Praca oryginalna *Original paper*

Analysis of stallion semiologic semen parameters

DOROTA BANASZEWSKA, KATARZYNA ANDRASZEK*, MAŁGORZATA SZOSTEK*, EWA WÓJCIK*, AGATA DANIELEWICZ*, RENATA WALCZAK-JĘDRZEJOWSKA**

Department of Breeding Methods and Poultry and Small Ruminant Breeding,
*Department of Animal Genetics and Horse Breeding, Institute of Bioengineering and Animal Breeding,
Siedlce University of Natural Sciences and Humanities, B. Prusa 14, 08-110 Siedlce
**Department of Andrology and Reproductive Endocrinology, Medical University in Łódź , Sterlinga 5, 91-425 Łódź

Received 15.07.2014 *Accepted 26.08.2014*

Banaszewska D., Andraszek K., Szostek M., Wójcik E., Danielewicz A., Walczak-Jędrzejowska R.

Analysis of stallion semiologic semen parameters

Summary

Routine semen examination is an important tool in diagnostics and fertility evaluation in humans and animals, both in vivo and in vitro. Among the most important parameters in semen evaluation are sperm motility and morphology. The aim of the study was a detailed morphological analysis of stallion sperm with particular attention to the parameters of the sperm head. The subject of the study was the sperm of Polish Half Bred stallions, isolated post mortem from the tail of the epididymis. The smears obtained were stained with silver nitrate Tygerberg's criteria, which most precisely characterize the sperm head, and were used in the morphological evaluation of the sperm. The evaluation was expanded to include characterization of the tail, particularly the midpiece. The silver nitrate staining used in the present study enables staining of sperm structures which are not visible when other methods of smear preparation are used. In further research this method will be compared with others routinely used in laboratories in order to determine the extent to which the staining method affects sperm morphology and artefact formation.

Keywords: stallion, sperm, silver nitrate, strict Tygerberg criteria

Semen evaluation is essential in predicting male fertility and plays a significant role in maximizing reproductive performance, both in natural conditions and in assisted reproduction. This is particularly important in the case of horses, as there are many valuable stallions that via insemination can affect the selection response by siring foals with outstanding breeding and performance value. Moreover, accurate classification and quantitative determination of a particular sperm defect can provide valuable information on the potential fertility of the stallion and contribute to a diagnosis of reproductive problems (5). Routine semen examination is an important tool in diagnostics and fertility evaluation in humans and animals, both *in vivo* and *in vitro* (26). Among the most important parameters in semen evaluation are sperm motility and morphology (15). Sperm cells acquire the ability to move after passing through the ductus epididymidis. Motility is a fundamental morphological parameter enabling sperm cells to reach the site of fertilization, and thus is a particularly important factor in fertility potential (36). Routine morphological evaluation of semen, however, does not provide a clear indication of its fertilization capacity, with such exceptions as asthenozoospermia, azoospermia or teratozoospermia (22). For this reason the search continues for ways to more precisely evaluate semen in terms of its functional characteristics. Staining of sperm with $AgNO_3$, after certain modifications, reveals elements of the sperm structure which are not visible during routine staining Andraszek and Smalec (1). Some authors confirm the sensitivity and usefulness of morphological evaluation of the sperm cell as a prognostic factor for fertility, particularly when Tygerberg's strict criteria are applied (19). Thus sperm cell morphology remains the most reliable indicator of fertilization potential, and the fertilization capacity of particular individuals is positively correlated with the percentage of morphologically normal sperm (31, 37). Sperm ultrastructure abnormalities can be a marker of pathology in the spermatogenesis process, resulting in reduced fertilization capacity and increased risk of embryonic death.

The aim of the study was a detailed morphological analysis of stallion sperm with particular attention to the parameters of the sperm head.

