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Morphometric dimensions of the stallion sperm head depending on the staining method used

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Abstract

Semen was collected from Polish Halfbred stallions. Twenty individuals from 3 to 4 years of age were selected for the study. At least one ejaculate from each stallion was collected and assessed. Sperm morphology was evaluated using Papanicolaou stain, SpermBlue®, and a complex of eosin and gentian stain, whereas unstained slides were used as control samples. Morphometric measurements were performed on 100 randomly selected sperm heads in each ejaculate. The length, width, perimeter, and surface area of the sperm head were measured. The frequency of nuclear vacuoles was determined as well. Tygerberg's strict criteria, which most precisely characterise the sperm head, were used in the morphological evaluation of the sperm. The results obtained indicate that in the case of staining with SpermBlue® and with eosin+gentian complex, the morphometry of the sperm head seems to be most similar to that observed in unstained smears. It also seems that neither shrinking nor swelling is uniform in the various staining techniques (Papanicolaou stain). Moreover, it appears that in comparison with unstained sperm, all methods caused the width of the head to increase as a result of swelling.

Keywords: stallion, sperm head, staining method, morphology, morphometry.

Introduction

Sperm morphology is an important parameter in predicting fertility in humans and animals (19, 30). While there are other significant semen parameters, such as sperm motility and sperm concentration, in the era of *in vitro* fertilisation, insemination, and natural mating service, morphological structure of spermatozoa is regarded as the most important. Even though sperm cells motility might be normal, head defects may render them incapable of fertilisation (44).

Sperm cells are considered to be normal if their appearance is within the classification for a given species, which includes the shape and size of the head, midpiece, and tail. Abnormalities may be associated with anomalies in DNA structure, which can directly lead to reduced fertilisation capacity or reduced embryo quality, thus increasing the risk of embryonic death (42). Thurston *et al.* (46) concluded that the shape of the sperm head is determined genetically. Some authors indicate that the shape of the sperm head depends on certain factors that may emerge during spermatogenesis. It has been shown that morphologically varied gametes may appear as early as during the spermatogenesis process when a genetic factor significantly influences the structure and size of the cell (46). Scientists explain that an abnormal sperm head shape linked to poor chromatin condensation may result in the presence of sperm cells with an elongated, narrow head in the semen. Sperm cells with such morphology may lead to functional disturbances in the form of immature chromatin and fragmented DNA, causing a potential disadvantage for embryo development (4, 40). The size and shape of the sperm

cell have also been shown to have a significant influence on its functionality, including the course of the acrosomal reaction (36) and interactions with the zona pellucida of the oocyte (18). In this context determination of the normality of sperm head size and shape becomes particularly important, as they are important criteria in classifying sperm as having normal or abnormal morphological structure. A number of studies have shown that the average size of sperm heads in semen with abnormal morphology is larger than in reproductive cells from normal ejaculates (7).

A variety of staining methods for evaluating sperm morphology are used in laboratory practice to predict male fertilising ability. In the case of diagnosis of human semen, preparation of samples by the Papanicolaou staining method is often preferred (36). To evaluate animal semen, a simple staining method using a complex of eosin with gentian stain is common (5, 26), while SpermBlue® stain is used for the analysis of both human and animal semen (48). Accurate assessment of sperm morphology depends on meticulous preparation, fixation, and staining of sperm cells, as this affects the morphometry of the sperm head and of the entire cell (34). This makes the choice of staining technique especially important. The method used should interfere as little as possible with stained cells (32) and clearly show the boundaries of the sperm head and the remaining elements of the sperm cell.

The aim of the study was to determine the effect of semen staining by three different techniques on the morphometry of sperm heads in stallions.

