. Sperm samples corresponding to thirty-six cats exhibiting total motility ≥ 40% were used.

Assessment of sperm morphology. Sperm morphology was evaluated using two different staining methods, an air-dried stain (Giemsa-Wright) and a wet stain (eosin-nigrosin), to determine if the proportions of normal and abnormal spermatozoa were over- or underestimated. For the Giemsa-Wright stain, a 5 µl aliquot of sperm diluted in DPBS was smeared and allowed to air-dry. Then, samples were dipped for 1 min in each solution (fixative and stains; Panreac, Barcelona, Spain) (Howard et al. 1990). At the end, smears were cleaned with distilled water. Similarly, for the eosin-nigrosin stain, a 5 µl-volume sample was mixed with one droplet (the same volume) of the eosin-nigrosin staining solution. The suspension was incubated for 30 s at room temperature (20 °C) and one droplet was transferred to a labelled microscope slide, where it was smeared by sliding a cover slip in front of it. The smears were examined directly. At least 200 sperm were assessed at a magnification of × 1000 under oil immersion. All smears were mounted and coded before assessment.

Morphological defects were classified attending to the affected region of spermatozoa, i.e. head, midpiece and tail. Head anomalies were classified as macrocephalic, microcephalic, pear-shape head or spermatid. Midpiece anomalies were classified as detached head, bent neck, proximal and distal cytoplasmic droplet, bent midpiece or coiled midpiece. Bent, coiled and split tails were also anomalies observed in the sperm tail.

In order to evaluate the effect of sperm centrifugation on the morphology of spermatozoa, epididymal sperm samples from 12 domestic tomcats were assessed upon collection and after centrifugation. For this purpose, sperm samples were diluted in DPBS medium as described above, and morphology was assessed. Then, sperm samples were centrifuged at 300 × *g* for 8 min, the sperm pellet was re-suspended in 700 µl DPBS medium at 20 °C, and morphological assessment was carried out.

To evaluate the effect of cold shock during the sperm manipulation, sperm samples from eight domestic tomcats were refrigerated. Epididymal sperm samples were diluted in DPBS medium and centrifuged at 300 × *g* for 8 min. The sperm pellet was resuspended in 700 µl TRIS-based extender at 20 °C and then cooled to 4 °C (at a rate of 0.3 °C/min) using a controlled refrigerator with a digital thermometer. Two hours later, sperm morphology was assessed.

Statistical analysis. Giemsa-Wright and eosin-nigrosin (n = 36) were compared in order to evaluate if the staining method affects sperm morphology. Also, samples were centrifuged (n = 12) to evaluate if the centrifugation process affects sperm morphology. Finally, samples were refrigerated (n = 8) to determine if cold shock during sperm manipulation affects sperm morphology. Statistical analysis was performed using analysis of variance (ANOVA). The data assumed a normal distribution. The influence of staining method on percentage of morphologically normal spermatozoa, percentage of spermatozoa with abnormal heads, percentage with abnormal midpieces, and percentage with abnormal tails was assessed, with a P-value

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of less than 0.05 considered significant. Pair-wise comparisons were made using the least significant difference test. Data are expressed as mean ± SEM.

RESULTS

In this study, the percentage of normal spermatozoa was significantly affected by staining technique, with lower values assessed in Giemsa-Wright samples compared to samples stained with eosin-nigrosin (46.4 ± 1.7 vs 53.4 ± 1.4; $P < 0.01$).

While head abnormalities were significantly higher when Giemsa-Wright stain was used (5.6 ± 0.9 vs 1.2 ± 0.3; $P < 0.01$), the evaluation of midpiece and tail revealed significant lower values with this staining technique (20.3 ± 1.7 vs 27.9 ± 1.5; $P < 0.01$ and 20.6 ± 1.1 vs 24.0 ± 1.2; $P < 0.05$, respectively). The most frequent abnormalities detected in the spermatozoa heads were detached heads and macrocephalia, although the latter was significantly reduced when the eosin-nigrosin stain was used. Bent neck and midpiece, coiled midpiece, and distal and proximal cytoplasmic droplets were the abnormalities detected in the midpiece. Cytoplasmic droplets were significantly increased when the eosin-nigrosin stain was used (Table 1). The most frequently observed abnormalities affecting the spermatozoa tail in cat were bent and coiled tails.

