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Morphological Image of Fresh and Cryopreserved Dog Semen Evaluted by the Strict Analysis of Sperm Morphology Method, Using Sperm Quality Analyzer (SQA IIc) Evaluation

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Abstract

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Thirty fresh ejaculates from 15 dogs were cryopreserved in Tris-fructose-citric acid-egg-yolk extender with a glycerol content of 6%. Semen samples were examined by the methods of routine sperm analysis and by the SQA IIc device. The routine semen examination focused on the evaluation of parameters determining the quality of sperm membranes. The significance of monitoring semen quality in the course of the short-term survival test for predicting dog semen quality after thawing was assessed. Relevance of the assessment of sperm morphology, and above all the percentage of sperm with membrane changes in the acrosomal region was documented. The fact that the SQA device analyses semen quality by evaluating the mass of moving cells was confirmed. The results provided by the SQA IIc device appear insufficient for the needs of deeper dog semen analysis, especially morphology assessment.

Dog, sperm analysis, morphological assessment, Sperm Quality Analyser (SQA)

The testicles of breeding males are marked by their high cell production, accompanied by the natural production of insufficiently high-quality cells. A series of factors in the internal and external environment influence the production of this cell population. The proportion of abnormal cells in semen is linked to its fertilisation capacity. Morphological abnormalities of sperm in different animal species have been evaluated in connection with declines in fertility by a number of authors (Hancock 1959; Held et al. 1991; Oettlé 1993) . The limit values for the occurrence of morphologically abnormal sperm in semen have been established according to convention in different species of livestock, or in the human being, and are variable. Johnston et al. (2001) and Stockner and Bardwick (1991) set the percentage of morphologically abnormal sperm in normal dog ejaculate at below 20%. Threlfall (2003) considers acceptable a semen sample containing more than 70% morphologically normal sperm. Věžník et al. (2003) agreed with this value, with the proviso that primary defects in the ejaculate should not exceed 10%. According to Oettlé (1993) the fertility of fresh dog semen is markedly reduced when the percentage of morphologically abnormal cells in the semen is higher than 40%. For semen samples with a morphologically abnormal sperm below 40% the author obtained a pregnancy rate of 61%; when morphological defects exceeded 40%, this rate fell to 13%. Cryopreservation intervention reduces dog ejaculate quality and thus also the fertilisation success rate (Johnston et al. 2001). Feldman and Nelson (1987) consider fresh dog semen containing more than 70% of normal sperm and less than 20% of sperm with primary defects suitable for cryopreservation.

It is essential for the AI doses that donor semen quality is good because fertility of frozen dog semen, in additon to other factors, is associated with the quality of the fresh ejaculate

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after collection (Peňa et al. 1999). Nevertheless, the quality of sperm of some dogs declines more after cryopreservation than sperm of other dogs. This may be caused by variations in the quality of cell membranes (Eilts 2005). Due to the fact that resistance of dog spermatozoa to the process of cryopreservation is low (Tsutsui et al. 2000) it is important to monitor the integrity of the sperm plasma membrane when performing resistance tests in fresh semen. Acrosomal membranes are pre-programmed for membrane fusion. Accordingly, they are the most labile of all sperm membranes (Graham 2001). Changes in the character of membrane swelling and loosening or loss of acrosome are evaluated during the assessment of morphological characteristics of sperm acrosomal membrane integrity. Foote (1975) views morphological assessment of acrosomal integrity as the most reliable method for prediction of semen fertility.

