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Comparison between different markers for sperm quality in the cat: Diff-Quik as a simple optical technique to assess changes in the DNA of feline epididymal sperm

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Abstract

The majority of wild felids, as well as some domestic cats, have low sperm concentration in their ejaculates, and a high proportion of abnormal spermatozoa. We have employed several possible semen quality markers to further characterize cat epididymal sperm. Methods included possible apoptotic reporters, such as the annexin V assay to monitor exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane, as well as cell integrity; and the TUNEL assay to quantify DNA breaks. Sperm surface ubiquitination, another putative marker of sperm quality, was also monitored. The annexin V assay revealed a high percentage of sperm with PS exposure, and the TUNEL assay pointed to high levels ($13 \pm 12\%$) of sperm with DNA breaks. Correlations were found between apoptotic markers (but not ubiquitination) and semen parameters. In parallel to this analysis, cat sperm morphology was evaluated using the Diff-Quik optical stain, which has been used in human reproduction laboratories. Several types of abnormalities could be characterized with this method. Remarkably, head staining abnormalities detected using the Diff-Quik staining method were strongly correlated with, and could accurately predict, sperm DNA defects detected in the same sample using the TUNEL assay. We therefore suggest that sperm morphology analysis using Diff-Quik could be used in field conditions to assess sperm status, due to the simplicity of the procedure and the equipment involved.

Keywords: Feline sperm; Apoptosis; Diff-Quik; Sperm abnormalities; DNA breaks

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1. Introduction

Most of the 36 wild species of felids are classified as threatened, vulnerable or endangered, and the domestic cat has been widely used as the main research model for wild felids [1]. It is known that some domestic cats, and the majority of wild felines, have low sperm concentration in the ejaculate [2], and a high proportion of abnormal spermatozoa [3]. If the percentage of abnormal spermatozoa exceeds 60% the ejaculate is considered teratospermic. Interestingly, although abnormal spermatozoa from teratospermic ejaculates have more difficulties in penetrating the zona pellucida than normal sperm cells from the same ejaculate [4,5], cats with a high proportion of abnormal sperm have been used for reproductive purposes with good results [6].

Many studies have been carried out with feline sperm, including research on the ability of sperm to respond to osmotic stress and cold-induced damage during cryopreservation [7–9], characterization of capacitation and acrosomal reaction in different populations of sperm [10], or the state of sperm DNA [11]. These studies permitted a further understanding of cat sperm characteristics, and the development of more efficient cryopreservation protocols. Interestingly, to our knowledge, there have been no studies focusing on feline sperm programmed cell death (apoptosis), or on the possible use of apoptotic markers to assess feline sperm quality. Apoptosis is characterized by distinct ultrastructural and biochemical changes in affected cells, which can be monitored by different biochemical assays. In early apoptosis the cell loses its plasma membrane asymmetry, and exposes phosphatidylserine (PS) in the outer leaflet, thus tagging these cells for phagocytosis. In late apoptosis DNA suffers internucleosomal cleavage by specific endonucleases originating DNA fragments of approximately 180-base pairs [12]. Annexin V (A), an early apoptosis marker, is a Ca^{2+}-dependent phospholipid binding-protein that has a high affinity for PS [13]. Together with markers for cell permeability, such as propidium iodide (PI), this test is able to discriminate four different populations of spermatozoa: viable sperm (A−/PI−), early apoptosis (A+/PI−), late apoptosis or early necrosis (A+/PI+), and necrosis (A−/PI+) [12,14]. On the other hand, the TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling) assay detects the endonuclease digestion of DNA into oligonucleosomal fragments. Namely, it quantifies the incorporation of deoxyuridine triphosphate (dUTP) in the 3′ terminal of single and double-stranded DNA breaks, catalyzed by terminal deoxynucleotidyl transferase (TdT) [15]. Research carried out in other species has described correlations between apoptotic alterations and standard semen parameters, with possible influence in the outcome of Assisted Reproduction Techniques (ART). For example, working with sub fertile men, Shen et al. [14] found negative correlations between TUNEL positive cells and late apoptotic cells with both motility and vitality; as well as positive correlations with abnormal sperm morphology. Furthermore, TUNEL was also used to study germ cell apoptosis in cats, allowing the identification of different spermatogenic stages with high apoptotic rates [16]. Other authors have used the SCSA (Sperm Chromatin Structure Assay) to evaluate DNA fragmentation in ejaculated sperm from teratospermic and normospermic domestic cats [11]. An increase in DNA fragmentation in samples from teratospermic males was observed, but it was not associated with poor fertility or early embryo development, as long as the zona pellucida was bypassed using assisted sperm injection [11].

Another assay proposed to monitor sperm quality is the sperm surface ubiquitination assay [17]. Ubiquitination is a universal mechanism for protein recycling involving tagging of substrates via the covalent attachment of one or more molecules of ubiquitin. Defective sperm in the semen from several species (domestic bulls, wild cattle, rhesus monkey, human and mouse) seem to be strongly ubiquitinated on the cell surface, possibly marking them for phagocytosis/degradation [17]. Furthermore, besides recognizing TUNEL-positive spermatozoa, surface ubiquitination seems to detect a wide range of other abnormalities, and was shown to correlate negatively with sperm count, motility and normal morphology [18,19].

The common denominator of all the techniques described above is that they are all elaborate and time consuming, requiring some specialized reagents and equipment, and are therefore less than adequate for field conditions. On the other hand Diff-Quik is a rapid and simple staining method that stains the sperm head light blue/violet, the acrosome pale blue, the tail and midpiece blue or reddish [20]. It can be used to assess sperm morphology in field conditions using a bright field microscope, and is already used in veterinary clinics to stain blood smears.

These markers and staining procedures were used in this work to characterize cat epididymal sperm in an attempt to both test the possible use of apoptotic markers in this system, and obtain further knowledge on sperm quality and functionality, which could then be used when discriminating samples for assisted reproductive techniques in wild felids. The most interesting, and unexpected, result of all was the fact that staining abnormalities revealed by Diff-Quik could predict results from the TUNEL assay in the same sample, suggesting Diff-Quik as a possible tool in assessing sperm DNA status, especially in field-work conditions.

