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THE ROLE OF STAINING TECHNIQUES IN SEMINOLOGICAL ANALYSIS OF MAMMALIAN SEMEN

ROLA TECHNIK BARWIENIA W OCENIE SEMINOLOGICZNEJ NASIENIA SSAKÓW

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Streszczenie. Najważniejszymi parametrami oceny nasienia są koncentracja, ruchliwość i morfologia plemników. Morfologia plemników jest uznawana za najbardziej wiarygodny parametr w przewidywaniu płodności samców. Problemem w ocenie morfologii i morfometrii nasienia jest brak standaryzacji w odniesieniu do stosowanych technik barwienia. Procedura barwienia oraz zastosowane odczynniki mogą w istotny sposób wpływać na wartość parametrów morfometrycznych plemnika. Stosowanie barwników o różnym pH, różnej osmolarności oraz czas trwania procedury mogą wpływać na kształt i rozmiar plemników, a tym samym na wynik oceny morfologicznej nasienia. Konieczne jest opracowanie procedury oceny morfologii i morfometrii plemników, która w minimalnym stopniu zmienia strukturę nasienia ocenianego w stosunku do nasienia natywnego.

Key words: morphology, morphometry, semen, spermatozoa, staining techniques.
Słowa kluczowe: morfologia, morfometria, nasienie, plemnik, techniki barwienia.

INTRODUCTION

The most important parameters of semen analysis are sperm concentration, motility and morphology. Sperm morphology is probably the best source of information on male fertility. Therefore, studies on sperm morphology for *in vitro* and *in vivo* fertilization are developing very intensively (Nikolettos et al. 1999; Buendia et al. 2002; McAlister 2010). Spermatozoa are a unique type of cells, since – unlike other mammalian tissue-specific cells – sperm cells occur in a variety of sizes and shapes (Gage 1998; Gage and Freckleton 2003). Over many years of sperm research it has been observed that the morphology of sperm is varied, even within the same species. The same ejaculate contains sperm cells of various shapes, sizes and forms. Therefore, sperm morphology determination needs standardization of the sperm that are considered normal. Reference values developed for each species would be a helpful

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tool in diagnosing sperm defects and, consequently, fertility disorders. Microscopic evaluation of sperm morphology is a relatively simple and inexpensive method, yet capable of producing results similar to those obtained with more sophisticated and expensive systems (Buendia et al. 2002). So far, not all animal species have been assigned the criteria of various sperm forms considered as morphologically altered. The criteria have been compiled for bovine semen (Blom 1981, 1983; Rosłanowski 1987). This classification is also frequently used for porcine sperm assessment (Kondracki et al. 2006). A slightly different classification of sperm abnormalities was compiled for stallion semen (Kosiniak-Kamysz and Wierzbowski 2004). Also major sperm abnormalities were defined for domestic birds (Chełmońska and Dymkowska 1993).

Two basic sperm morphology categorization systems have been developed for human sperm. One was developed by the World Health Organization (WHO) and, although developed for human sperm, it is applied for animal semen analysis as well. Another system is the Tygerberg categorization defining much more strict criteria, primarily in relation to the head of the sperm, in which the border forms are considered abnormal (Kruger et al. 2004). An example of the differences between the two systems is the fact is that the WHO indicates teratozoospermia if the percentage of normal sperm is lower than 30%, whereas under Tygerberg criteria this threshold is reduced to 14%. This threshold was determined in IVF--related studies (McAlister 2010). Sperm morphology categorization according to Tygerberg strict criteria is also used in assisted reproduction technologies, identification of biomarkers of sperm dysfunction, and predicting male fertility potential. Numerous studies have shown that sperm morphology varies greatly depending on the reproductive capacity of the male. There is a positive correlation between the percentage of morphologically normal sperm and fertility. This enables the identification of sperm morphological abnormalities in a larger group of breeding males that reveal conception problems, particularly if the semen is evaluated according to strict Tygerberg criteria (Tasdemir et al. 2002; McAlister 2010).

Morphologically abnormal sperm has probably no chance to cover the distance to the oocyte and, as a result, has no fertilizing ability (Tasdemir et al. 2002). This is confirmed by studies of Kazerooni et al. (2009) on the correlation between the percentage of normal sperm and their motility in the semen. Some studies show that the *zona pellucida* of the ovum is able to distinguish between normal and abnormal sperm, and also recognizes sperm with a relatively lowest amount of cytoplasm (Parinaud et al. 1996; McAlister 2010).