Material and Methods

The material for the study was semen collected from Polish Halfbred stallions. Twenty individuals from 3 to 4 years of age were selected for the study. At least one ejaculate from each stallion was collected and assessed. The ejaculates were collected by means of the artificial vagina technique at a temperature of about 40-46°C. The semen was kept at room temperature until needed for slide preparation for morphology and morphometry analysis. Slides were prepared within 15 min after collection. Sperm morphology was evaluated using Papanicolaou stain, SpermBlue®, and a complex of eosin and gentian stain. Unstained slides were used as control samples. At first a routine sperm smear was made and allowed to air-dry. For the Papanicolau staining method, the air-dried slides were placed in 96% ethanol for fixation for 15 min and then stained using the routine protocol recommended by WHO (50) (reagents from Sigma Chemical Co., USA). At the end of the procedure, the slides were dehydrated with equal parts of absolute ethanol and xylene, then cleared with xylene alone for 1 min and mounted with DPX medium. The SpermBlue® staining method was carried out as previously described, using a commercially available kit (Microptic S.L., Spain) (48). The slides were placed horizontally on a staining tray and covered with 1 mL of SpermBlue® fixative for 10 min. Then the fixative was gently removed. Immediately afterwards, without washing or drying the slides, 0.5 mL of SpermBlue® stain was applied to each fixed sperm smear for 12-15 min. Care was taken to spread the stain equally across the smear surface. After the stain was removed by gently running it off, the slides were slowly dipped in distilled water (one or two dips lasting for 3 s). Then the slides were left in an upright position to air-dry. Finally, the slides were mounted with DPX medium. All chemicals in this procedure were purchased from Sigma Chemical Company (USA). For the eosin+gentian complex staining method, smears were prepared by careful dragging a drop of fresh sperm across a degreased microscopic slide heated to 37°C (26). The slides were allowed to air-dry for a minimum of 2 h, and were then prepared and preserved in 96% ethanol for 5 min. After 30 min, the preserved slides were washed in distilled water, and then lightly stained with 10% aqueous solution of eosin for 20 to 60 s. The coloured slides were then washed in distilled water and stained with gentian pigment for 3 to 5 min. After staining, the slides were gently rinsed with distilled water for 2 min to remove debris and dried at room temperature. This procedure provided a clean background and thus good contrast against the stained spermatozoa. All reagents used were purchased from Sigma Chemical (Germany). The slides were prepared and assessed at the same time, by the same person, using a microscope. As a control sample, unstained smears were prepared from fresh semen on a microscopic slide heated to 37°C, and then air-dried.

The sperm cells were evaluated with an Olympus BX50 fluorescence microscope and the MultiScan image analysis system and measurement software from Computer Scanning. Phase contrast microscopy was used for evaluation of the unstained smears. Morphometric measurements were performed on 100 randomly selected sperm heads in each ejaculate. The sperm heads were clearly visible in the field of view of the microscope. A total of 8000 sperm heads were evaluated. The sperm heads were analysed, evaluated, and measured at 100× magnification. The length, width, perimeter, and surface area of the sperm head were measured according to the method described by Kondracki *et al.* (26). The frequency of nuclear vacuoles in the sperm head was determined as well. Tygerberg's strict criteria, which most precisely characterise the sperm head, were used in the morphological evaluation of the sperm (Table 1).

The data for the morphometric measurements of the spermatozoa were stored in a database and exported for further statistical analysis. Statistical differences between the samples were tested using Tukey's test (STATISTICA, version 10.0, StatSoft Inc., PL). The level of significance was set at $P \leq 0.01$.

Results

In the case of Papanicolaou staining, the stallion sperm heads took on a pale violet colour (Fig. 1a). The acrosomal part was lighter, gradually becoming darker towards the tail, so the boundary of the acrosome was rather difficult to identify precisely. The outline of the head was sufficiently clear, smooth, and easy to identify. The nuclear vacuoles were clearly visible and easy to identify. The midpiece and tail were pale pink, the end of the tail was difficult to distinguish and the boundary between the midpiece and the tail could not be detected. The background of the smear was light and unstained and did not hinder the evaluation. Staining by the SpermBlue® technique coloured the sperm heads blue. The acrosomal part was lighter. In some cells a clear acrosome boundary could be seen (Fig. 1b). The outline of the head was distinct enough to identify. The vacuoles were also rather transparent and easy to analyse. The boundary of the midpiece and the remainder of the tail were difficult to distinguish; both parts were pale greyish blue. The background of the smear was lighter and did not hinder the analysis. In the case of staining with eosin+gentian complex, the sperm heads were very distinctly coloured dark violet (Fig. 1c). The acrosomal part was somewhat lighter, with a blurred boundary. The outline of the head was very distinct and easy to identify. The vacuoles were visible, but not as clearly as in the case of Papanicolaou and SpermBlue® staining. The midpiece and tail were very well stained with a violet colour, but the boundary of the midpiece was difficult to identify. The background of the smear was lighter and did not hinder the analysis. In contrast, in the case of the control sample – unstained smears analysed under phase contrast microscopy – the sperm heads were pale and the outline in the acrosome part was not entirely distinct (Fig. 1d). The nuclear vacuoles were not well visible. The midpiece and tail were also pale and not well visible. The background of the smear was rather dark and made analysis of the cells more difficult.