2. Materials and methods

All fluorescent probes were acquired from Molecular Probes (Eugene, OR, USA), unless stated otherwise. The antibody for the ubiquitin assay (clone KM691) was from Kamiya Biomedical (Seattle, WA, USA), and the remaining reagents were from Sigma–Aldrich (St. Louis MO, USA).

Cat testes from 36 males were collected immediately after castration performed in four veterinary clinics. The epididymides were separated from the testis, minced and incubated for 30 min in 2 ml of Ham's F10 medium supplemented with 20 mM Hepes, 100 UI penicillin/ml and 1 mg bovine serum albumin (BSA)/ml at 37 °C [3,11,21].

Sperm motility was measured, at the end of the 30 min incubation, in a hemocytometer, using a bright field microscope with closed diaphragm, to allow an easier assessment. The motility was divided into four categories: rapid progressive movement, slow progressive movement, nonprogressive motility and non-motile sperm. Total motility was calculated adding the first three categories. The sperm was allowed to rest in the hemocytometer until no movement was detected to determine sperm count.

We observed 200 spermatozoa for each sample in all assays. All images were taken with a 100× oil immersion objective.

2.1. Diff-Quik assay

Morphology and staining feature were assessed using a Diff-Quik kit (Medion Diagnostics, Germany). This kit is composed by a fixative (methanol), an anionic/acidic dye (eosin) that stains positively charged/basic proteins (red), and a cationic dye (methylene blue and derivatives) that stains nuclei and negatively charged molecules (blue). The smears were done using 10 μl of sperm suspension dragged with a coverslip and allowed to air dry. The slide was immersed in each solution of the staining kit for 1 min and dipped rapidly in water, air dried and observed in a bright field microscope. The time needed for each solution is dependent of each observer's ability to discriminate sperm structures, and may vary also with different Diff-Quik kits. After establishing these parameters the Diff-Quik assay is very reproducible.

The staining characteristics were always analyzed within each sample. Spermatozoa with staining intensities differing from the normal situation (light blue/violet—see Results) were included in the abnormal staining category. This was done whether the spermatozoa in question showed a darker blue color, or were more reddish (or a mixture of both).

2.2. Annexin V assay

The annexin V assay incorporates annexin V conjugated with Alexa Fluor 568 (red fluorescence), a green-fluorescent nuclear and chromosome counterstain that is impermeant to live cells (SYTOX green) and Hoechst 33342, a cell-permeant nucleic acid stain that emits blue fluorescence when bound to double stranded DNA. To carry out the assay 100 μl of the sperm suspension was centrifuged at 800 × *g* for 10 min and the pellet resuspended in 100 μl Hepes buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Annexin V (5 μl), SYTOX green (10 μl of a 5 nM solution) and Hoechst 33342 were added to the mixture and incubated for 15 min at room temperature. The suspension was then centrifuged at 800 × *g* for 10 min, the pellet mounted on a slide with antifade (Vectashield, Vector Labs, Burlingame CA, USA) and observed with a Zeiss AxioPlan 2 Fluorescence Microscope.

2.3. TUNEL assay

The APO-BrdU™ TUNEL Assay Kit (Molecular Probes) was used to detect nicked DNA in epididymal sperm of male cats, with some minor modifications. Sperm were allowed to adhere to poly-L-lysine coated coverslips, which were then fixed in 2 ml of a 2%(v/v) formaldehyde solution in PBS (200 mg/l KCl; 20 mg/l KH_2PO_4; 1150 mg/l Na_2HPO_4; 8000 mg/l NaCl; 500 mg/l NaN_3; pH 7.2) for 1 h at room temperature. Coverslips were then placed in a permeabilizing solution containing 1% (v/v) Triton X-100 in PBS for 20 min. To store the samples, coverslips were placed in blocking solution (100 mM glycine; 1 mg/ml BSA in PBS) and kept at 4 °C.

To carry out the TUNEL assay coverslips were incubated with 50 μl of DNA-labeling solution [31.25 μl distilled water; 50 μl reaction buffer; 0.75 μl Tdt (terminal

deoxynucleotidyl transferase); 8 μl BrdUTP] for 1 h at 37 °C in the dark. After a washing step with Rinse buffer for 10 min they were covered with 100 μl of the anti-BrdUTP antibody solution (97.5 μl Rinse buffer; 2.5 μl Alexa Fluor 488 dye labeled anti-BrdUTP antibody) and incubated for 2 h at 37 °C in the dark. Following the incubation the sample was washed with 0.1% (v/v) Triton X-100 for 30 min. TUNEL positive cells stain green, while DAPI was used as a DNA counterstain (blue). The coverslips were mounted with antifade and observed with a Zeiss AxioPlan 2 Fluorescence Microscope. For a negative control the same procedure was applied but omitting Tdt.

2.4. Ubiquitin assay

Sperm were allowed to adhere to poly-L-lysine coated coverslips, which were then fixed in 2 ml of a 2% (v/v) formaldehyde solution in PBS for 1 h at room temperature. The coverslips were stored in blocking solution at 4 °C until used. The primary antibody was prepared in blocking solution at a 1:400 dilution, placed over the coverslip and incubated at 37 °C for 30 min. The coverslips were then washed in 0.1% (v/v) Triton X-100 in PBS for 30 min and then stained with an appropriate mouse IgM secondary antibody tagged with Alexa 568 (red) prepared in blocking solution, and incubated at 37 °C, for 20 min in the dark. The coverslip was washed again in 0.1% (v/v) Triton X-100 in PBS for 30 min, incubated with DAPI for DNA counterstaining, mounted with antifade and observed with a Zeiss AxioPlan 2 Fluorescence Microscope. For negative controls the same procedure was applied omitting the primary antibody.