Morphology also involves sperm morphometry, which is also an important determinant of the male reproductive capacity (Gosh et al. 2010). According to clinical studies, sperm of infertile men have a head of larger dimensions. Also the ratio of length to width of the sperm head was higher in men with fertility problems compared to fertile men (Katz et al. 1986). The results of observations of human spermatozoa correspond with the data revealed in animal studies. Many authors seek relationships between sperm morphometry and male fertility (Casey et al. 1997; Chan et al. 1999; Hirai et al. 2001; Esteso et al. 2006; Nunez-Martinez et al. 2007). A considerable difference in sperm head sizes between fertile and infertile males was found in different species (Katz et al.1986; Casey et al. 1997; Antończyk et al. 2012). Males with the semen that had smaller sperm heads were more fertile. The study revealed that the head size was not the only factor affecting the efficacy of fertilization; the dimensions

of the midpiece and the tail were also important. The sperm with longer tails have greater possibility of fertilization due to the increased motor abilities (Antończyk 2012). Information on sperm morphometry extends the knowledge on the actual ability of sperm to fertilize *in vitro* and *in vivo*, and also allows determination of the suitability of the semen to cryopreservation (Aitken et al.1985; Jeulin et al. 1986; Hirano et al. 2001; Antończyk 2012). The normal structure of the acrosome ensures the success and the proper sequence of the steps in the process known as the acrosome reaction, in which hydrolytic enzymes are activated to allow the sperm to bind to the glycoproteins of the *zona pellucida* (Grøndahl et al. 1994; Nikolettos et al. 1999). It has been demonstrated that evaluation of acrosome integrity allows better prediction of fertilization (Menkveld et al. 2003; McAlister 2010; Menkveld et al. 2011). Namely, a significant correlation has been found between the percentage of sperm with an intact acrosome and the effectiveness of *in vitro* fertilization (Ozguner et al. 2009).

SELECTED SEMEN ANALYSIS TECHNIQUES

Semen analysis can be carried out by many microscopic methods, using the simplest light microscopes or sophisticated techniques like fluorescence, electron, or scanning microscopes, or flow cytometry (Ramalho-Santos et al. 2007). Evaluation of sperm morphology involves various staining techniques. Most staining methods used for sperm morphology are suitable for light microscopy. The quest for the best method of semen analysis produced a number of sperm staining techniques. None of them, however, is an error-free method, which pertains primarily to result interpretation. The differences in the results of the assessment – which are partly due to differences in the preparation of the material, its fixation, staining technique used, temperature during the test, and the quality of the equipment – can be as high as 30–60% (Iguer-Ouada 2001; Rijsselaere et al. 2004, 2007). The problem in the evaluation of sperm morphology and morphometry is the lack of standardization of the staining techniques. Preferences vary when it comes to sperm of different species. According to the guidelines issued by the Society for Theriogenology (SFT), stallion sperm morphology should be performed in wet, unstained smears using the phase contrast microscopy (Kenney et al. 1983). However, veterinary laboratories often do not possess such microscopes and staining of stallion semen is carried out using various techniques, often those recommended for other species. An example is the eosin-nigrosin stain recommended by the SFT for bovine sperm (Chenoweth et al. 1992) or the Papanicolaou staining technique recommended for human sperm analysis by the World Health Organization (WHO 2010).

One of the simplest methods of preparing semen smears for analysis is India ink dyeing. As a result of this method, the uncoloured sperm are clearly seen against the black background. The morphology of the sperm can be easily determined under an optical microscope (Ramalho-Santos et al. 2007). Other methods of sperm morphology assessment use various types of dyes and reagents. The Diff-Quik staining kit, approved by WHO for determining human sperm quality, is quite easy to use. The kit consists of a fixative reagent, usually methanol, and an acidic dye that stains basic sperm proteins red (Ramalho-Santos et al. 2007; McAlister 2010). Another simple method of assessing semen is negative staining,

in which the background is coloured, rather than the objects. This is done using acidic dyes, whose negative ions avoid the cell walls. The dyes are black technical ink and 10% solution of nigrosin or 10% solution of opal blue. Negative staining can be used to distinguish immature sperm with protoplasmic droplets. Staining is used particularly for frozen-thawed semen samples. The live sperm acrosome is visible on the head as a glowing band, whereas the dead spermatozoa have a blurry outline of the front part of the head (Bielański 1977). Differential staining is a common method of semen smear preparation for analysis. This technique is used to monitor sperm of almost all species (O'Connell et al. 2002; Łukaszewicz et al. 2008). Differential staining is a live-cell staining method. It enables the identification of live and dead sperm. The head of a live sperm is uncoloured, whereas a dead sperm head is stained pink (Fig. 1).