The morphological examination of semen constitutes one of the most objective methods of assessment and great attention has been devoted to it in a number of studies (Bartlett 1962; Check et al. 1992; Oettlé 1993; Menkveld and Kruger 1995; Root Kustritz et al. 1998; Kuster et al. 2004). The first description of dog sperm was published by Leeuwenhoek in 1679 (Oettlé 1993). One of the first to characterise and classify morphologically abnormal sperm was Lagerlöf (1936). This classic form of evaluation was also used with small variations by Bretschneider (1948), Bonadona (1956) and a number of others. Some studies place most importance on establishing the time, species and site of noxious substance on the characteristics of changes (Menger and Menger 1981). The development of the so-called strict morphological analyses (Kruger et al. 1986; Menkveld and Kruger 1995) allowed expanded and more accurate morphological diagnosis. For classification, the entire spermatozoon is taken into account and emphasis is placed on the multiparametric examination of spermatozoa, i.e. all changes present in the spermatozoon are evaluated. Any deviations from normal shape and structure are considered abnormal. Relating abnormalities to individual parts of the spermatozoon and dividing them into primary (developmental, major) and secondary (obtained, minor) enables us to better orientate ourselves in determining the place and time of the emergence of the abnormalities. This separation was noted while developing the computer programme SASMO (Strict Analysis of Sperm Morphology, Věžník et al. 2001).

The SQA IIc was developed for human semen analysis. The device assesses the quality of semen by creating the SMI (sperm motility index) parameter. Parameters of the device designated as sperm concentration, percentage of motile spermatozoa and percentage of sperm with normal morphology are derived by the device from SMI values on the basis of a conversion by means of specific algorithms (Comodo et al. 1997). The relationship between dog sperm motility and concentration and the SMI values has been studied by some authors (Iguer-Ouada and Verstegen 2001), but the relationship between percentages of morphologically aberrant sperm relative to the SMI values has not yet been investigated.

The aim of our research was to show the importance of morphological examination, focusing on establishing the secondary sperm abnormalities characterised as damage to the acrosome. We tested the hypothesis that it is important to monitor the parameters of dog sperm analysis in the course of the short-term survival test in order to predict semen quality after thawing.

We have also verified the possibility of using the SQA IIc device for detailed dog semen analysis. Most importantly, usability of the SQA IIc device for the assessment of dog semen morphology was investigated.

Materials and Methods

The animals and the methodology for collecting semen

Fifteen dogs of different ages from 2 to 11 years (average age 5) were included in the experiment, from the following breeds: Afghan Hound, Basset, Beagle, Doberman, Yorkshire Terrier, Labrador, German Shepherd,

Rottweiler, Leonberger, Shetland Sheepdog and Irish Wolfhound. The dogs were owned by private keepers who brought them to the Clinic of diseases of dogs and cats for experimental semen collection and examination. The semen was collected repeatedly by manual manipulation into plastic test-tubes with a funnel, without presence of a bitch. Clenching the collector in the palm sufficed to minimize the thermal shock. The second sperm rich fraction of each semen sample was collected. In total, 30 fresh semen samples were included for examination.

Examining the semen

After collection, the sperm concentration, percentage of motile and live sperm and the morphological image were established. The concentration was examined haemocytometrically in a Bürker Chamber, the motility was examined using the counting method in a microscope under 200 - 400 × magnification. A drop of semen was placed on a microscope slide and covered with a cover-slip, at 35 °C. Eosin-nigrosin stain was used for distinguishing live from dead sperm. The morphological assessment was carried out by monitoring the samples stained according to Karras (Gamčík et al. 1976). The samples were examined under an optical microscope at magnification 1000 × using oil immersion, and were evaluated using the multiparametric method of evaluation with the SASMO (Strict Analysis of Sperm Morphology) programme (Věžník et al. 2001). The evaluation concentrated on the percentage of morphologically abnormal sperm, the percentage of sperm with primary defects, the percentage of secondary acrosomal defects and the percentage of secondary defects of tail.

The parameters of the SQA IIc device (SQA IIc, Medical Electronic Systems LTD, San Diego, CA, USA) were set as follows: SMI (sperm motility index), sperm motility, sperm concentration and normospermia. The last three indicators have been named "WHO parameters" by the device manufacturer, because they are listed in the WHO manual as criteria for the qualitative assessment of semen (WHO 1999).