Table 1. Formulas used to calculate sperm head morphometry measurements

Variable	Formula
Head length (µm)	L
Head width (µm)	W
Head perimeter (µm)	P
Head area (µm^2)	A
Head ellipticity	L/W
Head elongation	(L-W)/(L+W)
Head roughness	$4\pi(A/P^2)$
Head regularity	π(L*W/4*A)

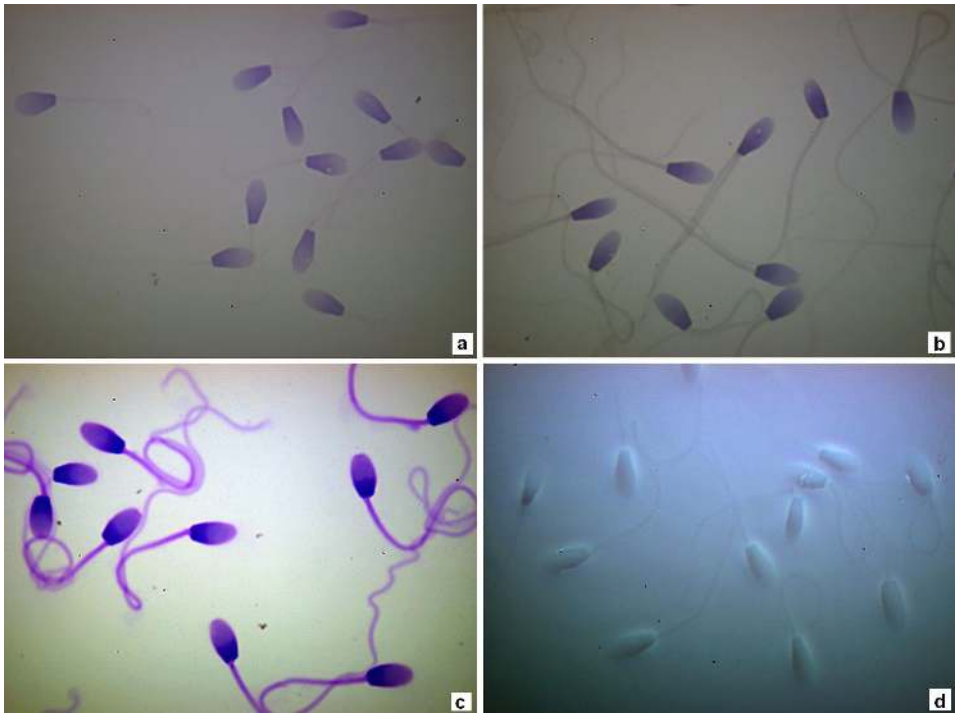

Fig. 1. Stallion sperm head: Papanicolaou staining (a), SpermBlue® staining (b), eosin+gentian complex staining (c), unstained semen – phase contrast (d)