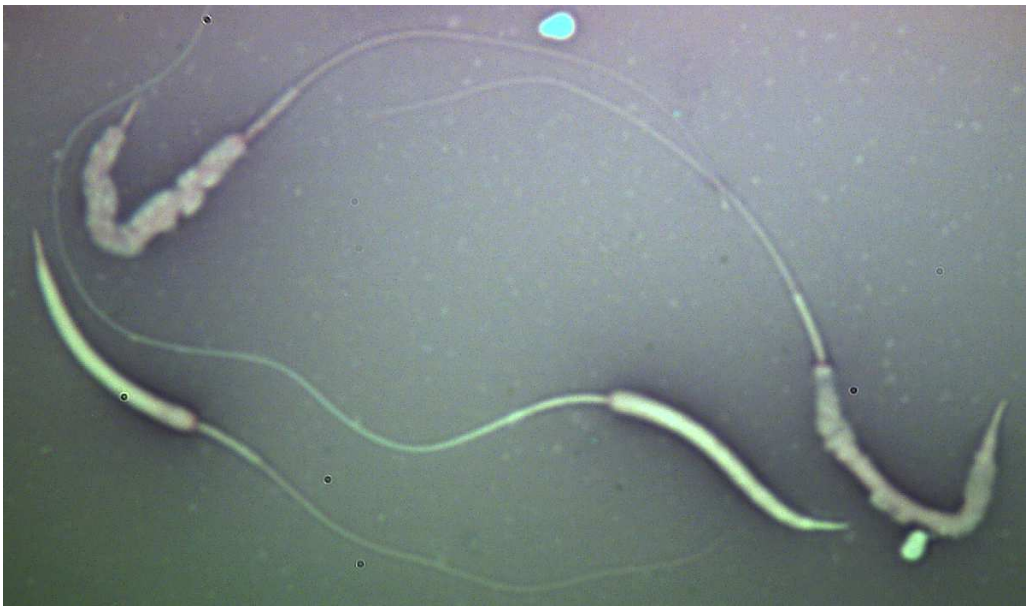

Fig. 1. Rooster spermatozoa – eosin + nigrosin complex staining
Ryc. 1. Plemniki koguta – barwienie kompleksem eozyna + nigrozyna

This staining method produces the least artefacts. The dye used in this technique is the eosin-nigrosin stain (Bielański 1977). Another technique uses nigrosin alone. This method is ideal for evaluating thawed semen and it reveals changes in the acrosome. Normal sperm are recognized by a bright band visible around the anterior part of the cell. A damaged acrosome, on the other hand, is visible as a bright part of the head with a flattened frontal edge (Bielański 1977). In routine tests of sperm morphology, the Animal Breeding and Insemination Centres (SHiUZ) in Poland most commonly use the eosin-gentian stain, which is an acidic dye, recommended for bull semen, but also used in other species (Blom 1981; Kondracki et al. 2005; Banaszewska et al. 2015) – Fig. 2.

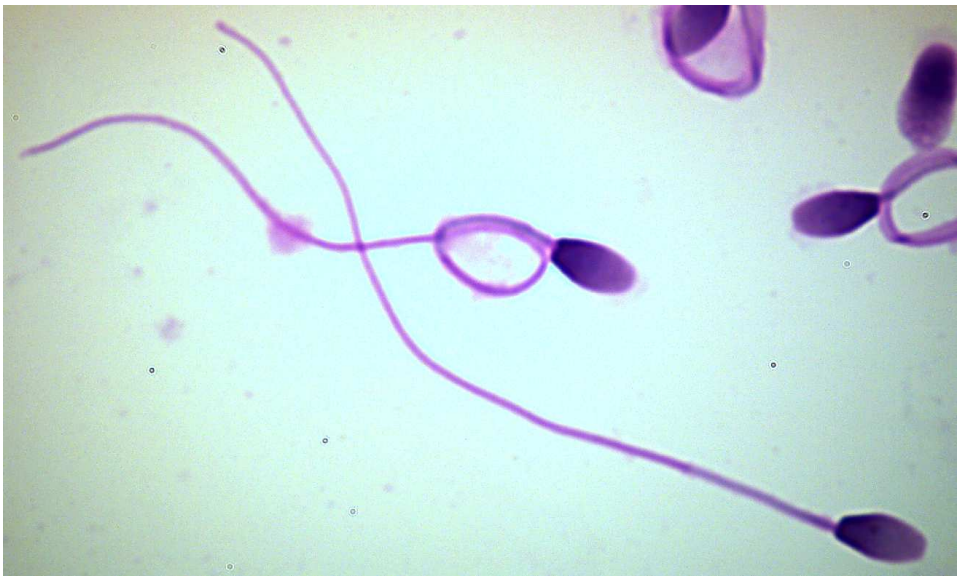

Fig. 2. Stallion spermatozoa – eosin + gentian complex staining
Ryc. 2. Plemniki ogiera – barwienie kompleksem eozyna + barwnik gencjanowy

The most common method used in human sperm analysis is Papanicolaou staining (PAP) – Fig 3.