The functional, morphological and SQA parameters were also established after the conclusion of the 120 min survival test. The test was carried out at laboratory temperature (22 °C) in semen diluted with physiological saline buffered with phosphate at a pH of 7.2. This pH was selected because of its activating effect on sperm motility. The ejaculates were diluted at a concentration of 100×10^6 spermatozoa/ml. After 120 min, the semen samples were heated to 35 °C and then evaluated.

Long-term cryopreservation and thawing of semen

Individual ejaculates were centrifuged at 500 *g* for 10 min; semen plasma was separated and then frozen and stored at -18 °C. The semen was diluted with Tris-fructose-citric acid-egg-yolk extender (modified according to Andersen 1975) with a glycerol content of 6% at a final concentration of $250 - 750 \times 10^6$ spermatozoa/ml. The diluted semen was poured into 0.5 ml labelled straws and the ends were sealed with polyvinylalcohol. The straws were equilibrated at 4 °C for 2 h and then frozen in liquid nitrogen steam in a metal straw-holder frozen to -100 °C. After 10 min they were stored in liquid nitrogen.

After thawing in a water bath at a temperature of 65 °C for 6 s, the semen samples were diluted to an approximate concentration of 100×10^6 spermatozoa/ml in physiological saline buffered with phosphate to a pH of 7.2 at 35 °C, and containing 20% autologous semen plasma. The parameters of sperm analysis described above were again established.

The quality evaluation of fresh ejaculates was performed by comparing the obtained values with the conventional limits of currently accepted criteria for fresh dog ejaculate quality (Věžník et al. 2000). These authors suggest that the values of evaluated parameters should not decrease by more than 10% from those of fresh semen during the short-time survival test. The parameters of semen samples after thawing were compared with the limit values of thawed dog semen samples based on the results of Linde-Forsberg and Forsberg (1989).

The relationships of the semen quality parameters were evaluated by statistically assessing changes during the survival test and during cryopreservation. Changes in the parameter values of sperm analysis during cryopreservation were established as a percentage of the increase or decline in values after thawing compared with the initial values of the fresh semen after collection. Similarly, the changes in values during the survival test were established by comparing the values obtained after 120 min with the initial values after collection. The parameter values for routine sperm analysis were statistically compared with the parameters of the SQA IIc device.

Statistical methods

Statistical analysis was performed using the Stat Plus software (Stat Plus, version 1.01, 1991, VRI Brno). The Pearson Correlation test was used for evaluation of correlations for parametric data. The Spearman test was used for evaluation of correlations for non-parametric data. Data were evaluated for differences between groups using the Paired t-test for parametric data and Wilcoxon Signed Rank Test for non-parametric data. The level of significance was set at $p < 0.05$. The Kruskal-Wallis Test was used for analysis of variances.

Results

Tables 1 and 2 show the average values for the parameters monitored of fresh and thawed semen.

The lower limits of the qualitative criteria and the proportion of ejaculates corresponding to the conventional values in the given indicators are shown in Table 3. Table 4 shows comparison of the values of assessed parameters with the limit values that determine the quality of thawed dog semen.

Table 1. Average values of parameters of the routine analysis of semen during the course of the short-term survival test and after thawing (n = 30)

		Concentration	Motility	Live sperm	Morphologically abnormal sperm	Normospermia	Sperm with primary defects	Sperm with secondary defects of acrosome	Sperm with secondary defects of tail
		$\times 10^6$/ml	%	%	%	%	%	%	%
1	Average±SD	276.3±233.51	78.0±11.79	83.8±10.23	27.0±12.52	73.0±12.52	10.5±5.85	8.5±8.28	8.7±5.39
	Max	1225.6	95.0	96.2	53.5	92.0	28.5	34.0	25.0
	Min	51.2	55.0	56.9	8.0	46.5	3.5	1.0	2.5
2	Average±SD	nd	52.3±18.70	75.8±11.13	36.1±17.98	63.9±17.98	10.3±6.81	15.42±15.14	13.4±10.78
	Max	nd	90.0	91.8	82.6	87.0	35.5	65.2	49.8
	Min	nd	10.0	48.3	13.0	17.4	2.0	3.0	3.0
3	Average±SD	91.4±28.72*	41.5±12.74	55.5±10.75	57.5±12.92	42.5±12.92	9.1±4.89	41.3±11.34	18.7±8.90
	Max	150.4	70.0	88.2	78.5	67.5	19.5	68.5	38.0
	Min	49.6	5.0	38.0	32.5	21.5	1.5	21.0	2.0