Table 2 presents the data comparing the morphological characteristics of the stallion sperm heads stained with three different techniques, as well as the unstained control slides for comparison. The data show that the staining method, and thus the chemical reagents used, affected the dimensions of the stallion sperm head. The sperm cells had the smallest heads in the case of the Papanicolaou staining. This was the result of the smallest sperm head length, width, surface area, and perimeter. The heads of the sperm stained with Papanicolaou stain were 0.33 μm shorter than the heads of sperm stained with eosin+gentian complex and 0.14 μm shorter than the heads of the sperm stained with SpermBlue® ($P \leq 0.01$). The heads of the sperm stained with Papanicolaou stain were also narrower by 0.17-0.25 μm than the heads of the sperm stained by the other methods ($P \leq 0.01$). The largest sperm head size was observed in the case of staining with eosin+gentian complex; the perimeter of the sperm head was as much as 1.16 μm greater than in the case of Papanicolaou stain and 0.97 μm greater than in the case of staining with SpermBlue® ($P \leq 0.01$). The results were similar for the surface area of the sperm head. The surface area of the sperm heads stained with eosin+gentian complex was the largest, at 18.66 μm^2. This was as much as 2.24 μm^2 greater than the area of sperm heads stained with Papanicolaou stain and 1.06 μm^2 greater than in the case of SpermBlue® ($P \leq 0.01$). In comparison with the control sample of unstained sperm, the data show that each of the staining methods affected the dimensions of the sperm head differently. The width, perimeter, and surface area of the sperm head were all smaller under phase contrast microscopy than in the case of the stained cells. Only the length of the sperm head was greater than when Papanicolaou and SpermBlue® were used.

Table 3 presents parameters taking into account standard sperm head measurements (length, width, perimeter, and surface area) in the form of Tygerberg's strict criteria, which describe the relative shape of the sperm head, allowing the semen to be classified not only morphometrically but also morphologically. The data in Table 3 show that the sperm cells stained by the Papanicolaou method were substantially more elliptical and elongated than the sperm stained with SpermBlue® and eosin+gentian complex ($P \leq 0.01$). The heads of sperm stained with SpermBlue® were characterised by greater roughness, by 0.03 in comparison with Papanicolaou staining and by 0.02 in comparison with eosin+gentian complex ($P \leq 0.01$). Regularity of the shape of the head differed only in the case of staining with eosin+gentian complex, where the indicator was 0.01 lower than in the case of sperm heads stained with Papanicolaou or SpermBlue® ($P \leq 0.01$). As an additional parameter of the morphology of sperm heads, the percentage of sperm heads with nuclear vacuoles was determined. The number of vacuoles in the heads ranged from 0.09% to 0.14%. Vacuoles were best identified by Papanicolaou staining, in which the highest number of them was found. The comparison of indicators characterising the sperm head in the case of each staining method and the control shows that the heads of the sperm that did not undergo staining had a more oval and rounded shape, which was indicated by their greater ellipticity and elongation values. The heads of the control sperm were also more symmetrical, as evidenced by the higher regularity value. The lower roughness value in the unstained cells indicates a lower degree of interference and damage caused by the stains. It is difficult to identify vacuoles using phase contrast microscopy, as evidenced by the lowest percentage of vacuoles observed (0.05%) in comparison with the stained semen.

Table 2. Morphometric variables of the stallion sperm head measured manually with Multiscan software

Morphometric parameter	Papanicolaou	SpermBlue®	Eosin+gentian	Unstained semen - phase contrast
Number of sperm cells	2.000	2.000	2.000	2.000
Head length (μm)	$6.61^{A} \pm 0.40$	$6.75^{B} \pm 0.38$	$6.94^{C} \pm 0.34$	6.85 ± 0.34
Head width (μm)	$3.22^{A} \pm 0.24$	$3.39^{B} \pm 0.26$	$3.47^{C} \pm 0.23$	3.06 ± 0.23
Head perimeter (μm)	$19.95^{A} \pm 1.19$	$20.14^{B} \pm 1.18$	$21.11^{C} \pm 1.15$	19.91 ± 0.75
Head area (μm^2)	$16.42^{A} \pm 1.50$	$17.60^{B} \pm 1.69$	$18.66^{C} \pm 1.66$	15.90 ± 1.47

Different superscripts designate significant differences between means within rows; upper-case letters: $P \leq 0.01$.

