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Research Article

Evaluation of the Canine Epididymal Sperm Morphology with two Different Staining Methods, One Fixative Solution and Motile Sperm Organelle Morphology Examination (MSOME) [1]

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Abstract

The aim of this study was to compare the use and effectiveness of two different stains, one fixative solution and Motile Sperm Organelle Morphology Examination (MSOME) on morphological characteristics of spermatozoa in fresh dog semen samples from epididymis. After routine castration, cauda epididymides were collected from 20 dog testes. Morphological abnormalities were evaluated by using Hancock's buffered formol saline solution, the aniline blue stain, Diff-Quik stain and MSOME analysis in epididymal semen. Conventional semen analysies and MSOME were simultaneously performed on the same sample from each dog. Percentage of abnormal spermatozoa, head abnormality and acrosomal defects were significantly higher in samples with two different staining methods and one fixative solution than MSOME technique. It was concluded that the usage of the Aniline blue stain may be an efficient method for evaluating the sperm morphology of dog semen. Although no correlation was established amoung four different methods on sperm morphology assessment.

Keywords: *Canine, Morphology, MSOME, Semen, Stain*

Motil Sperm Organel Morfolojisi Muayenesi (MSOME) ve Fiksatif Solüsyon, İki Farklı Boyama Tekniği İle Köpek Epididimal Spermatozoa Morfolojisinin Değerlendirilmesi

Özet

Bu çalışmanın amacı, taze köpek sperması örneklerinde spermatozoonların morfolojik özellikleri üzerine Motil Sperm Organel Morfolojisi Muayenesi (MSOME), bir fiksatif solüsyon ve iki farklı boyanın kullanımını ve etkinliğini karşılaştırmaktı. Kastrasyon işleminden sonra, 20 adet köpek testisinden kauda epididimisler elde edildi. Epididimal spermada morfolojik bozukluklar, Hancock sıvı fikzasyon yöntemi, aniline blue boyama yöntemi, Diff Quik boyama yöntemi ve MSOME tekniği ile değerlendirildi. Her bir köpekten bir örnek alınarak, konvensiyonel semen analizi ve MSOME analizi aynı örnekte eş zamanlı olarak incelendi. Morfolojik olarak ortalama abnormal baş ve akrozom defekti MSOME tekniğine göre diğer üç farklı methodta önemli derecede yüksek bulunmuştur. MSOME tekniği ile 3 farklı yöntem karşılaştırıldığında, anilin boyama yönteminde baş bozukluk oranı bulunan anormal spermatozoa oranı önemli derecede yüksek bulunurken, köpek spermatozoa morfolojisi değerlendirilmesinde Aniline blue boyama yönteminin etkili bir yöntem olduğu sonucuna varıldı. Spermatozoa morfolojisinin değerlendirilmesinde üç farklı method ile MSOME tekniği arasında herhangi bir paralellik bulunmadı.

Anahtar sözcükler: *Boya, Köpek, Morfoloji, MSOME, Sperma*

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INTRODUCTION

The male factor is considered a major contributory factor to infertility [1]. Sperm morphology has become an area of great interest to assess of male infertility, since observation of normal and abnormal morphological sperm forms in semen samples [2]. Evaluation of sperm morphology displays a potential impact on male fertility [3] and has been recognized to be the best predictor of outcome of natural fertilization [4], intrauterine insemination [5], and conventional *in vitro* fertilization [6]. Nowadays, many conventional and advanced methods exist to assess semen quality [7]. To evaluate the morphology of mammalian spermatozoa, many stains and staining combinations have been used, for example, Papanicolaou, Hematoxiline, Toluidin blue-pironin, Giemsa and Nigrosin. But conventional light microscopic analysis of spermatozoa has limitations in evaluating the fine structures, such as the acrosome and nucleus [8,9]. New possibilities have arisen because the latest technical facilities improvements are available in the IVF-laboratories allow to assess the sperm morphology in details. The introduction of MSOME (Motile Sperm Organelle Morphology Examination) by Bartoov et al.[4] allows the examination of subcellular disorders like nuclear vacuoles at high magnification (6000-12500x) in real time on motile sperm. Oliveira et al.[10] evaluated the correlation between MSOME classification and sperm morphology classification according to the Tygerberg criteria in 97 semen samples from an unselected group of couples undergoing infertility investigation. The study showed a strong positive correlation between the percentage of normal sperm forms according to the Tygerberg criteria and MSOME. The main aim of this study was to test the effectiveness of two different staining techniques (Aniline blue, Diff-Quick), one fixative solution (Hancock's method), traditionally used for the assessment of sperm morphological analysis, and to determine their correlation with Motile Sperm Organelle Morphology Evaluation (MSOME).