1 initial values of fresh semen
2 values after 120-min survival test of fresh semen
3 values of the parameters of semen after thawing
SD standard deviation
* sperm concentration after diluting thawed sample at an assumed concentration of 100×10^6 spermatozoa /ml
nd not determined

Table 3. Proportion of ejaculates corresponding to conventional limits accepted for the qualitative evaluation of dog semen during the survival test (n = 30)

Parameter		Motility		Live sperm		Morphologically abnormal sperm	
Time after collection		0 min	120 min	0 min	120 min	0 min	120 min
Conventional limits for the parameters (%)		>70	>63	>70	>63	<30	<36
Proportion of ejaculates corresponding to the conventional limits (%)	established by routine sperm analysis	76.7	26.7	90.0	86.7	66.7	66.7
	established by the SQA device	96.7	56.7	nd	nd	0	0

nd not determined

The relationship between the values of examined parameters obtained during cryopreservation and the values obtained during the survival test was investigated. A highly significant correlation was found between the decline in the live sperm percentage during the survival test of fresh semen and the decline of this parameter values during cryopreservation ($r = 0.519$, $p < 0.01$).

There was a statistically non-significant correlation between the increase of secondary acrosomal defects during the survival test of fresh semen and the increase of these changes during cryopreservation. However, regression analysis showed an obvious positive trend. A statistically significant negative correlation ($r = -0.441$, $p < 0.05$) was found between the percentages of sperm with acrosomes showing membrane changes after the 120 min survival test and percentages of live sperm after thawing. Different levels of disturbed membrane integrity in the area of the acrosome as assessed by the viability test using eosin-nigrosin stain are shown in Fig. 1, 2 and 3 (see Plate II and III).

Table 2. Average values of SQA parameters of semen monitored in the course of the short-term survival test and after thawing (n = 30)

		Concentration	Motility	Normospermia	SMI
		$\times 10^6$/ml	%	%	%
1	Average±SD	203.1±24.24	84.7±6.10	53.5±3.05	520.1±62.91
	Max	225.0	91.0	57.0	582.0
	Min	107.0	62.0	43.0	293.0
2	Average±SD	nd	64.3±19.20	42.5±11.46	334.8±153.20
	Max	nd	89.0	56.0	564.0
	Min	nd	17.0	17.0	45.0
3	Average±SD	97.2±49.93*	54.7±18.06	36.6±11.85	253.3±119.74
	Max	183.0	79.0	51.0	458.0
	Min	0.0	0.0	0.0	26.0

1 initial value of fresh semen
2 value after 120-minute survival test of fresh semen
3 value of the parameters of semen after thawing
SD standard deviation
SMI sperm motility index
* sperm concentration after diluting thawed sample at an assumed concentration of 100×10^6 spermatozoa /ml
nd not determined

Table 4. Proportion of semen samples corresponding to the limits selected by our laboratory for the use in dog semen after thawing (n = 30)

Parameter		Motility	Live sperm	Morphologically abnormal sperm
Chosen limits for the parameters (%)		>40	>40	>60
Proportion of ejaculates corresponding to the limits (%)	established by routine sperm analysis	73.3	96.7	60.0
	established by SQA device	80.0	nd	53.3

nd not determined

The relationship between the decline of fresh sperm motility during the survival test and the decline of this parameter values during cryopreservation, and the relationship between the increase of morphologically abnormal sperm during the survival test and their increase during cryopreservation was statistically non-significant.