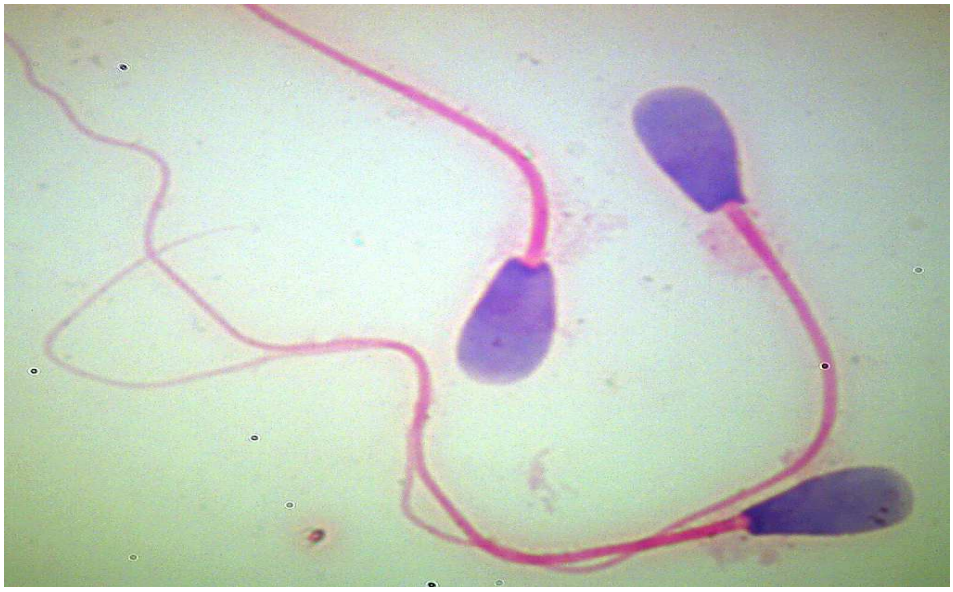

Fig. 3. Bull spermatozoa – Papanicolaou staining
Ryc. 3. Plemniki buhaja – barwienie Papanicolaou

The Papanicolaou stain is recommended by the WHO and widely used in andrology laboratories and fertility clinics. Staining takes several steps and consists in immersing the slide in a series of reagents in 20 consecutive steps (varying concentrations of alcohol and dyes). Ethanol and xylene are used, which are hyperosmotic and may cause contraction of the sperm head (Maree et al. 2010). Papanicolaou staining allows identification of the acrosome and post-acrosome regions within the sperm head, cytoplasmic droplets, midpiece

and tail (WHO 1999). The nuclei are stained intensively blue, and the cytoplasm in various shades of purple. The disadvantage of this method is its time-consuming character and an impact of many chemicals used during the staining, which can affect primarily sperm morphometric dimensions (Kellogg et al.1996). Another staining method used for human semen is Rapidiff® (RD). It is a quick and simple technique. The procedure was introduced by Kruger et al. (1987), since it turned out that it was comparable with the Papanicolaou staining method (Maree et al. 2010). A drawback of this method is a strong background coloration, which can hamper the analysis (Henkel et al. 2008). In addition, differences in the osmotic pressure of sperm may result in a significant number of swollen heads detected by Rapidiff® (Maree et al. 2010).

SpermBlue® (SB) is in turn a staining technique used for both human and animal semen analysis (Fig. 4).