MATERIAL and METHODS

This experiment was conducted to compare the effects of two stains, one fixative solution and MSOME analysis on morphological characteristics of spermatozoa in fresh semen samples. Testicles were obtained from 20 privately owned mixed-breed dogs (age range: 2-8 years, body weight<10 kg) after routine castration at local veterinary practices. Ethics committee approval for this study was given by Ethical Committee of Poland University Veterinary Faculty (Ike 72/2009).

Sample Collection

Samples were kept in phosphate buffered saline (PBS) at room temperature for transport to the laboratory. All tissue was processed within 2 h of collection. The cauda epididymis and vas deferens were dissected from each testis and placed in a clean and dry petri dish. After removal and dissection of the testicles, samples were obtained from the distal portion of the epididymis by cutting the tail of epididymis with a scalpel blade and placing it into 1 ml Human Tubal Fluid (HTF). The semen samples were washed with Sperm Washing Medium (Irvine Biologicals) by centrifugation at 800 g for 10 min and supernatant was discarded. Pellet was suspended in the same solution and thereafter centrifuged once more. The pellet was gently over-layered with medium in the tube which was sealed, inclined at 45°C and kept at 37°C for 60-90 min in 5% CO_2. A sterile Pasteur pipette was used to remove the supernatant containing actively motile sperms [11]. After diluted sperm samples, morphological abnormalities were evaluated by using Hancock's buffered formol saline solution, the aniline blue stain, Diff-Quik stain and MSOME analysis. Since the main objective of this experiment was only to compare the effects of the two staining solutions, fixative solution and ultramorphological analyses upon the morphological characteristics of spermatozoa, no additional control group was included. Conventional semen analysis (Hancock's method, aniline blue stain, Diff-Quik stain) and MSOME exploration were performed simultaneously on the same sample from each dog. Sperm abnormalities were categorized as abnormal heads (including pear shaped, small heads, narrow, heads alone, or large heads), acrosome defects, abnormal midpieces and proximal cytoplasmic droplets.

Diff Quick

A modified Diff Quick method was used as follows. Thin smears of the well-mixed diluted sperm samples were prepared in duplicate by placing 10 µL on clean slides. After air-drying, the slides were stained using Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL). Afterwards the smears were consecutively stained with solution 1 (10 min), then air-dried and stained with solution 2 (10 min). Finally, the slides were washed in running tap water to remove the excess stain (10 to 15 times). The stained slides were evaluated at x1000 magnification with oil immersion (Leica Microsystems). For each smear, at least 200 spermatozoa were examined [12].

Aniline Blue

Ten-microliter drops of diluted semen samples were spread onto glass slides and allowed to dry. These smears were then fixed at room temperature in buffered 3% glutaraldehyde in phosphate - buffered saline (PBS) for 30 min and air-dried. After fixation, the slides were stained with 5% aqueous aniline blue mixed with 2% acetic acid (pH = 3.5) for 5 min, washed with distilled water and air-dried. Briefly, the staining solution was prepared by adding 5 g of aniline blue (Water blue, Fluka, Buchs, Switzerland) to 100 mL of PBS, filtering, and adjusting the pH to 3.5 with

2% glacial acetic acid (Merck, Darmstadt, Germany). For each stained smear, 200 spermatozoa were evaluated with light microscope in oil immersion magnification (100x objective) [13].