2.5. Statistical analysis

All variables were checked for normal distribution. Bivariate correlation was evaluated by calculating the Pearson's correlation coefficient (r) with a two-tailed significance (P). Linear regression analysis was performed to establish predictive value of a variable. For the variable ''age'', which does not have a normal distribution, a Mann–Whitney test was performed. All statistical analyses were carried out using the SPSS, version 12.0, software for Windows (SPSS Inc., Chicago, IL, USA).

Table 1
Sperm number and motility in the cat epididymis

	Sperm number ($\times 10^6$)	Sperm displaying				
		Total motility (%)	Progressive motility (%)	Slow motility (%)	Non- progressive motility (%)	Non-motile sperm (%)
Mean ± S.D.	18 ± 10	47 ± 16	31 ± 19	5 ± 4	12 ± 9	52 ± 16
Maximum	48	72	65	15	33	100
Minimum	3	0	0	0	0	28

$n = 36$.

3. Results

The values for sperm number and motility are shown in Table 1. The morphology parameters were quantified using Diff-Quik and standards normally employed for equine sperm, with slight modifications [36]. Results are shown in Table 2. If several

Table 2
Sperm morphology and abnormal staining in cat epididymal sperm, assayed with Diff-Quik

Morphology parameter	Mean ± S.D.	Rank
Head abnormalities	46.86 ± 12.98	*A*
Detached head	5.17 ± 4.24	2
Narrow head	3.09 ± 3.07	1
Elongated head	6.09 ± 6.86	1
Pear shaped head	0.46 ± 0.95	1
Small head	0.74 ± 0.98	1
Double head	0.60 ± 0.74	1
Macrocephalic head	2.89 ± 3.77	1
Abnormal contour	14.49 ± 9.68	1
Abnormal staining[&]	21.70 ± 10.61	1
Midpiece abnormalities	10.74 ± 10.00	*B*
Thin midpiece	1.14 ± 6.59	1
Separated fibers	0.03 ± 0.17	5
Abnormal neck	0.20 ± 0.47	4
Broken neck	0.40 ± 0.69	5
Broken midpiece	0.43 ± 0.78	5
Double midpiece	0.26 ± 0.51	4
Thick midpiece	0.31 ± 0.93	3
Short midpiece	0.09 ± 0.37	3
Proximal cytoplasmatic residue	1.20 ± 1.53	1
Distal cytoplasmatic residue	2.63 ± 3.34	2
Bent or double bent midpiece	4.09 ± 3.66	2
Tail abnormalities	7.14 ± 5.41	*C*
Bent tail	3.09 ± 3.72	3
Double bent tail	0.54 ± 0.78	3
Coiled tail	1.57 ± 1.72	2
Tail coiled around the head	0.34 ± 0.54	2
Thin tail	0.00 ± 0.00	3
Broken tail	0.74 ± 1.04	3
Coiled end tail	0.57 ± 1.36	3
Double tail	0.06 ± 0.24	1
Short tail	0.00 ± 0.00	3
Detached tail	0.17 ± 0.45	2
Acrosome abnormalities	13.74 ± 6.88	*B*
Total abnormalities	78.14 ± 8.66	

$n = 35$, except for staining abnormalities (&) where $n = 23$. The rank varied with origin of defect (testicular or epididymal), frequency and relevance for the functionality of the spermatozoa, adapting equine standards [36]. The abnormalities were classified first by major location. Thus, head abnormalities (A) were considered more important than midpiece and acrosome defects (B), which, in turn were deemed more important than tail abnormalities (C). Inside each area rank varied between 1 (most important) and 5 (least important).

morphological abnormalities were observed in the same spermatozoon, this spermatozoon was included in the most important abnormal parameter category considering the origin (testicular or epididymal), relevance or prevalence of the defect (rank shown in Table 2).

The percentage of abnormal cells was quite high (Table 2). Figs. 1 and 2 depict representative examples of the more typical characteristics found, which are included in the categories quantified in Table 2. The use of stained smears allowed the detection of a greater number of head abnormalities, but without altering the recognition of other types of sperm defects. Notably, using the Diff-Quik stain we determined that, within the same sample, stronger hues of dark blue/reddish label could be seen on the sperm head in some cases (Fig. 2a, compare normal sperm (arrowheads) with abnormal staining (arrow)). Thus we created a staining abnormality parameter, including all spermatozoa with a greater intensity of blue or red staining, starting with more subtle differences (Fig. 2a, arrow) to more evident staining differences (Fig. 2c). Increased staining intensities were also associated with other morphological abnormalities, the most common being cytoplasmatic residues, giant or abnormal heads or bent midpieces.

Figs. 3–5 show the labeling patterns found with the annexin V, TUNEL and ubiquitin assays, respectively. The sperm population was divided into four subpopulations, according to the patterns found with annexin V: viable sperm (A−/S−/H+), early apoptotic (A+/S−/H+), late apoptotic/early necrotic (A+/S+/H+) and necrotic (A−/S+/H+). Annexin V labeling was observed in abnormal sperm but also in apparently normal sperm (Fig. 3). On the other hand, the TUNEL assay only detects sperm with DNA breaks. In this case the staining obtained was not always homogeneous, and fluorescence could often be observed exclusively either on the apical or basal portions of the sperm head (Fig. 4). Finally, ubiquitinated sperm showed very variable fluorescent labelling, but no signal was ever observed on the sperm head (Fig. 5). Besides abnormal sperm, cells that were apparently normal also tested positive in this assay. Table 3 shows values of labeled sperm using all three fluorescence-based assays.