Table 5. Correlations between the values of routine analysis of semen and of the SQA device and the statistical significance of the differences in the averages of these values (n = 30)

		Motility vs. motility (SQA).	Concentration vs concentration (SQA)	Normospermia vs. normospermia (SQA)	Morphologically abnormal sperm vs. SMI
1	*r*	0.239[b]	0.570[a]	0.260[b]	-0.261[b]
	t-test	a	b	a	nd
2	*r*	0.792[a]	nd	0.241[b]	-0.223[b]
	t-test	a	nd	a	nd
3	*r*	0.666[a]	0.215[b]	0.098[b]	-0.072[b]
	t-test	a	b	b	nd

1 initial value of fresh semen
2 value after 120-minute survival test
3 value after thawing
SMI sperm motility index
a statistical significance where $p < 0.01$
b statistically nosignificant
nd not determined
r correlation coefficient

Analysis of variance between the values of sperm with primary defects after collection and the values after the 120 min survival test and after thawing was carried out in order to verify the diagnostic relevance of morphological examination. Statistical non-significance of variances demonstrated concordance in these findings.

The statistically significant correlation between the increase in the percentages of morphologically abnormal sperm and sperm with secondary acrosomal defects during the survival test ($r = 0.508$, $p < 0.01$) and also during cryopreservation ($r = 0.843$, $p < 0.01$) was detected.

The correlations between values of determined parameters established by routine sperm analysis and the SQA device and the differences between the groups are shown in Table 5. Regression analysis of the relationship between routinely determined sperm concentrations and those determined by the SQA device showed a logarithmic character of correlation at the initial value.

Discussion

The integrity and normal resistance of the plasma membrane of sperm is a necessary prerequisite of successful fertilization, as only sperm with intact plasma membranes are capable of fertilization (Peňa et al. 1999). The importance of the sperm membrane quality assessment during the survival test for the prediction of semen quality after thawing has been documented by statistical evaluation of the results. This is consistent with the results obtained by Věžník et al. (1986) who confirmed that the increase in secondary alterations in bull sperm during the 120 min survival test significantly correlated with sperm quality after thawing (freezability).

The assessment of the percentage of sperm with secondary changes reveals sperm with disturbed membrane integrity in the area of the acrosome, but perhaps not so substantially disturbed that it manifests during the viability test with the eosin-nigrosin stain. On the other hand, a number of sperm shown by intravital dye to be dead need not have a microscopically disturbed membrane in the area of the acrosome, and therefore both parameters of investigation are important. Different levels of disturbed membrane integrity in the area of the acrosome assessed by the viability test with eosin-nigrosin stain are shown in Fig. 1, 2 and 3. Sperm with moderately disturbed membrane integrity in the area of the

acrosome may even be motile, as long as they are not also damaged in the tail area. Nevertheless from the point of view of their future success in the fertilisation process, such sperm are not promising because their life is limited. This conclusion corresponds to Oettlé's (1986) assertion concerning the inadvisability of evaluating semen quality solely on the basis of motility itself, because motile sperm need not always be fertile.

The higher occurrence of the secondary acrosomal defects may be partly due to methods employing the morphological dyes. Stains emphasising the structures of the acrosome reveal more damaged cells than overall methods of staining. As Root Kustritz et al. (1998) and Root and Johnston (1994) state, methods of staining and the preparation technique also have an influence on the results of the morphological assessment. Any staining method causes the occurrence of artefacts and may mask genuine defects. It is more difficult to assess stained samples of thawed semen than samples of fresh semen because elements of the extender, like egg-yolk or milk, may interfere with most stains (Oettlé 1986).

The results of our evaluation demonstrate the dependence of the increase in the proportion of morphologically abnormal sperm during storage or cryopreservation predominantly on the growth of secondary acrosomal changes, which corresponds to the assertion of Věžník et al. (2005). The lower percentage of ejaculates corresponding to the established limits for percentage of sperm with morphological defects in fresh and thawed samples (Table 3, 4) demonstrated the relevance of sperm morphology assessment in addition to the parameters of motility and percentage of live sperm. It follows from the present results that morphology of live or even motile spermatozoa may be abnormal.