Hancock's Method

According to the Hancock's method, 0.5 ml of each sample were added to an Eppendorf test- tube which containing 1 ml Hancock's solution [62.5 ml formalin (37%), 150 ml saline solution, 150 ml buffer solution and 500 ml double distilled water] [14]. One drop of the semen mixture was dropped on a slide and covered with a cover slip. Sperm morphology was determined by counting a total of 200 sperm cells under phase contrast microscope with an oil immersion objective.

Determination of Morphology by MSOME

An aliquot of 1 µL of diluted sperm suspension was transferred to a 5 µL microdroplet of modified HTF medium containing 7% polyvinylpyrrolidone (PVP medium; Irvine Scientific). This microdroplet was placed in a sterile glass dish (Fluorodish; World Precision Instruments, USA) under sterile paraffin oil (Light Mineral Oil for Embryo Culture, Irvine Scientific, USA). The sperm cells, which were suspended in the microdroplet, were placed on a microscope stage covered by a droplet of immersion oil. The examination was performed with Leica DMI 6000B inverted microscope equipped with DIC/Nomarski optics using a Leica objective HC χ PL FLUOTAR 100 χ/1.30, under oil immersion. Spermatozoa were analyzed at magnifications greater than or equal to 6600× Classification of four categories was done according to Vanderzwalmen et al.[15] for each sperm sample. MSOME involves the grading of spermatozoa according to the presence of nuclear vacuoles:

– Grade I, oval shaped sperm head without vacuoles.

– Grade II, oval shaped sperm head with 1-2 small vacuole < 4% of the head area

– Grade III, oval normal shape and size sperm head with one large vacuole > 4% of the head area or several small vacuoles

– Grade IV sperm head with abnormal morphology with or without vacuoles.

At least 200 motile spermatozoa per semen sample were evaluated and percentage of abnormal spermatozoa was determined [16]. *Fig. 1A* and *Fig. 1B* shows normal spermatozoa and spermatozoa with large vacuoles analysed by MSOME.

Statistical Analyses

All data were checked for normal distribution with Shapiro-Wilk and homogenity of variance with Levene's test. Data were not normally distributed. The non-parametric Kruskal Wallis test was used to determine the differences between the methods. Post hoc analysis of pairwise difference between methods was performed using Mann Whitney U test with Bonferonni correction. P values <0.05 were considered to be significant. The results were presented as the mean ± SEM.

RESULTS

The values of the morphological changes observed from the use of each technique are presented in *Table 1*. Significantly increased morphological head defects and abnormal acrosome were observed in the Aniline blue method when compared with Diff-Quik and Hancock's methods. Average percentages of morphologically abnormal head defects and abnormal acrosome were significantly higher in samples with 3 different methods than MSOME technique. For abnormal heads alteration,

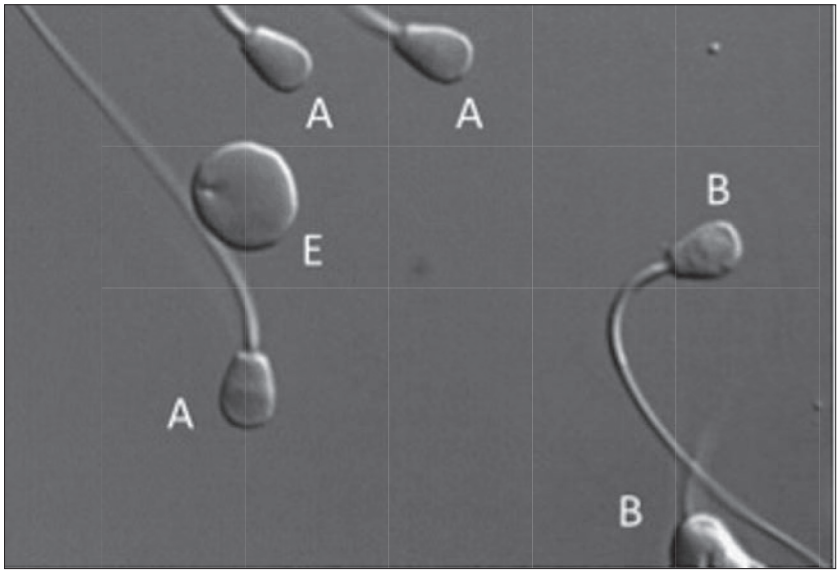

Fig 1. A- Normal spermatozoa observed at high magnification (×8400); **B-** Spermatozoa with large nuclear vacuoles observed at high magnification (×8400), **E-** Erythrocyte