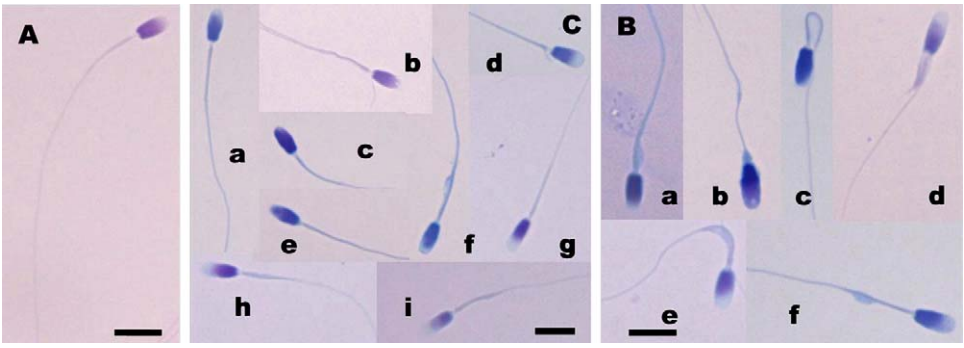

Fig. 1. Cat sperm morphology using Diff-Quik. (A) Represents a typical normal spermatozoa from cat epididymis, while (B) and (C) show different types of acrosomal and midpiece abnormalities. (B) (a)–(c) represent acrosomal vacuolization; (d) shows a large acrosome and (e) a small acrosome; (f) represents a reacting acrosome with the flattened apical portion; (g)–(i) represents knobbed acrosomes. (C) (a) sperm with proximal droplet; (b) thin midpiece with a proximal droplet; (c) bent midpiece; (d) double midpiece; (e) bent midpiece with cytoplasmatic residues; (f) distal droplet. Bars represent 5 μm.

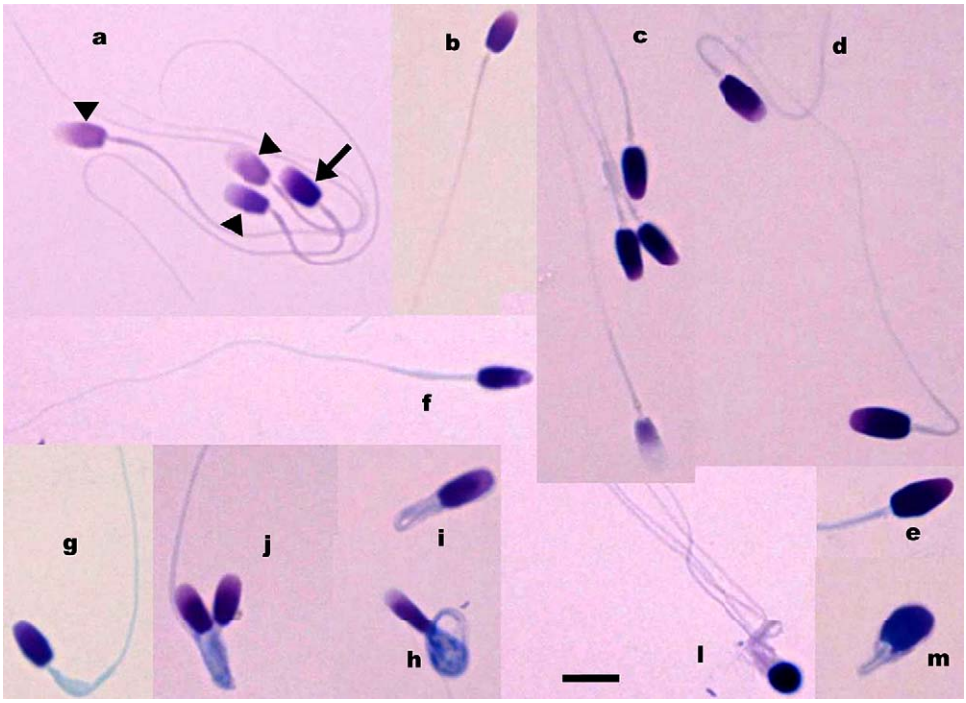

Fig. 2. Sperm morphology and abnormal staining using Diff-Quik. (a) Represents spermatozoa with different normal staining patterns (arrowheads), as well as a sperm cell representative of borderline abnormal staining (arrow); (b) and (c) represent cells with different levels of color intensity (stronger hue when compared with 1A), but without any other striking alteration; (d) and (e) represent abnormally stained macrocephalic sperm heads; (f) represents an abnormal stained sperm with acrosome abnormalities in color and shape; (g) represents one of the most common association seen between staining abnormalities and other abnormalities, a bent midpiece with cytoplasmatic residues; (h)–(j), (l) and (m) represent other major alterations seen in association with abnormal staining. Bar represents 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

After determining that all assays may be used in cat epididymal sperm, and that labeling patterns were similar to what has been previously reported in other species, we performed statistical analysis on our data. Samples were grouped as young males (less than 12 months; $n = 11$) and adult males (12+ months; $n = 20$) to analyze the influence of age on semen parameters. We observed that age influenced the number of TUNEL positive cells (Mann–Whitney test; $P = 0.030$) and showed a tendency to influence ubiquitin positive cells ($P = 0.083$). Young males had an average of 6.50 ± 3.41 (mean $\pm$ S.D.) of TUNEL positive cells, while in mature males the value increased to 15.38 ± 14.69. When analyzing the data obtained with the ubiquitin-based assay no correlations between the level of ubiquitination and other semen parameters or populations defined by the annexin V or TUNEL assays were found (data not shown).