The statistical non-significance of the correlation between the values of the decline in motility during the survival test and during cryopreservation is caused by the high drop in sperm motility during the 120 min survival test. This marked sperm motility decrease may be the result of using a physiological saline buffered to a pH of 7.2 as a dilution medium. This pH, chosen to activate sperm motility, may be less suitable for certain ejaculates because of its high alkalinity, which by supporting higher movement activity results in faster exhaustion. It is possible to explain the non-significance of the correlation between the increase of morphologically abnormal sperm during cryopreservation and the increase of these changes during the short-term survival test by the differential occurrence of secondary changes to the tail after collection, during the 120 min survival test and after thawing. The highest occurrence and also increase in secondarily changed tails in comparison with the values after collection was found in semen after thawing.

The SQA device analyses semen quality by evaluating the mass of moving cells i.e. it assesses a semen sample on the basis of two parameters: motility and concentration. By evaluation of the results obtained by the SQA, the relationships between concentration and motility of spermatozoa were demonstrated. In cases of marked differences between concentration and motility, distorted SMI values were found. For this reason, a mutual correlation between the motility values established by the classic counting method and using the SQA in the whole set of ejaculates at the initial value after collection was not attained. After eliminating semen samples with extreme differences between concentration (higher than 200×10^6 spermatozoa/ml) and motility (lower than 70%), a correction of the relations occurred and the motility values established by the SQA correlated with the motility established by counting ($r = 0.679$; $p < 0.01$; n = 20). Also, the differences in means were statistically non-significant. Regression analysis of the initial concentration values established routinely and by the SQA device showed a logarithmic character of the relationship. This is influenced by the maximum concentration values that can be evaluated by SQA. The maximum possible concentration given by the device was 225×10^6 spermatozoa/ml. Iguer-Ouada and Verstegen (2001) did not observe

a statistically significant correlation over the tested 200×10^6 cells/ml, possibly because of a saturation of the system.

A semen sample with very low sperm motility is given a low SMI index by the device, but sets zeroes for the so-called WHO parameters. The results recalculated from SMI using the device algorithms and designated as sperm motility and normospermia have been modulated according to the limit values. Therefore, the percentage of motility established by the SQA device ranged from zero to 91%. The quantity of normal cells is set from zero to 57%, given the maximum SMI value is obtained. These restricted values of normospermia assessed by SQA device caused that the obtained ejaculates were not of acceptable quality according to the mentioned conventional quality limits (Table 3).

Conclusion

The hypothesis that it is important to monitor the parameters of sperm analysis during the survival test in order to predict semen quality after thawing was tested.

The relevance of evaluation of sperm morphology, especially the percentage of sperm with membrane changes in the acrosomal area, was documented.

While using the SQA IIc device for evaluating the quality of dog semen samples, it was confirmed that the SQA device analyses ejaculate quality by evaluating the mass of moving cells. The results provided by the SQA IIc device appear insufficient for the needs of deeper dog semen analysis, especially morphology assessment.

Morfologický obraz nativního a konzervovaného semene psa a jeho hodnocení metodou Striktní analýzy morfologie spermií, s využitím hodnocení přístrojem Sperm Quality Analyzer (SQA IIc)

30 nativních ejakulátů od 15 psů bylo konzervováno Tris-fruktoso-citrát-žloutkovým ředidlem s obsahem glycerolu 6 %. Semeno bylo vyšetřeno metodami rutinní spermatoanalýzy a přístrojem SQA IIc. Rutinní vyšetření semene bylo zaměřeno na hodnocení parametrů určující kvalitu membrán spermie.