Table 1. Morphological sperm evaluation of epididymal samples stained with two different techniques (mean ± SEM; $n = 36$)

Defects	Giemsa-Wright	Eosin-nigrosin	*P*-value
Normal	53.4 ± 1.4	46.5 ± 1.7	< 0.01
Abnormal			
Macrocephalic	3.4 ± 0.8	0.3 ± 0.8	< 0.01
Microcephalic	1.3 ± 0.3	0.6 ± 0.2	n.s.
Pear shape	0.4 ± 0.1	0.8 ± 0.2	n.s.
Spermatides	0.3 ± 0.1	0.1 ± 0.1	n.s.
Detached heads	6.6 ± 0.9	4.7 ± 0.8	n.s.
Bent neck	1.3 ± 0.3	1.0 ± 0.2	n.s.
Bent midpiece	6.7 ± 1.1	8.6 ± 1.0	n.s.
Coiled midpiece	1.1 ± 0.3	1.8 ± 0.4	n.s.
Proximal droplets	2.9 ± 0.5	7.7 ± 1.1	< 0.01
Distal droplets	1.3 ± 0.3	4.1 ± 0.6	< 0.01
Bent tail	15.4 ± 1.1	19.4 ± 1.2	< 0.05
Coiled tail	2.9 ± 0.6	3.9 ± 0.8	n.s.

The eosin-nigrosin stain allows differentiation of live and death spermatozoa. Using this stain 90% of normal spermatozoa were considered as "alive". The more common abnormalities in live spermatozoa were bent midpiece, proximal droplet and bent or coiled tails. Dead spermatozoa showed a significantly higher percentage of detached heads (Table 2).

The percentage of morphological sperm abnormalities was similar before and after centrifugation ($P > 0.05$) for both staining methods. For this analysis, defects were also grouped according to the part of the spermatozoa affected (i.e., head, midpiece or tail; Table 3), but significant differences were not detected.

Epididymal sperm were diluted in TRIS-based egg-yolk extender for refrigeration at 4 °C and were then compared with initial values. The morphological sperm defects did not vary in fresh or cooled samples, except for head abnormalities in Giemsa-Wright samples, where cooled values were significantly lower. Differences between staining methods were observed, with a higher percentage of head defects and lower percentage of midpiece and tail defects in samples stained with Giemsa-Wright than in eosin-nigrosin-stained samples (Table 3).

DISCUSSION

Table 2. Proportions of different abnormal sperm forms detected using eosin-nigrosin vital staining in live and death spermatozoa recovered from the epididymides (mean ± SEM; $n = 36$)

Defects	Live	Death	*P*-value
Normal	41.9 ± 1.8	4.9 ± 0.9	< 0.01
Abnormal			
Macrocephalic	0.2 ± 0.1	0.1 ± 0.1	n.s.
Microcephalic	0.2 ± 0.2	0.4 ± 0.1	n.s.
Pear shape	0.1 ± 0.1	0.03 ± 0.1	n.s.
Spermatides	0	0.08 ± 0.1	n.s.
Detached head	1.1 ± 0.3	3.6 ± 0.6	< 0.01
Bent neck	0.8 ± 0.2	0.3 ± 0.1	< 0.05
Bent midpiece	7.5 ± 0.9	1.3 ± 0.4	< 0.01
Coiled midpiece	1.6 ± 0.4	0.2 ± 0.1	< 0.01
Proximal droplet	7.1 ± 1.0	0.6 ± 0.1	< 0.01
Distal droplet	3.7 ± 0.6	0.4 ± 0.2	< 0.01
Bent tail	15.3 ± 1.3	3.8 ± 0.6	< 0.01
Coiled tail	3.1 ± 0.6	0.8 ± 0.3	< 0.01

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Table 3. Comparison of Giemsa-Wright and eosin-nigrosin stains for evaluation of the effect of centrifugation (at recovery and after centrifugation; n = 12) and the effect of cold shock (at the recovery and after cooling; n = 8) on the morphology of epididymal sperm samples

	Centrifugation effect			Cooling effect		
	after collection	after centrifugation	*P*-value	fresh	cooled	*P*-value
Normal sperm						
Giemsa-Wright stain	56.3 ± 1.7[a]	56.5 ± 1.5[a]	n.s.	55.5 ± 1.7[a]	54.3 ± 3.5[a]	n.s.
Eosin-nigrosin stain	50.3 ± 1.8[b]	46.5 ± 1.9[b]	n.s.	49.6 ± 1.9[b]	41.7 ± 2.8[b]	n.s.
Abnormal sperm – head defects						
Giemsa-Wright stain	6.0 ± 1.9[a]	3.8 ± 0.9[a]	n.s.	7.1 ± 2.5[a]	1.6 ± 0.8	0.05
Eosin-nigrosin stain	1.0 ± 0.2[b]	1.0 ± 0.3[b]	n.s.	1.1 ± 0.3[b]	0.9 ± 0.3	n.s.
Abnormal sperm – mid-piece defects						
Giemsa-Wright stain	18.7 ± 3.7	19.7 ± 2.4[a]	n.s.	16.1 ± 2.5[a]	23.3 ± 3.1	n.s.
Eosin-nigrosin stain	26.5 ± 3.3	28.1 ± 1.7[b]	n.s.	26.3 ± 2.5[b]	30.1 ± 3.8	n.s.
Abnormal sperm – tail defects						
Giemsa-Wright stain	19.1 ± 1.6	20.1 ± 1.8	n.s.	19.3 ± 2.3	20.9 ± 1.7[a]	n.s.
Eosin-nigrosin stain	21.2 ± 2.2	24.3 ± 2.1	n.s.	22.9 ± 2.3	27.3 ± 2.3[b]	n.s.