Table 3. Sperm morphology according to Tygerberg's strict criteria and the frequency of nuclear vacuoles in the stallion sperm

Morphometric parameter	Papanicolaou	SpermBlue®	Eosin+gentian	Unstained semen - phase contrast
Number of sperm cells	2.000	2.000	2.000	2.000
Ellipticity	$2.06^{A} \pm 0.18$	$2.00^{B} \pm 0.18$	$2.01^{B} \pm 0.14$	2.25 ± 0.20
Elongation	$0.34^{A} \pm 0.04$	$0.33^{B} \pm 0.04$	$0.33^{B} \pm 0.03$	0.38 ± 0.04
Roughness	$0.52^{A} \pm 0.04$	$0.55^{B} \pm 0.05$	$0.53^{C} \pm 0.04$	0.50 ± 0.03
Regularity	$1.02^{A} \pm 0.03$	$1.02^{A} \pm 0.05$	$1.01^{B} \pm 0.03$	1.04 ± 0.03
Nuclear vacuoles (%)	$0.14^{A} \pm 0.63$	$0.09^{B} \pm 0.44$	$0.10^{B} \pm 0.45$	0.05 ± 0.22

Different superscripts designate significant differences between means within rows; upper-case letters: $P \leq 0.01$.

Discussion

The lack of standardisation in the preparation, assessment, and use of a suitable staining technique necessitates the selection or development of one that will minimally alter semen parameters, thereby improving the accuracy of diagnosis. An additional difficulty in diagnosing fertility is the fact that microscopic analysis of sperm shows that sperm morphology is highly heterogeneous, as sperm cells with many different shapes, sizes, and forms are observed in one ejaculate (5, 16). This has led scientists to identify and describe the morphological characteristics of well-shaped sperm cells. According to Vaissaire (47), the dimensions of the sperm head in the stallion are normal when its length is 6-7 µm, its width about 3-4 µm, and its thickness about 2 µm. A well-formed sperm head should be oval with a smooth surface. In this study the dimensions fell within this range in the case of all staining techniques applied. The neck of the stallion sperm should be about 0.5 µm in length (47). The midpiece, containing a certain number of mitochondria, should be uniform (37), without defects or folds. It should be about 8-10 µm long (47), and its length should be about 1.5 times the head length. The tail should be straight, uniform, and thinner than the midpiece, and its length should be 41-42 µm in the stallion (47).

In the case of human semen analysis, the criteria established by WHO are applied, and for detailed clinical diagnostics Tygerberg's strict criteria are used as well (50). In contrast with standard assessment, Tygerberg's strict criteria specify abnormalities in the sperm head in a more rigorous manner (28, 35). Sperm morphology additionally evaluated using Tygerberg's strict criteria is a good biomarker of sperm dysfunction specifying the cause of male infertility (8). This is confirmed by the positive correlation found between fertilisation capacity in humans and the percentage of sperm with well-formed heads (10). Correct classification and quantitative determination of a specific defect in sperm structure can provide valuable information on the potential fertility of the stallion and may be helpful in diagnosing and prognosticating fertility problems (7). Not only abnormalities in sperm morphology were shown to be important for migration of sperm to the ovum cell and at the site of fertilisation, but their DNA quality is also significant, as it affects to some extent maintenance of pregnancy (33). It is therefore important during assessment of sperm morphology to choose a staining technique allowing the most accurate possible determination of male fertility potential.

The Papanicolaou method is a commonly used technique for staining semen in andrological laboratories and clinics treating infertility in humans. This technique is regarded as very reliable, but is also time-consuming due to the multiple stages of staining. It does, however, enable identification of the acrosome, cytoplasmic drop, midpiece, and tail (29). Observations by Brito *et al.* (6) indicate that despite WHO's recommendation of the Papanicolaou method for assessment of human semen, it does not produce the desired results in the case of stallion semen. Difficulties in interpretation of smears have resulted from insufficient colouring of cells, which has made it difficult to identify more subtle sperm defects. This was confirmed in the present study, particularly in the case of the midpiece and tail, which were not well visible on the slide. A much simpler and faster staining procedure, SpermBlue®, which also identifies individual structures in the sperm cell, has been relatively recently introduced to the market. There are studies suggesting that this method is more effective than the complicated Papanicolaou staining method (48).