Table 4 shows the relevant correlations found between some morphology parameters (Table 2), motility and TUNEL, using all samples. The correlation found between total motility and midpiece abnormalities ($r = -0.481$; $P = 0.003$) as well as between non-

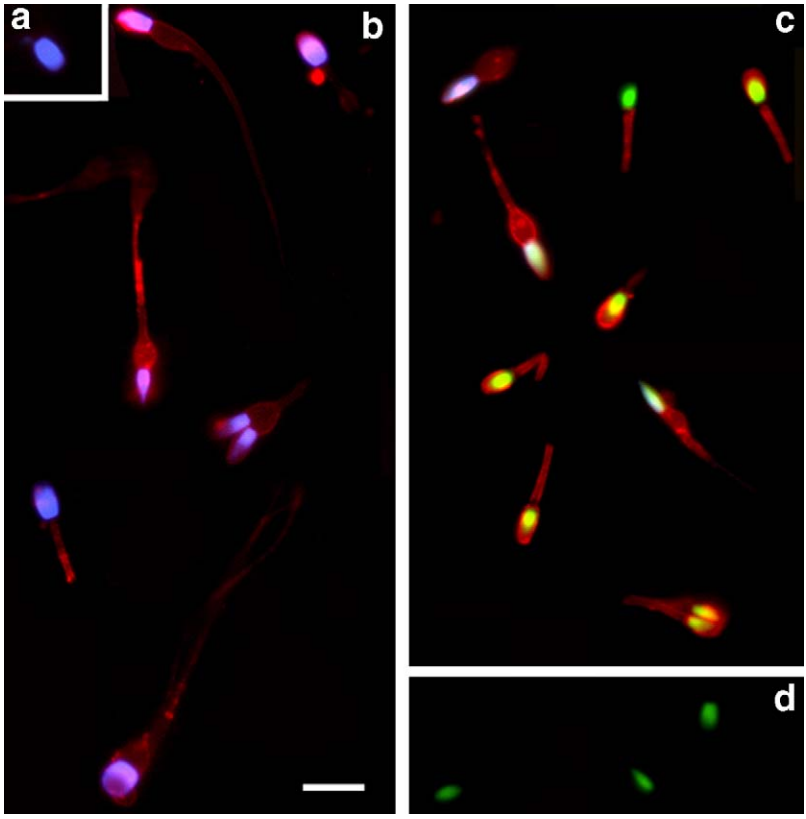

Fig. 3. Annexin V assay. Annexin V (red)/Sytox Green/Hoechst (blue) labeling allowed the characterization of four distinct populations in each sperm sample: (a) viable sperm (A−/S−/H+); (b) early apoptosis (A+/S−/H+); (c) late apoptosis (A+/S+/H−) and (d) necrosis (A−/S+/H+). Bar represents 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

motile sperm and midpiece abnormalities ($r = 0.533$; $P = 0.001$), confirms the capacity of the Diff-Quik assay to discriminate midpiece and tail abnormalities. Furthermore it reflects the functional relevance of midpiece defects. The presence of different types of abnormalities in one spermatozoon, as well as the relation between different types of sperm abnormalities, could be appreciated by smear observation, and by considering the correlations found between head abnormalities and midpiece/tail abnormalities ($r = -0.483$, $P = 0.003$ and $r = -0.433$, $P = 0.008$, respectively).

There were several correlations found between apoptotic markers, motility and morphology. Early apoptotic sperm was positively correlated with acrosome abnormalities ($r = 0.360$; $P = 0.033$), while late apoptosis was positively correlated with sperm count ($r = 0.355$; $P = 0.034$). TUNEL was negatively correlated with motility parameters, especially progressive motility ($r = -0.429$; $P = 0.009$, Table 4). The most striking and unexpected correlation, shown in Fig. 6, was the positive, strong and very significant correlation between staining abnormalities detected using Diff-Quik (all staining diverging from the normal staining) and TUNEL positive labeling ($r = 0.701$; $r^2 = 0.492$; $P = 0.000$).

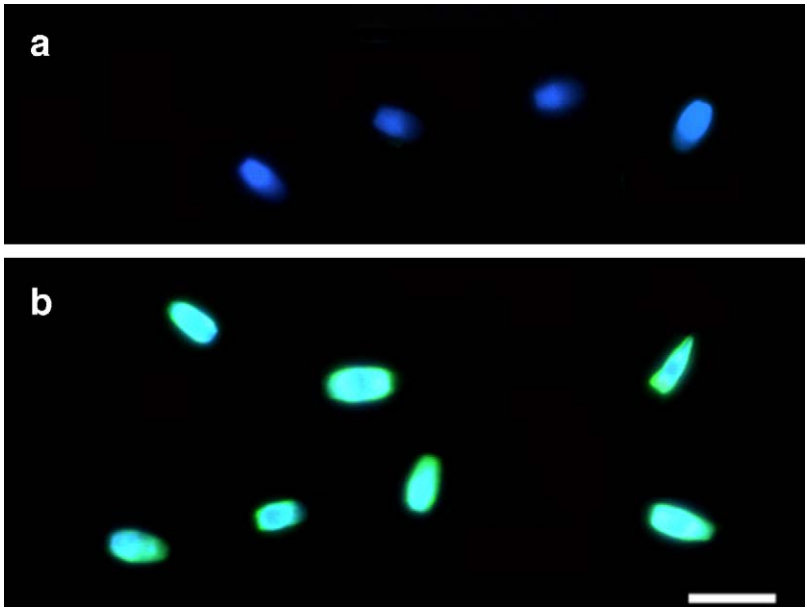

Fig. 4. TUNEL assay. The different labeling patterns obtained with the TUNEL assay are shown. (a) No labeling (DAPI+/TUNEL−). (b) TUNEL-positive cells (green) with different fluorescence intensity labeling (DAPI+/TUNEL+). DNA (DAPI) is in blue. Bar represents 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

In this study we first analyzed basic semen parameters (motility, sperm number, morphology) in cat epididymal sperm. The low motility found in epididymal sperm was expected since sperm was collected from total epididymis and it is known that the male gametes only acquire motility in the corpus region [22]. The percentage of normal sperm ($22 \pm 9\%$) present in these samples was inferior to that obtained by other researchers. Using phase contrast microscopy analysis of sperm taken from the cauda epididymis and ductus deferens, Neubauer et al. [21] quantified $66 \pm 2\%$ of structurally normal sperm in a normospermic population, and $11 \pm 1\%$ in a teratospermic population. The values obtained here may be explained by the use of Diff-Quik, that allows assessment to all kinds of abnormalities, especially head abnormalities, which are more difficult to detect by phase contrast microscopy [23]. Another explanation may be the use of sperm from the entire epididymis. As Axnér et al. [22] have demonstrated, there is a decrease in head abnormalities and an increase in tail abnormalities during epididymal traffic. This is in agreement with a much higher level of head abnormalities and a lower level of tail abnormalities observed in this work. The use of more restricted definitions of what constitutes a normal spermatozoon and the creation of another abnormality category, denominated abnormal staining, may explain the remaining increased abnormalities.