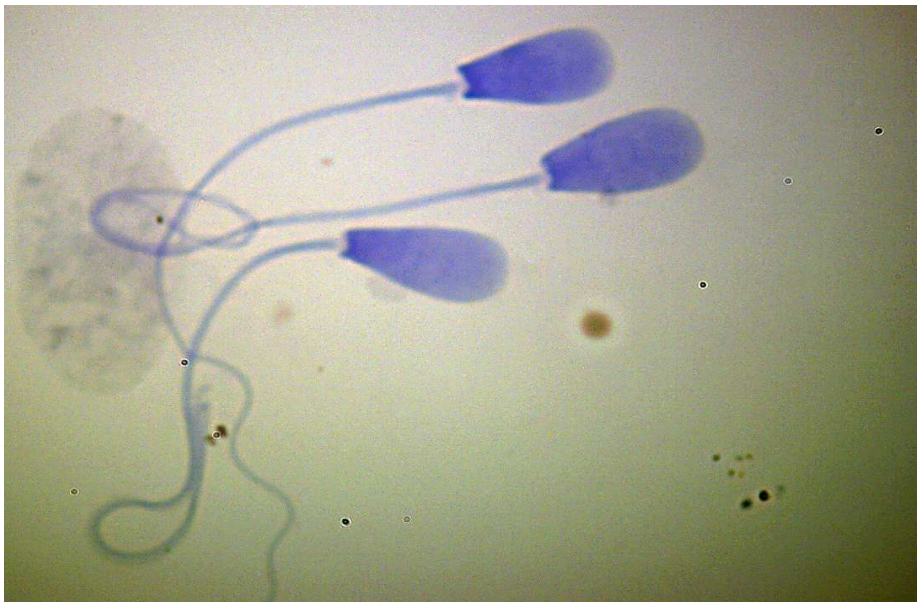

Fig. 4. Bull spermatozoa – SpermBlue® staining
Ryc. 4. Plemniki buhaja – barwienie SpermBlue®

No negative effects on the size of human spermatozoa have been found in relation to this method of staining, which is probably caused by substances that are isoosmotic in relation to semen. SB is a quick and easy two-step procedure, comparable in efficacy to the Papanicolaou technique. Unlike RD and Papanicolaou, SB staining has a positive effect on acrosome staining efficiency, and all the structures of the sperm are stained in various shades of blue. SpermBlue® is recommended for both fresh and frozen semen. Research conducted by van der Horst and Maree (2009) suggests that this technique of staining yields better results than the Papanicolaou staining and other techniques. It has also been found that sperm stained with SpermBlue® showed morphometric values most similar to the results in the fresh, unstained semen (McAlister 2010).

In addition to these methods of semen analysis, fluorescent techniques are also used. Fluorescent staining is performed using fluorochromes. For viewing, a microscope with

a fluorescent attachment or a flow cytometer can be used. Semen analysis using flow cytometry consists in performing multiple measurements within a short time, which give a precise and accurate results (Klimowicz et al. 2005). Fluorochromes enable, among others, evaluation of metabolic activity of the spermatozoa, acrosomes, and capacitation, or assessment of cell membranes.

The potential of the mitochondria located in the midpiece of the sperm can be determined by Rhodamine 123 and JC-1 fluorochrome. JC-1 accumulates in the mitochondria; orange shows a high membrane potential, low is marked in green, and moderate in green-orange. To evaluate the mitochondrial transmembrane potential MitoTracker Green dye can be used. It labels mitochondria green and enables detection of sperm structure diversity (Ramalho--Santos et al. 2007).

The acrosomal status can be determined by a combination of fluorescent dye and lectin. Due to its high sensitivity, the test is increasingly being used in staining equine, porcine, and canine semen. The most frequently used fluorescent dyes linked with lectin include phycoerythrin, fluorescein isothiocyanate, AlexaFluor®, and FITC-PSA (Antończyk 2012). The lectin – FITC-PSA complex is used to monitor the acrosome matrix mainly in humans and horses. As a result of labelling, the acrosomal part of the sperm becomes green with a fluorescent band on the sperm head. Semen of other species may require a different lectin (Ramalho-Santos et al. 2007).

Dyes used to assess sperm membrane integrity are nucleic acid fluorescent stains, which do not penetrate the intact cell membrane (Bochenek and Smorąg 2007). These include Hoechst 33258 dye, propidium iodide (PI), ethidium bromide (EB), carboxyfluorescein diacetate, or SYBR-14 (Pintado et al. 2000; Bochenek and Smorąg 2007; Hossain et al. 2011; Antończyk 2012). These compounds are hydrolysed after penetrating living cells with the intact cell membrane. Under such a type of staining, only cells with a damaged membrane become fluorescent. Propidium iodide allows visualising plasmalemma damage by staining the sperm head red (Niżański and Klimowicz 2005; Hossain et al. 2011). Hoechst 33258 allows detection of both dead and living sperm in the semen. Stained are cells with a damaged cell membrane. This allows an easy and quick assessment of the number of dead sperm in the semen (Niżański and Klimowicz 2005; Niżański et al. 2006; Hossain et al. 2011). Fluorochrome SYBR-14 is most commonly used with propidium iodide and allows detection of normal sperm plasmalemma. The sperm with an intact cell membrane are coloured green, and those with a damaged plasmalemma – red (Niżański and Klimowicz 2005). This staining technique can be used to identify live, dead, and dying cells (Klimowicz et al. 2005). The effectiveness of SYBR-14 have been confirmed by Bolaños et al. (2012), who carried out observations on semen of stallions, determining the condition of sperm morphology and their ability to survive freezing. SYBR-14 staining is also used to assess cell membrane of human, boar, ram, dog, mice, birds and fish (Garner and Johnson 1995; Flajshans et al. 2004; Antończyk 2012). Another stain, Hoechst 33342, allows detection of such semen elements as somatic cells – in too concentrated or inappropriate diluents – or cell fragments, which may affect the results of the evaluation. Due to haploid chromatin, sperm fluorescence is very intensive, while somatic cells exhibit fluorescence that is suppressed (Niżański et al. 2006).