In the present study, the smears stained with SpermBlue® were characterised by fairly well coloured sperm heads, but as in the case of the Papanicolaou stain, the analysis of the midpiece and tail was hindered by the less intense colouring of these structures. A very simple technique that has been used for many years to evaluate mammalian semen is staining with a complex of eosin and gentian stain (26, 27). This method stains sperm heads very distinctly but makes observation of the boundary of the acrosome or the midpiece difficult, which was confirmed in the present study. Hence each of these methods has its advantages and disadvantages. The use of each of the stains has specific consequences in terms of the possibility of assessing individual structures in gametes (19). Although some studies indicate that alternative staining techniques are effective, others report that there are significant discrepancies in differentiation, intensity, and contrast, and more importantly, in the size and shape of the spermatozoa, and each of these characteristics can affect the final result of the morphological assessment in prognostication of male fertility (50). This is due to the effect of the various chemical reagents on the stained sex cells, as the stains can cause the sperm head to shrink or swell. These changes do not take place in a uniform manner, as one staining technique may cause a greater increase in the width of the sperm head, while another may increase its length. The Society for Theriogenology (SFT) recommends the use of wet-mount stallion semen preparations and phase contrast microscopy (25).

The comparison of the dimensions of unstained sperm and stained samples in the present study suggests that the most objective result is obtained in the case of staining with SpermBlue® and eosin+gentian complex, which seem to exert the least effect on the length of the sperm head. The width of the sperm head was increased by each of the staining methods in varying degrees.

Some studies have compared the evaluation of the shape of the heads of sperm stained by various methods according to Tygerberg's strict criteria (32, 33).The four software-calculated indices, *i.e.* ellipticity,

elongation, roughness, and regularity, take the standard morphometric measurements and give an indication of the sperm head shape into account. Ellipticity indicates whether the sperm head is thin or tapered. If the value for head ellipticity is high, this means that the sperm head is thin. Elongation indicates the roundness of the sperm head, where the closer to zero value, the rounder the head. Low values for the roughness index indicate amorphous or irregular heads, while the regularity index indicates pyriform sperm heads. In the present study, the sperm stained with Papanicolaou stain had substantially more oval heads than the sperm stained with SpermBlue® and eosin+gentian complex. However, the most oval and elongated shape of the sperm head was observed in the unstained control sample. When these criteria are applied to the results of the present study, sperm heads appear to be more symmetrical in the case of staining with Papanicolaou and SpermBlue®, which are more often used for human semen, than in the case of eosin+gentian complex, while the greatest symmetry of the sperm head was observed in the semen that did not undergo any staining.

An additional element of evaluation of the morphology of sperm heads is the presence of vacuoles, which are considered to be a defect in sperm structure (49). Vacuoles in the sperm head may be associated with DNA fragmentation (38) or abnormal chromatin condensation (13). However, the origin and exact cause of the emergence of vacuoles are still the subject of many studies (13, 38). Nuclear vacuoles have been described as a crater defect in the spermatozoa of stallions (23). Vacuoles are believed to arise during spermatogenesis and can already be present in the spermatids (24). It is also thought that the presence of vacuoles in human sperm heads can have a negative effect on fertilisation, and thus on the quality of embryos (31), as well as the later stages in the development of the zygote in ICSI cycles (12). For identification of vacuoles, a modification of Papanicolaou staining is recommended (39), which enables observation of pale blue spots in the acrosomal region and dark blue ones in the post-acrosomal region of the sperm head (50). Held *et al.* (20) report that in a 9-year-old infertile Arabian stallion 75% of sperm heads were observed to be abnormal, including 57% with one or more vacuoles. Although the presence of vacuoles in the sperm head is associated with abnormal semen morphology, in a study by Park *et al.* (39) the human semen with better morphology had more vacuoles in the sperm heads than the semen with a greater percentage of abnormal spermatozoa. This suggests that the presence of vacuoles in the sperm head may be a normal characteristic of its morphological structure (39). Scientists also presume that the presence of vacuoles in the acrosomal region may indicate the migration of limited amounts of acrosin to the sperm surface. This may be the beginning of the acrosomal reaction (10). A study by Brito *et al.* (6) indicates that wet-mount stallion semen preparations and the use of phase contrast microscopy facilitate observation of nuclear vacuoles, which is evidenced by the increased frequency of this defect in comparison with stained slides. This is not confirmed by the results of the present study, as in the unstained control sample the percentage of nuclear vacuoles observed was clearly the lowest, which indicates that they were more difficult to identify. Some authors suggest that the preparation of wet mounts can cause artefacts. This is unlikely, as in this case there is a considerably less interference with cell structures. However, this method does not affect all elements of sperm structure in the same way; for example, while a smaller percentage of sperm head abnormalities was observed in wet mounts, there were more sperm cells with folded tails than in the case of differential (eosin-nigrosin) and Papanicolaou staining (39). Such observations of differences in the occurrence of particular defects when different staining techniques are used have been made by many authors (2, 15). The discrepancies in the reaction of semen to the stains used in preparing slides may result from the interspecies differences in the resistance of semen to the effects of external factors (6, 50). The differences in the dimensions of the sperm head may also be determined by the structure and arrangement of microfibres occurring in it. The cytoskeleton of the sperm head consists of nuclear proteins and the nuclear membrane, which are partly responsible for nuclear shaping. Depending on the fixatives used and the stain applied, changes may occur in the arrangement of actin fibres in the sperm head (9). For some species of mammals classifications have been developed regarding sperm morphology (50). In the case of stallion semen, however, there are few studies dealing with this subject. One study on the morphology of stallion sperm stained by the differential method (eosin-nigrosin) was conducted by Brito (6). Hence an important factor that should be taken into account in selecting a staining technique is comparison of how the staining procedure changes the morphology and dimensions of the sperm cells with respect to cells in unstained semen (33). Factors affecting sperm dimensions include the osmolality and tonicity of the surrounding medium (33). The phenomenon of changes in sperm dimensions can also be observed during cryopreservation of semen. Preservation of semen at low temperatures has been found to decrease the dimensions of sperm heads in the semen of humans (45), stallions (3), bulls (41), boars (17), and goats (22). Sperm placed in hypotonic solutions will have a tendency to swell, while in hyperosmotic solutions they will shrink. Esteso *et al.* (11) found that the surface of sperm heads from the epididymis of red deer and the elongation (length to width) coefficient are statistically different in ejaculates with high and low cryo-resistance. The effect of the size and shape of the sperm head on semen quality after thawing has been shown in other studies as well (22,