Significant differences within a row ($P < 0.05$) are indicates in *P*-value column
[a,b]Significant differences between stains within a column ($P < 0.05$)

Results concerning sperm morphology in samples obtained from epididymides in domestic tomcats showed great inter-individual variability. The important effect of sperm morphology on fertility in the cat has been previously documented (Axner et al. 1998; Axner and Linde-Forsberg 2007). It was observed that the percentage of morphologically normal spermatozoa was 53.4% when the Giemsa-Wright stain was used, and only seven tomcats showed more than 40% of morphologically normal spermatozoa. The percentage of abnormal spermatozoa observed in this study was similar to that reported by Tebet et al. (2006) in epididymal spermatozoa, but slightly lower than that obtained by Axner and Linde-Forsberg (2007) in ejaculated spermatozoa. In the domestic tomcat, high proportions of abnormal spermatozoa (more than 60%) are usually produced, which is termed teratospermy. Moreover, normal spermatozoa obtained in these teratospermic toms take longer to capacitate *in vitro*, are compromised in their ability to undergo tyrosine phosphorylation, and have a reduced capability to undergo the acrosomal reaction (reviewed in Penfold et al. 2003). Although the aetiology of teratospermy in tomcats is unknown, it has been suggested that genetic, seasonal or nutritional factors are involved (Blottner and Jewgenow 2007; Jewgenow et al. 2009).

Also, morphological sperm differences have been noted between laboratories, as a consequence of different methods of fixing, staining and classification of spermatozoa (Axner et al. 1997). Chatdarong et al. (2010) reported that head abnormalities in frozen-thawed sperm samples do not fluctuate when different methods for sperm selection are used, although significant increases in normal midpieces and tails, and a lower percentage of proximal droplets was observed after single-layer centrifugation through colloid, in contrast with a simple washing method. In the present study, centrifugation did not significantly modify sperm morphology in epididymal cat sperm samples. Distal and proximal cytoplasmic droplets frequently appear in cat sperm when it is collected from the epididymal region close to the testicle. It has been reported that centrifugation (at 700 *g*) provokes the distal migration or the disintegration of droplets (Tebet et al. 2006). However, in the present study no variation in the cytoplasmic droplets was associated with centrifugation.

Spermatozoa are specialised cells that are related to the external environment through the cytoplasmic membrane. Modifications of habitual conditions require these cells to adapt to new circumstances. Emphasising the importance of diluents on sperm morphology, it has been demonstrated that spermatozoa undergo osmotic and pH changes when they are in contact with diluents (i.e. DPBS) or seminal fluid, and this interaction

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provokes the dissolution or breaking of cytoplasmic droplets, or the coiling or bending of spermatozoa tails (Axner et al. 1998). When spermatozoa are fixed before staining, their morphology is maintained. However, when cells are directly immersed into the dyes, as occurs in wet stains, they can suffer structural changes leading to differences in morphological assessment in comparison with spermatozoa samples that were previously fixed. In this sense, it has been reported that fewer sperm tail abnormalities are detected when the osmotic exchange rate is lower (Lengwinat and Blottner 1994), which supports the assertion that osmotic variations between solutions should be reduced when morphological assessment is carried out.

Interestingly, epididymal cat spermatozoa diluted in phosphate-buffered saline undergo morphological modifications comparable to those occurring during ejaculation, when spermatozoa are mixed with seminal fluids. Thus, morphological sperm assessment will be different if epididymal samples are collected but not diluted (Axner et al. 1998). It has been reported that the osmolality of cat semen after ejaculation is below 320 mOsm/kg (Glover and Watson 1985). Hypo-osmotic solutions induce cellular swelling and rupture of membranes, and also induce the disappearance of cytoplasmic droplets and lead to tail defects (Pukazhenthi et al. 2002; Cooper and Yeung 2003). Swelling is more harmful for spermatozoa than shrinking (and dehydration) induced by hypertonic solutions (such as occurs when cryoprotectors are added). It has been reported that the eosin-nigrosin stain with low osmolality is associated with a significant increase in bent tails in canine spermatozoa (Johnson et al. 1991). But, as described by Bjorndahl et al. (2004), staining solutions used for morphological assessment in human sperm show wide osmotic variations. Cells exposed to hypertonic solutions may suffer a net leak/influx of non-permeating solutes into the cells. This alteration is presumed to arise from the ability of the solute to change the conformation and stability of the hydrophilic portions of the plasma membrane (Pukazhenthi et al. 2000). On the other hand, hypotonic solutions may induce cellular lysis if the maximum volume is exceeded during the cell swelling (Pukazhenti et al. 2000).