Some of the dyes allow the evaluation of sperm chromatin structure. Basic dyes for monitoring the normality of the chromatin include aniline blue, chromomycin A3, and acridine orange. Aniline blue (Fig. 5). allows identification of sperm with excessive content of histones in the chromatin. Abnormal sperm are stained dark blue, while normal remain light blue.

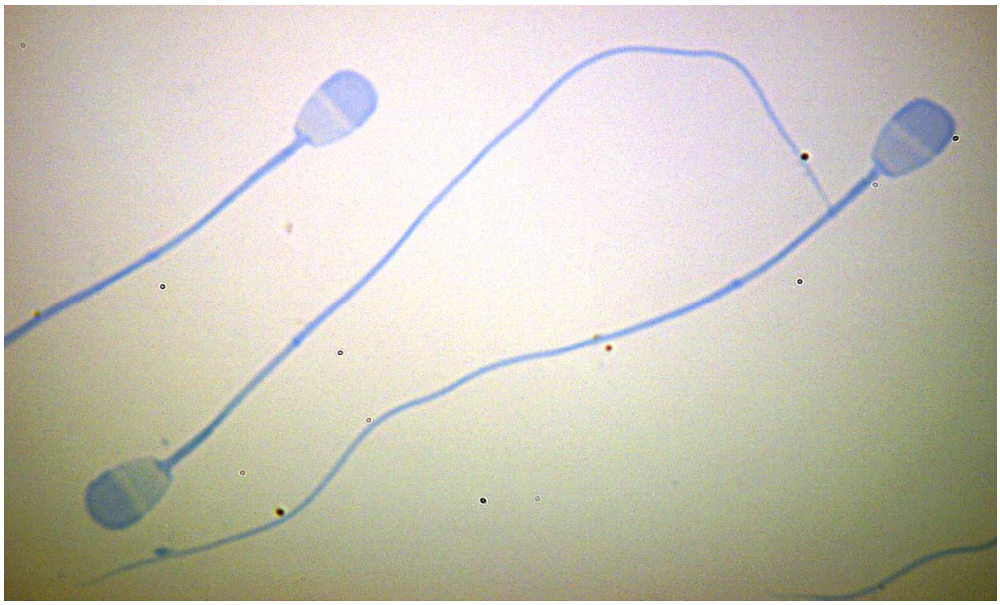

Fig. 5. Fox spermatozoa – aniline blue staining; normal spermatozoa
Ryc. 5. Plemniki lisa – barwienie aniliną blue; plemniki prawidłowe

Chromomycin is a fluorochrome used for the detection of sperm with impaired chromatin condensation caused by improper replacement of histones by protamines during spermatogenesis (Fig. 6).

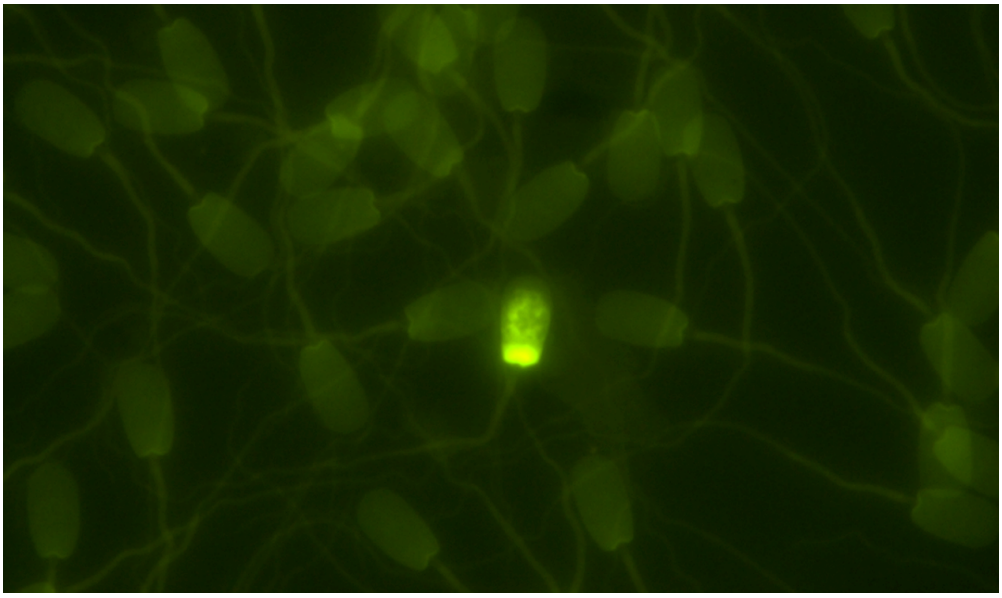

Fig. 6. Boar spermatozoa – chromomycin A3 staining
Ryc. 6. Plemniki knura – barwienie chromomycyną