33). It is obvious that any reagent used, whether in preservation processes or in semen staining, may cause changes in sperm dimensions, and the more of these compounds are applied, the greater the likelihood of interference and cell damage. Papanicolaou staining uses over 12 different chemical substances, whose effect has not yet been precisely determined (32). Some substances inducing dehydration, such as xylene and alcohol, cause germ cells to shrink, and fixatives induce changes in the dimensions of sperm heads (16, 43). Research by Aksoy *et al.* (1) has confirmed that the staining method affects the size of the sperm head (length and width). Other studies show that some staining techniques lead to an increase or decrease in the dimensions of the sperm head (14). A comparative study by Mc Alister (33) showed that the dimensions of human sperm stained with SpermBlue® were the most similar to the dimensions of sperm in unstained smears. In a study by van der Horst and Maree (48), when SpermBlue® staining was used, no clear signs of swelling were observed in human sperm cells or those of various animal species. This is a fast method consisting of a simple fixation and staining procedure (48). An additional advantage of sperm staining with SpermBlue® is that the background is not coloured, which might mask some boundaries in the sperm cells, hindering their analysis (48). This was confirmed by our observations. Other studies have found that staining with Rapidiff® caused excessive swelling in terms of the width of the sperm head, while Papanicolaou stain caused the length of the head to decrease in comparison with the sperm in unstained smears (33). Henkel *et al.* (21) also drew attention to the fact that the morphometric values obtained using different staining techniques differ, which should be taken into account in determining the values for normal sperm in individual laboratories.

To sum up, the differences in the dimensions of the sperm head when different staining techniques are used may be the result of the fixatives and chemical reagents used in the staining. The results obtained indicate that in the case of staining with SpermBlue® and with eosin+gentian complex, the morphometry of the sperm head seems to be most similar to that observed in unstained smears. It also seems that neither shrinking nor swelling is uniform in the various staining techniques. For example, Papanicolaou staining causes the length of the sperm head to shrink, but not its width. Moreover, it appears that in comparison with unstained sperm, all methods cause the width of the head to increase due to swelling. These observations lead to the conclusion that it is very important to determine the natural size of the sperm head for each staining technique so that accurate assessment and classification can be made in male fertility diagnosis. It is also very important to select an appropriate staining technique for a given animal species, as research by many authors indicates that some methods that work well for one species are not suitable for the analysis of another one.

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