Byl ověřen význam sledování parametrů spermatoanalýzy v průběhu krátkodobého testu přežitelnosti pro predikci kvality semene psa po rozmrazení. Byla doložena významnost posouzení morfologického obrazu semene, a to především zastoupení spermií s membránovými změnami v oblasti akrosomu. Byla potvrzena skutečnost, že přístroj SQA analyzuje kvalitu ejakulátu na základě zhodnocení masy pohybujících se buněk. Pro potřeby hlubší spermatologické diagnostiky semene psa, zvláště pak zaměřené na morfologickou analýzu semene, se jeví výsledky poskytnuté přístrojem SQA IIc jako nedostačující.

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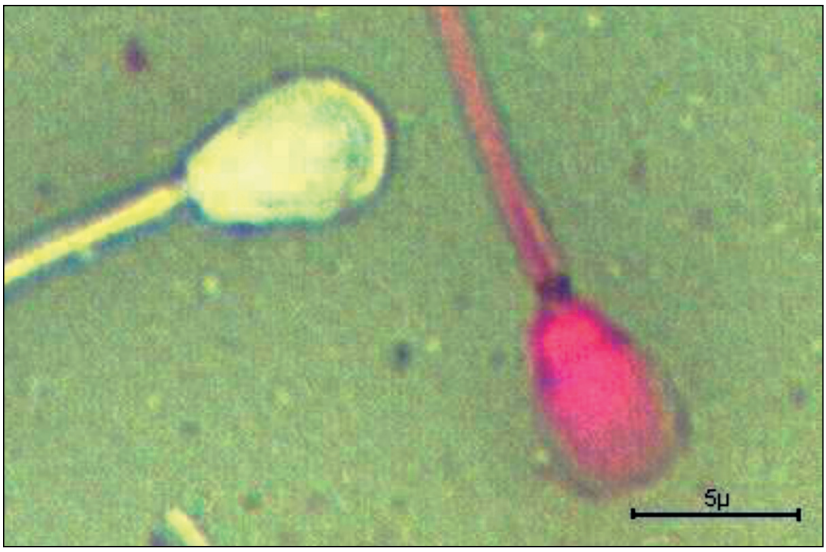

Fig. 1. Vital (white) spermatozoon with intact acrosome and dead (red) spermatozoon with swollen acrosome (eosin-nigrosin staining). Magnification × 1000.

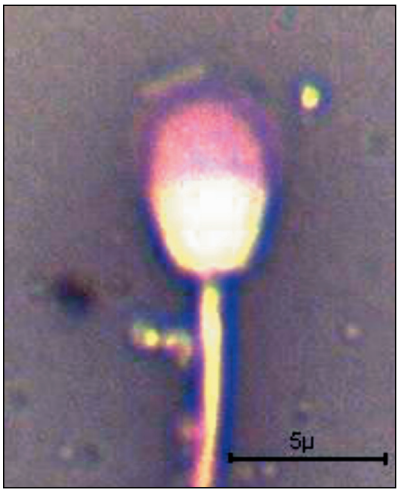

Fig. 2. Progressive penetration of the eosin stain in the cell across the impaired membrane in the area of acrosome (pink acrosomal cap; eosin-nigrosin staining). Magnification × 1000.

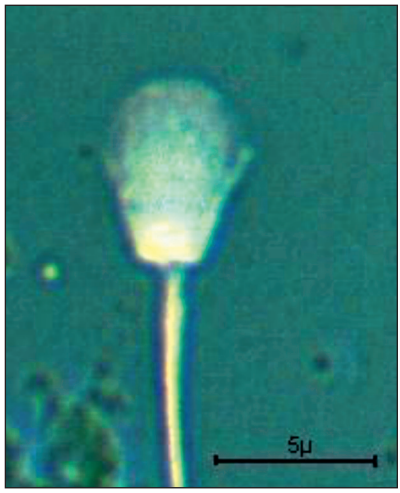

Fig. 3. Vital (white) spermatozoon with the impaired membrane in the area of acrosome morphologically expressed as swollen acrosome (eosin-nigrosin staining). Magnification × 1000.