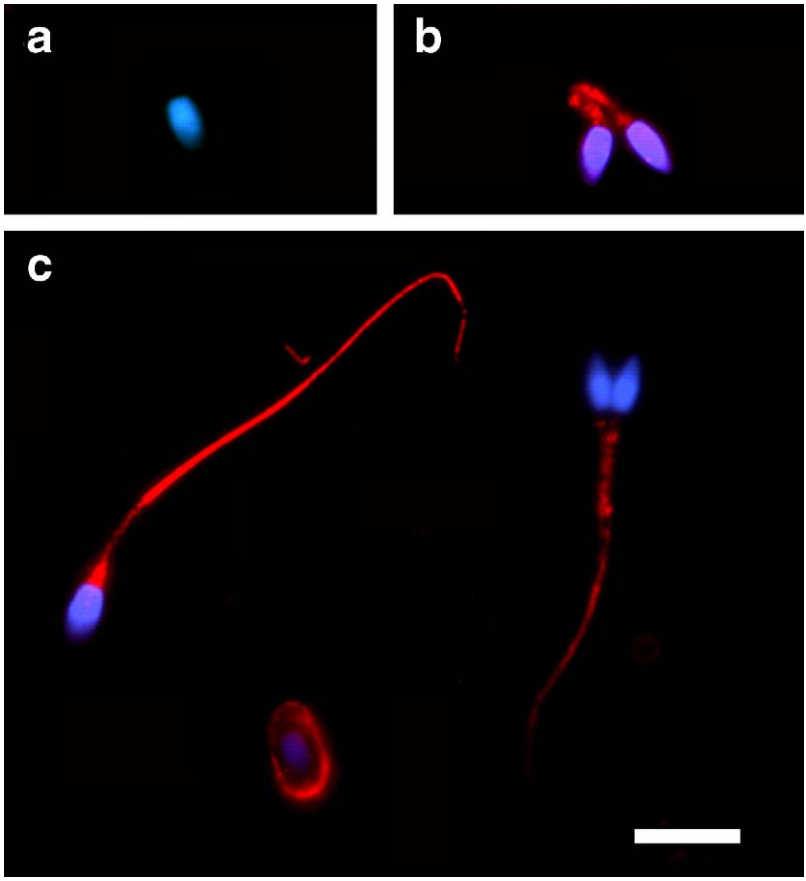

Fig. 5. Ubiquitin assay. Different patterns of ubiquitin labeling are shown. (a) Sperm with no labeling. Ubiquitin positive cells (red) may have different levels of staining, from only midpiece labeling (b) to total tail labeling (c). DNA (DAPI) is in blue. Bar represents 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 3
Mean parameters determined with the annexin V assay, TUNEL assay and ubiquitin assay

	Annexin V assay (% of marked cells)				TUNEL positive (% of marked cells)	Ubiquitin positive (% of marked cells)
	Viable	Early apoptosis	Late apoptosis	Necrosis		
Mean ± S.D.	39 ± 12[a]	4 ± 3[a]	30 ± 12[a]	27 ± 10[a]	13 ± 12[a]	28 ± 20[b]
Maximum	72	14	50	51	68	92
Minimum	14	0	0	11	0	5

[a] $n = 36$.
[b] $n = 31$.

Table 4
Correlations between motility, morphology and TUNEL

	Abnormal midpiece	Abnormal acrosome	TUNEL+
Total motility	$r = -0.481$ $P = 0.003$	$r = 0.470$ $P = 0.004$	$r = -0.360$ $P = 0.031$
Progressive motility	$r = 0.385$ $P = 0.022$	$r = 0.338$ $P = 0.022$	$r = -0.429$ $P = 0.009$
Non-motile sperm	$r = 0.533$ $P = 0.001$	$r = -0.517$ $P = 0.001$	$r = 0.364$ $P = 0.029$

Shown are the Pearson's correlation coefficient (r), calculated with a two-tailed significance (P).

Although ubiquitin staining detected both altered cells and apparently normal cells, as has been described [17,24], no correlation between this marker and other semen parameters was found, in contrast to published literature [19,25]. The lack of correlation may be due to different result presentation (percentage of ubiquitinated cells instead of fluorescence median), or to the fact that ubiquitination could be an ongoing process, and we may therefore be analyzing sperm that were not completely ubiquitinated because they were isolated from earlier parts of the epididymis. It should also be noted that the percentage of ubiquitinated sperm cells has been shown to negatively correlate with fertility in stallion sperm, while, at the same time, having no type of correlation with semen parameters [24]. In this case we have no information on the potential fertilization ability of our samples.

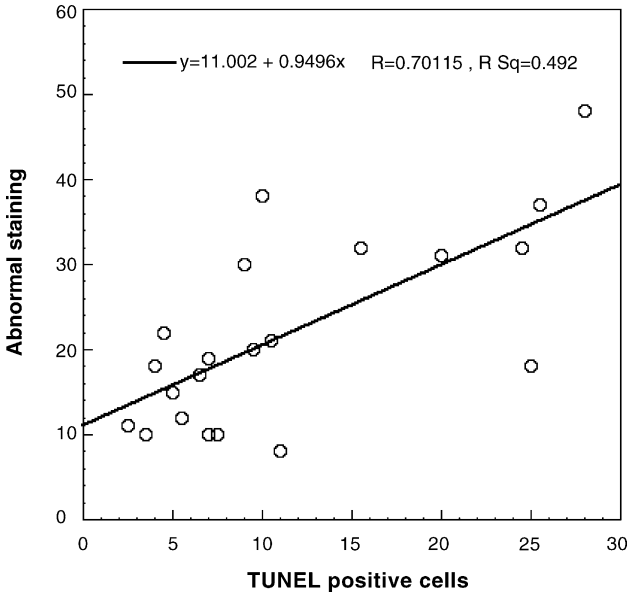

Fig. 6. Correlation between TUNEL and abnormal staining. The relation between TUNEL positive cells (with defects in sperm DNA) and abnormal staining is shown, including the linear regression performed on the data.