Şekil 1. A- Yüksek büyütme (×8400) altında gözlenen normal morfolojiye sahip sperm hücresi, **B-** Yüksek büyütme (×8400) altında gözlenen geniş vakuole sahip sperm hücresi, **E-** Eritrosit

Table 1. *Percentage data (mean ± SEM) of morphological sperm defects of dog semen using different staining, Hancock's solution and Motile Sperm Organelle Morphology Examination (MSOME) methods*

Tablo 1. *Motil Sperm Organel Morfolojisi Muayenesi (MSOME) methodu, Hancock solüsyonu ve farklı boyalar kullanılarak köpek spermasında saptanan morfolojik sperm defektlerinin ortalama verileri (ortalalama ± SEM)*

Method (n=20)	Abnormal Acrosomal Rate (%)	Abnormal Head Rate (%)	Abnormal Middle Piece Rate (%)	Presence of Cytoplasmic Droplet (%)
Diff Quick	9.50±1.2^{c}	17.60±1.4^{b}	2.00±0.7^{a}	17.00±0.5^{a}
Aniline Blue	18.50±1.8^{a}	24.7±1.4^{a}	2.00±0.6^{a}	15.00±0.3^{a}
Hancock's	12.00±1.2^{b}	19.10±1.4^{b}	1.00±0.6^{b}	15.00±0.4^{a}
MSOME	1.00±0.6^{d}	1.60±0.8^{c}	1.02±0.5^{b}	1.00±0.4^{b}

Groups with different letters (a,b,c,d) in the same column are significant different (P<0.05)

an average of 24.7±1.4% was obtained in aniline blue, a value that was higher (P<0.05) than the one found when using Diff Quick and Hancock methods. For middle piece changes, a higher average was obtained when using the smear with the conventional Diff-Quik and aniline blue compared with Hancoock and MSOME methods. In this study, statistically significant difference was observed for cytoplasmic droplet alteration when using the four different methods.

DISCUSSION

In most mammalian species, conventional semen analysis is mainly based on the assessment of the sperm concentration, the motility characteristics and the morphological classification of spermatozoa in the evaluation of male factor infertility [17,18]. Assessment of sperm morphology can be influenced by many factors, such as the fixation and staining technique (e.g. Diff-Quik, nigrosin/eosin) [17], sperm preparation methods procedures, quality of the microscope and examiner's skills [18]. A number of studies of sperm staining procedures used to assess sperm morphology for several animal species have reported that the same fixatives and stains have different reactions with the sperm of individual species [19]. Therefore, it is important to find the most suitable staining technique for each species [20]. Our results indicate that it is not suitable for clearly defining and indicating the boundaries of the acrosome for evaluating canine semen morphology, although Diff-Quik stain method is simple and easy to evaluate. Normally, it is sufficient to fixate the smear in Diff-Quik fixative for 30 sec, but we recommend leaving smears in each solution for 10 min to achieve the best result and it can be effect the results. Therefore, Diff Quik stain is not to be a useful alternative method to evaluate for canine semen morphology [13]. But the aniline blue staining is suitable for clearly defining the main components of sperm and allowed good visualization of canine spermatozoa morphology [13]. The present study is the first to describe aniline blue staining of canine sperm for sperm morphology. Aniline blue staining can be used to examine two different sperm parameters as integrity of the DNA and sperm morphology. The assessment of both sperm morphology and chromatin on the same slide would be suitable for andrology laboratories. Also, Hancoock's method is not suitable for clearly defining the main components of sperm because the sperm samples are not fixed.