Evaluation of protamination in sperm cell nuclei allows observing whether their genetic material is well organized. Its improper organization can lead to structural disturbances within the genetic material and to sperm malfunctioning, and – in consequence – problems with fertilization (Andraszek et al. 2014). Chromomycin stained normal sperm have light green colour, while damaged ones are characterized by an intensive green fluorescence. Acridine orange is used in the studies of sperm DNA stability. The fluorochrome in combination with native DNA emits green fluorescence, and in combination with damaged, single-stranded DNA, the label glows orange (Kellogg et al.1996; Andraszek et al. 2014).

Increasingly, sperm morphology determination is performed using computerized systems. Computer-assisted sperm analysis (CASA) goes beyond conventional methods of sperm analysis. The systems find application in male infertility treatment centres, but also in those dealing with animal reproduction (Niżański and Klimowicz 2005). This approach allows eliminating – to a large extent – the human factor in the evaluation process of sperm morphology. The analysis is done automatically. Infertility treatment centres use this system to test the semen for fertilizing ability. CASA applications in the centres for animal breeding and veterinary research institutes include testing of the effects of medicines and evaluation of semen quality and its fertilizing ability (Klimowicz et al. 2005; Niżański et al. 2006). CASA is very fast, give objective results and needs relatively little labour to assess the sperm morphology. The software also allows analysis of large batches of sperm, reducing the risk of error, and also enables a very detailed analysis of ejaculate. Despite a relatively complicated process of machine preparation and settings changes for different animal species, the system is very efficient, hence reproducible, high-precision evaluation results are attained (Niżański et al. 2006). However, the system may also be error-prone, which in this case can result from the automation of the analysis process. The main problem is that the use of different staining techniques for a particular material or type of analysis can affect the outcome of the number of morphologically normal sperm and cause discrepancies regarding their dimensions. In such circumstances a male can in one laboratory be classified as an individual with normal sperm morphology, whereas in another lab – as one with a fertility disorder (Gago et al. 1998). Comparing the results of semen analysis from laboratories that use different analysis techniques poses a particular difficulty for doctors of human medicine and veterinarians (McAlister 2010). Although some studies suggest that alternative staining techniques are effective and provide accurate results, other reports demonstrate considerable differences between staining methods with respect to staining intensity and contrast, but also, more importantly, with respect to the size and shape of the sperm. Each of these parameters can have a significant impact on the results of the assessment of morphology (Coetzee et al. 2001). The subtle differences in evaluated smears are particularly problematic with fertility analyses in the cases where the values of the sperm morphology vary within the reference range (Kruger et al. 1987).

Morphometric evaluation of sperm involves comparing certain characteristics of the structure with the reference minimum and maximum values. It defines the following parameters: length and width of the head, its surface area and degree of elongation, and the length of the tail. Normal sperm are determined by strict criteria and cannot deviate beyond the adopted limits. There should also be no protoplasmic droplets in either the proximal or distal locations, or defects of the flagellum (WHO 1999).

A comparison of the results of the Papanicolaou and DiffQuik staining by two independent laboratories showed no significant differences in the morphology of sperm stained with the two methods (Kruger et al. 1987). The analyses carried out in another laboratory revealed discrepancies in sperm morphology results between the DiffQuik and Papanicolaou stains (Henkel et al. 2008). Some studies have shown that DiffQuik causes significant swelling of the sperm and excessive background coloration of the slide, which can impede the analysis of sperm (Maree et al. 2010; WHO 1999, 2010). Despite these reports DiffQuik is still considered a valid staining technique in the evaluation of human sperm morphology (McAlister 2010; WHO 2010). To stain the semen of ganders (Chełmońska 1972) and roosters (Lukaszewicz 1988), some authors used the method according to Blom (1981), dedicated mainly for bull semen. It was established, however, that the heads of spermatozoa stained this way tended to swell, which disqualifies the method from poultry sperm analysis (Łukaszewicz et al. 2008).