The annexin V assay separated the sperm population into four subpopulations. The number of viable sperm (undamaged plasma membrane) was inferior to the number observed by Axnér et al. [2] using SYBR-14/propidium iodide and the same sperm extraction solution. This confirms the superior membrane integrity discriminating capability of annexin V described by Anzar et al. [12] in bull semen. The positive correlation found between sperm count and late apoptosis may indicate a diminished capability of annexin V positive sperm to be eliminated during epididymal traffic, or a greater difficulty of the epididymal epithelium to support a larger number of spermatozoa. Acrosomal abnormalities were positively correlated with early apoptosis and negatively correlated with viable sperm. These abnormalities may mark the cells for apoptosis or, in alternative, they could represent initial steps of the acrosome reaction. In this case, PS exposure might be a normal feature of capacitated sperm initiating the acrosome reaction [26]. In spite of the controversy surrounding PS exposure, the annexin V assay may be a useful tool to assess sperm plasma membrane integrity. In fact, Anzar et al. [12] applied this method to evaluate changes in bull sperm following freeze/thaw, and reported an increase (up to 40%) of annexin V marked cells after cryopreservation. In addition, the use of superparamagnetic microbeads conjugated with annexin V (MACS) [27] allowed apoptotic cell sorting, improving motility following cryopreservation-thawing, as well as cryosurvival rate [28].

In humans, some studies have pointed to a superior abortion rate after intracytoplasmatic sperm injection (ICSI), and a greater risk of birth defects in children conceived by both in vitro fertilization (IVF) and ICSI. This may reflect the use of altered sperm, possibly including structural changes in DNA [29]. The relevance and origin of DNA breaks in ejaculated sperm is unknown but work in human has raised three possible explanations. Thus, fragmented DNA may result from topoisomerase II, an enzyme that creates and ligates nicks during the elongating spermatid phase. Incomplete topoisomerase II activity could leave unligated nicks that can be detected by the TUNEL assay [30]. The second hypothesis considers that DNA breaks are the result of DNA cleavage during germinal cell apoptosis, suggesting that the process of cellular death is not completed, and that these maturing spermatids escape phagocytosis by the Sertoli cells [31]. Finally, DNA breaks may result from production of free radicals during ejaculation [32,33].

The presence of DNA breaks in ejaculated cat sperm has been analyzed through SCSA, and correlated with bad quality (teratospermic) ejaculates, but not with early embryonic development arrest or failure [11]. We have analyzed DNA breaks using the TUNEL assay, a marker for late stage apoptosis. A mean of 13 $\pm$ 12% sperm with fragmented DNA was found, very similar to what was reported in the normospermic population analyzed by Penfold et al. (13.8 $\pm$ 2.4 (mean $\pm$ S.E.M.)) [11], and in infertile men (15%) [14]. No correlation was found between TUNEL positive sperm and putative apoptotic sperm detected by the annexin V assay. This may be explained by a non-apoptotic origin of DNA breaks (TUNEL positive cells may represent immature sperm that did not complete DNA packaging) or by a phosphatidylserine exposure by other mechanisms, such as sample processing or capacitation [33].

Staining abnormalities were detected when comparing sperm from the same sample. Some of the heads were dark blue and others were reddish, when compared with the majority of spermatozoa present in the sample (light blue/violet), and were all included in

the same parameter. The staining abnormalities could be potentially explained by: (a) the presence of a larger amount of DNA; (b) easier access to the DNA; (c) presence of basic elements in the nucleus. The larger amount of DNA could be explained by aneuploidy, while DNA breaks marked by SCSA [11], or less compacted DNA [34], may be responsible for the easier access to sperm DNA. The reddish color observed in some cases may be due to basic elements, such as histones or transition proteins, that may not be correctly removed and that could thus stain with eosin [35]. Furthermore, eosinophily is increased in degenerated cells. The close correlation and predicting value of staining abnormalities in relation to TUNEL positivity may indicate that DNA breaks, as part of degenerating/apoptotic processes or as marker of immature sperm, may be responsible for part of the staining abnormalities observed. This relation allows a simple assay like Diff-Quik to be used, not only to characterize sperm morphology, but also to assess sperm functionality in terms of DNA status. This may be especially useful in the absence of more sophisticated methodology, such as in field conditions. Since we used epididymal sperm from castrated males, further studies will have to be carried out to determine if the correlations described are also found in the ejaculate, and, more importantly, if they relate to fertility, either in vitro or in vivo. We would expect that low-quality samples (evaluated using this new Diff-Quik methodology) would be impaired in their fertilization ability.

In conclusion, this work demonstrates the presence of large quantities of apoptotic sperm in the cat epididymis, which may explain difficulties observed in cryopreservation and ART and, mainly reveals a new tool, Diff-Quik, for discriminating functional sperm.

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References

[1] Farstad W. Current state in biotechnology in canine and feline reproduction. Anim Reprod Sci 2000;60–61:375–87.
[2] Wildt DE, Brown JL, Swanson WF. Cats. In: Knobil E, Neill J, editors. Encylcopedia of reproduction, vol. 1. New York: Academic Press; 1999. p. 497–510.
[3] Axnér E, Pukazhenthi B, Wildt D, Linde-Forsberg C, Spindler R. Creatine phosphokinase in domestic cat epididymal spermatozoa. Mol Reprod Dev 2002;62:265–70.
[4] Howard JG, Brown JL, Bush M, Wildt DE. Teratospermic and normospermic domestic cats: ejaculate traits, pituitary–gonadal hormones, and improvement of spermatozoal motility and morphology after swim-up processing. J Androl 1990;11:204–15.
[5] Howard JG, Bush M, Wildt DE. Teratospermia in domestic cats compromises penetration of zona-free hamster ova and cat zonae pellucidae. J Androl 1991;12:36–45.