With regard to the effect of cold shock on sperm sensitivity, Pukazhenti et al. (1999) demonstrated that spermatozoa are easily damaged when the cooling rate is fast, although they affirmed that a gradual drop of temperature minimises this negative effect. Some authors have proposed that the cholesterol-phospholipid ratio in the sperm membrane could be associated with this cooling sensitivity (Parks and Graham 1992). Cooling sperm is very important for the transport of samples or for maintaining them for hours or days before they are used. It has been reported that cat sperm are less cooling-susceptible than the sperm of other species (Glover and Watson 1985; Glover and Watson 1987), but that cooling rate could modify sperm survival (Pukazhenti et al. 1999). Axner et al. (2004) affirmed that acrosome status and cytoplasmic membranes are mildly affected by the cooling process, in contrast with the more detrimental effects observed after freezing.

Our study compared the competence of two different staining techniques to distinguish morpho-anomalies in epididymal feline spermatozoa. The interpretation of morphological results remains an important laboratory practice undertaken in the selection and diagnosis of subfertility. In domestic cats, morphological assessment can be used to determine the potential fertility of the obtained sperm (Howard 1990), and can also be used as a criterion for the acceptance or rejection of sperm samples, e.g. for freezing. However, great variations can be observed due to different classification systems, slide preparations and staining techniques, or level of experience of the technicians.

As described by Palmer et al. (2013) in the bull, a significantly higher incidence of sperm head abnormalities was recorded when semen samples were dried and fixed in formol-saline before they were stained with carbolfuchsin, in comparison with samples stained with eosin-nigrosin. Similar findings were observed in our study, and this discrepancy is worrying since head abnormalities inform about disturbances in testicular function. As has been recently discussed in other species, the system of morphological assessment should be systematised, in order to obtain more reproducible and comparable results in cats. For example, computer-based assessment could be implemented.

The present study revealed that the choice of staining method influences the morphological abnormalities that are detected when assessing feline epididymal sperm. The Giemsa-Wright stain identified a higher percentage of normal spermatozoa than the eosin-nigrosin stain (the latter has been recommended by the Society of Theriogenology), which demonstrates the importance of establishing

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a routine or standard staining method. Other studies comparing different staining techniques affirm that the number of protoplasmic droplets is lower when using Spermac stain in comparison with Hancock stain, and has been attributed to cell degeneration during the staining process (Schafer and Holzmann 2000).

Another complication in feline morphological semen assessment is the use of different classification systems. Different studies have variously classified primary, secondary and tertiary abnormalities as occuring in the testis, in the epididymides or vas deferens, or as being caused by improper handling of the semen after collection (Platz and Seager 1978; Zambelli and Cunto 2006). Other authors prefer to classify morphological abnormalities as primary and secondary, reflecting a spermatogenic versus epididymal origin of these defects (Axner 1998). And finally, abnormalities have also been classified according to the parts of the spermatozoa that are affected, i.e. head, midpiece or tail (Axner et al. 1998).

It has been demonstrated that the choice of staining or preparation technique affects the morphology of canine ejaculated spermatozoa, revealing higher average percentages of morphologically normal spermatozoa in samples stained with Giemsa-Wright than in samples stained with eosin-nigrosin (Root Kustritz et al. 1998). Our results obtained in feline epididymal sperm samples are in agreement with these authors. In the present study, a lower number of macrocephalic spermatozoa was counted after eosin-nigrosin staining. It could be suggested that macrocephalic spermatozoa might exhibit altered membranes and higher sensitivity to osmotic stress than normal spermatozoa, which could provoke spermatozoa rupture. Our results suggest that a wet stain (such as eosin-nigrosin) can affect sperm membranes therefore leading to the disappearance of macrocephalic sperm. A lower number of cytoplasmic droplets was observed after Giemsa-Wright staining. Similar observations were obtained after Spermac® staining (Schafer and Holzmann 2000), in which case the elimination of droplets was attributed to the fixation with alcohol. The alcohol fixation during Giemsa-Wright staining could explain the reduced incidence of cytoplasmic droplets in the present study. In any case, distal cytoplasmic droplets are considered as normal in epididymal feline sperm (Tebet et al. 2006).

Our results suggest that staining methods may alter the morphology of feline spermatozoa artifactually, especially in epididymal sperm that have more cytoplasmic droplets than ejaculated sperm. The staining method should be carefully considered in order to minimise variations and to improve reproducibility between different laboratories.

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