A variety in the interpretations of the results of sperm evaluation in relation to different staining techniques resulted in the fact that some doctors choose a method depending on the objective of the examination. There is the opinion that routine human sperm analysis should involve Papanicolaou staining, whereas DiffQuik is a better solution when a rapid assessment of sperm morphology is needed. Despite this recommendation, the interest in how the particular staining techniques affect the morphometry results does not cease to exist. An additional problem around the assessment of morphology is the time for sample preparation. Papanicolaou staining preparation takes a long time, which delays semen analysis and postpones the possible examinations (Henkel et al. 2008). Another aspect is that various chemicals are used for different staining techniques. In many cases, the mere preservation of semen on a microscope slide can change the structure of the sperm. Often the alcohol used at different concentrations can lead to dehydration and shrinkage of the sperm head. Preincubation of the sample in physiological saline solution may act hypotonically and cause swelling of the head, the midpiece, and tail. According to the literature, the morphometry of the sperm may also be influenced by the osmotic pressure, staining time, freezing, and thawing. The changes may affect not only the dimensions of the sperm, which can falsify the results, but also can affect the structure of chromatin (Azis et al. 1998; Andraszek et al. 2014). The osmotic pressure of human spermatozoa remains in the range 330 to 370 mOsm/kg (Rossato et al. 2002). The osmotic coefficient of water permeability for human sperm membranes is very high, which indicates the presence of numerous pores in the cytoplasmic membrane. Under various factors, water enters into the sperm so as the osmotic equilibrium be attained. This influx of water into the sperm increases the size of the head, leading to a bulge on the membrane, disturbing the head surface to volume ratio. If the sperm is placed in hyperosmotic conditions, the opposite takes place; water loss occurs and the head shrinks (Abraham-Peskir et al. 2002; Maree et al. 2010).

The varying dimensions of the sperm head can be also due to the structure and arrangement of microfibres in the sperm head. The sperm head cytoskeleton consists of a nuclear proteins and nuclear envelope, which are partly responsible for the formation of the nucleus. Depending on the smear preservation and staining (fixation) method, the orientation of actin fibres in the sperm head can vary (Dvorakova et al. 2005). The shape of the sperm

head is an important factor in terms of hydrodynamics and presumably, sperm with more slender and oval heads in shape are characterized by greater efficiency of movement. A relationship between the shape of the head and motility of the sperm can be sought; we should study whether the sperm with a more oval head also has a longer midpiece, organelles of which are important for the sperm movement (Gage 1998; Gage and Freckleton 2003).

Some authors suggest – in terms of semen cryopreservation – that the sperm head morphometry may be an indicator of fertilizing capacity of the sperm qualified for freezing (Watson 2000). It is believed that sperm with smaller and more elongated heads survive cryopreservation better (Esteso et al. 2006), which may be a matter for consideration aimed to improve the storage of frozen semen (Phetudomsinsuk et al. 2008). During semen cryopreservation sperm chromatin structure may change resulting in the reduction of the surface area of the head, which again may lead to sperm morphology abnormalities (Arruda et al. 2002). The freezing of sperm also affects the functionality of the mitochondria and the acrosome, and disturbs chromatin stability (Vlasiu et al. 2008), as well as causes unfavourable changes in the sperm cytoskeleton plasma membrane (Gutierrez-Perez et al. 2011). A high percentage of spermatozoa with changes within the head in stallion semen was found to be correlated with embryonic mortality during pregnancy (Blottner et al. 2001).

CONCLUSIONS

Despite the extensive knowledge concerning semen, the issue of gametes with high fertilization potential remains open. The differences in sperm size and shape within different species, breeds, and individuals, as well as the fact that spermatozoa are always heterogeneous in a single ejaculate which includes both functionally normal and damaged sperm are major obstacles to proper analysis. As a result, smear staining and the way of their evaluation can significantly affect the results of morphometric measurements and, consequently, the assessment of semen. Lack of established standards for different staining techniques is a very topical issue raised in the current subject literature. The literature points out that the need to establish or develop the staining technique that will in a clear and precise way allow the analysis of sperm morphology and morphometry, both in humans and animals. Furthermore, a standard of slide formulation for morphological evaluation should be also developed. This would allow the comparability of results between laboratories, increasing the value of sperm morphology analysis in terms of predicting and assessing male fertility.

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Abstract. The most important semen parameters are the concentration, motility and morphology of sperm cells. Sperm morphology is regarded as the most reliable parameter for predicting fertility in males. A problem in evaluating sperm morphology and morphometry is the lack of standardization of staining techniques. The staining procedure and reagents used can significantly affect the morphometric parameters of the sperm cell. The use of stains with different pH or osmotic pressure, as well as the duration of the procedure, may influence the shape and size of the sperm, and thus the result of the morphological evaluation of the semen. It is necessary to develop an evaluation procedure for sperm morphology and morphometry that will minimize the changes in the structure of the evaluated semen in relation to the native semen.