[6] Axnér E, Holst BS, Linde-Forsberg C. Morphology of spermatozoa in the cauda epididymidis before and after electroejaculation and a comparison with ejaculated spermatozoa in the domestic cat. Theriogenology 1998;50:973–9.
[7] Pukazhenthi B, Pelican K, Wildt DE, Howard JG. Sensitivity of domestic cat (*Felis catus*) sperm from normospermic versus teratospermic donors to cold-induced acrosomal damage. Biol Reprod 1999;61:135–41.
[8] Pukazhenthi BS, Noiles E, Pelican K, Donoghue A, Wildt DE, Howard JG. Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors. Cryobiology 2000;40:139–50.
[9] Pukazhenthi B, Spindler R, Wildt DE, Bush LM, Howard JG. Osmotic properties of spermatozoa from felids producing different proportions of pleiomorphism: influence of adding and removing cryoprotectant. Cryobiology 2002;44:288–300.
[10] Pukazhenthi BS, Wildt DE, Ottinger MA, Howard JG. Compromised sperm protein phosphorylation after capacitation, swim-up, and ZP exposure in teratospermic domestic cats. J Androl 1996;17:409–19.
[11] Penfold LM, Jost L, Evenson DP, Wildt D. Normo versus teratospermic domestic cat sperm chromatin integrity evaluated by flow cytometry and intracytoplasmatic sperm injection. Biol Reprod 2003;69:1730–5.
[12] Anzar M, He L, Buhr MM, Kroetsch T, Pauls KP. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod 2002;66:354–60.
[13] Vermes I, Haanen C, Reutelingsperger CPM. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labeled annexin V. J Immunol Meth 1995;180:39–52.
[14] Shen H, Dai J, Chia S, Lim A, Ong C. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. Hum Reprod 2002;17:1266–73.
[15] Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331–45.
[16] Blanco-Rodriguez J, Martinez-Garcia C, Porras A. Correlation between DNA synthesis in the second, third and fourth generations of spermatogonia and the occurrence of apoptosis in both spermatogonia and spermatocytes. Reproduction 2003;126:661–8.
[17] Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. J Cell Sci 2001;114:1665–75.
[18] Sutovsky P, Neuber E, Schatten G. Ubiquitin-dependent sperm quality control mechanism recognizes spermatozoa with DNA defects as revealed by dual ubiquitin-TUNEL assay. Mol Reprod Dev 2002;61:406–13.
[19] Sutovsky P, Hauser R, Sutovsky M. Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology laboratory clinic population. Hum Reprod 2004;19:628–38.
[20] World Health Organization (WHO) Laboratory Manual. Cambridge University Press:1999;p. 17–23.
[21] Neubauer K, Jewgenow K, Blottner S, Wildt DE, Pukazhenthi BS. Quantity rather than quality in teratospermic males: a histomorphometric and flow cytometric evaluation of spermatogenesis in the domestic cat (*Felis catus*). Biol Reprod 2004;71:1517–24.
[22] Axnér E, Linde-Forsberg C, Einarsson S. Morphology and motility of spermatozoa from different regions of the epididymal duct in the domestic cat. Theriogenology 1999;52:767–78.
[23] Axnér E, Linde-Forsberg C. Semen collection and assessment, and artificial insemination in the cat. In: Concannon PW, England G, Verstegen III J, editors. Recent advances in small animal reproduction. Ithaca: International Veterinary Information Service (http://www.ivis.org/), 2002 Document No. A1228.0702 (accessed May 31, 2005)http://www.ivis.org/advances/Concannon/axner/chapter_frm.asp?LA=1.
[24] Gamboa S, Ramalho-Santos J. SNARE proteins and caveolin-1 in stallion spermatozoa: possible implications for fertility. Theriogenology 2005;64:275–91.
[25] Muratori M, Marchiani S, Forti G, Baldi E. Sperm ubiquitination positively correlates to normal morphology in human semen. Hum Reprod 2005;20:1035–43.
[26] Gadella BM, Harrison RAP. Capacitation induces cyclic adenosine 3',5'- monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. Biol Reprod 2002;67:340–50.
[27] Paasch U, Grunewald S, Fitzl G, Glander H. Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. J Androl 2003;24:246–52.

[28] Said TM, Grunewald S, Paasch U, Rasch M, Agarwal A, Glander HJ. Effects of magnetic-activated cell sorting on sperm motility and cryosurvival rates. Fertil Steril 2005;83:1442–6.
[29] Hanson M, Kurinczuk JJ, Bower C, Webb S. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. N Engl J Med 2002;346:725–30.
[30] Laberge R-M, Boissonneault G. On the nature and origin of DNA strand breaks in elongating spermatids. Biol Reprod 2005;10:1095.
[31] Sakkas D, Mariethoz E, St. John JC. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Exp Cell Res 1999;251:350–5.
[32] Aitken RJ, Gordon E, Harkiss D. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 1998;59:1037–46.
[33] Barroso G, Morshedi M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidadtive stress in human spermatozoa. Hum Reprod 2000;15:1338–44.
[34] Anzalone CR, Bench GS, Balhorn B, Wildt DE. Chromatin alteration in sperm of teratospermic domestic cats. Biol Reprod Suppl 1998;58.
[35] Hingst O, Blottner S, Franz C. Chromatin condensation in cat spermatozoa during epididymal transit as studied by aniline blue and acridine orange staining. Andrologia 1995;27:275–9.
[36] Bielanski W, Dudek E, Bittmar A, Kosiniak K. Some characteristics of common abnormal forms of spermatozoa in highly fertile stallions. J Reprod Fert Suppl 1982;32:21–6.