The resolving power offered by MSOME enables the identification of spermatozoa showing shape and size changes and intranuclear vacuoles as well, that would not be detected with conventional evaluation methods [21]. The presence of vacuoles on sperm head (size, number, localization and frequency) can be revealed during sperm movement [21,22]. Thus, the analysis of only motile spermatozoa by MSOME provides an advantage for morphological observation [21]. Higher magnification provided by the 100× DIC objectives are more appropriate to allow more detailed analysis of small cells [21]. We were the first to describe nuclear vacuoles in canine spermatozoa. In this study, the relationship between normal sperm morphology obtained by the conventional method and MSOME was assessed in 20 male dogs. No significant correlation was found between the frequency of morphologically normal spermatozoa as defined by MSOME and the frequency of morphologically normal spermatozoa using conventional method. The incidence of sperm normalcy by conventional sperm analysis was significantly lower than that by MSOME in this study. It should be stressed that MSOME focuses only on motile spermatozoa, unfixed motile sperm fraction, while the conventional morphological examination is applied to the entire semen sample post-fixation. The methods used (fixation and staining) do not allow the selective analysis of the motile sperm fraction alone [21]. Thus, the usage of MSOME might show a potential improvement in the morphological diagnosis of the sperm. But, a positive correlation has been observed between normal MSOME spermatozoa and normal spermatozoa using Tygerberg criteria (r 1⁄4 0.83, 0.0001) [10]. Oliveira et al.[10] evaluated the correlation between MSOME classification and sperm morphology classification according to the Tygerberg criteria [6] in 97 semen samples from an unselected group of couples undergoing infertility investigation. The study showed a strong positive correlation between the percentage of normal sperm forms according to the

Tygerberg criteria and MSOME (r=0.83; P<0.001). Conversely, the frequency of abnormal MSOME spermatozoa was negatively correlated with sperm concentration, sperm motility, and the percentage of spermatozoa with normal morphology [23]. The relationships between spermatozoa with size and number of nuclear vacuoles and conventional semen parameters have been more debated. Vacuoles in the sperm head have been reported to be associated with low sperm concentration, low sperm motility [24] or high teratozoospermia [25]. No correlation was reported between the rate of spermatozoa with large vacuoles and sperm morphology in the study. Also, Bartoov et al.[4] investigated the relationship between normal spermatozoa according to the WHO reference values [26] and MSOME in 20 patients. The authors found no significant correlation between the percentage of morphologically normal spermatozoa as defined by the WHO and the percentage of morphologically normal spermatozoa as defined by MSOME, since the incidence of sperm normalcy by routine sperm analysis was significantly higher than that by MSOME (26.1±7.2% and 2.9±0.5%, respectively). Perdrix et al.[27] analysed semen samples from 440 males, aged between 24 and 66 years, consulting for infertility investigation. The presence of vacuoles in the sperm head was significantly larger in poor semen samples (P<0.001). Relative vacuolar area (RVA), defined as vacuole area (l μm^2)/head area (l μm^2) X100, was the most discriminative MSOME criterion between normal and abnormal semen samples, and was negatively correlated with poor sperm morphology (r=0.53; P<0.001). It is noteworthy that conventional morphological examination is applied to semen sample including both alive and dead sperms, whereas the most remarkable feature of MSOME is the focused on motile sperm fractions, providing information about the sample fraction referred for ICSI (intra cytoplasmic sperm injection treatment) [4].

In the light of these findings, Aniline blue stain is an efficient method for evaluating the sperm morphology of canine semen. MSOME has been proposed as much stricter criterion of sperm morphology evaluation as compared to the conventional semen analysis [10]. But MSOME seems to be not a more strict technique for the classification of morphologically normal spermatozoa in this study. The sperm nuclear vacuoles evaluated at high magnification can be routine use of MSOME for ICSI as a criterion for semen analysis. It should be noted that more studies performed in greater number of infertile dogs are required to confirm the usefulness of MSOME in dog sperm morphology analysis.

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