Material and methods

The subject of the study was the sperm of Polish Half Bred stallions, isolated post mortem from the tail of the epididymis. Ten individuals aged at about 3-4 years were

selected for the study. The sperm were isolated according to the method described by Evans et al. (10). Fixed cells were suspended in a small volume of fresh Carnoy's solution, spread over degreased, cold microscope slides, and air-dried at room temperature. The smears obtained were stained with silver nitrate – $AgNO_3$ (17). A modification introduced by Andraszek and Smalec (1) was applied in both the isolation and staining of the sperm cells. All chemicals used in the study were purchased from Sigma Chemical Company. The sperm cells were evaluated using an Olympus BX50 fluorescence microscope and the MultiScan image analysis system together with measurement software from Computer Scanning Systems. From each stallion 50 morphologically normal sperm cells, clearly visible in the field of view of the microscope, were selected for analysis. A total of 500 sperm cells were evaluated. The following measurements were made: the length, width, perimeter and area of the sperm head; the area of the acrosome; and the length of the midpiece, tail, and the entire sperm (Fig. 1).

Tygerberg's criteria, which most precisely characterize the sperm head, were used in the morphological evaluation of the sperm. The evaluation was expanded to include characterization of the tail, particularly the midpiece. Statistical differences between the samples were determined using Tukey's test and ANOVA (STATISTICA version 10.0, StatSoft Inc., PL). The level of significance was set at $P \leq 0.05$ or $P \leq 0.01$. The study was carried out according to the guidelines of the III Ethical Committee in Warszawa (No 37/2011 from the 22 June 2011).

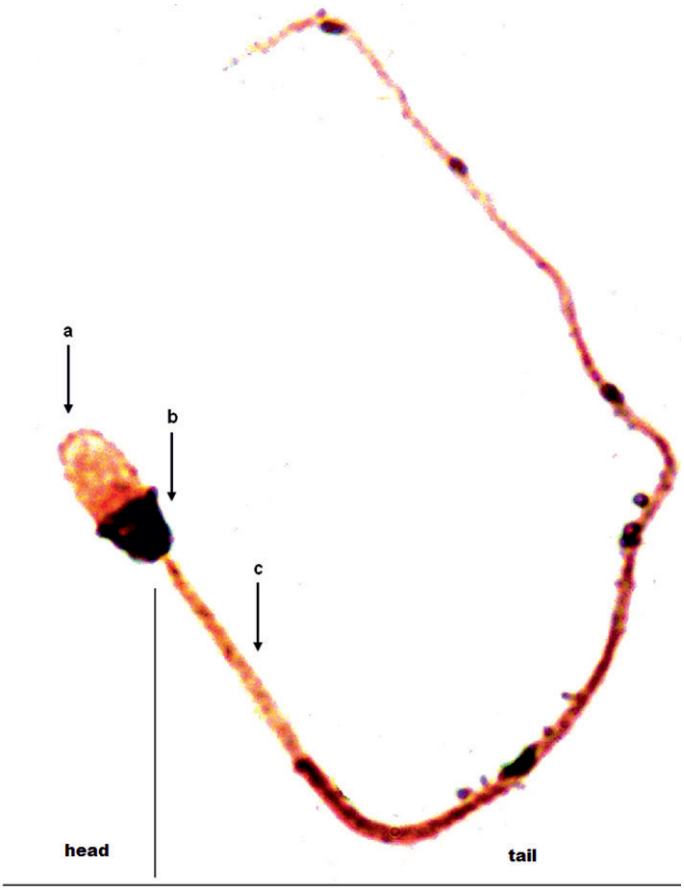

Fig. 1. Stallion spermatozoon stained with $AgNO_3$
Explanations: a – acrosome; b – post-acrosomal region (cap); c – midpiece

Results and discussion

Tab. 1 presents data on the morphological characteristics of the sperm from each stallion. The data show that sperm cells from different individuals vary in size and shape. The length of the head ranged from 7.40 μm (stallion no. 4) to 8.14 μm (no. 9) ($P \leq 0.01$). Apart from one stallion (no. 9), the average length of the sperm head did not exceed 8 μm. The width of the head also varied between individual stallions. It is not always the case that in stallions producing sperm with longer heads the heads are wider as well. The sperm heads of stallion no. 1 were widest, exceeding 4 μm, while those of stallion no. 10, for example, were 0.65 μm narrower ($P \leq 0.01$), but the length of the sperm heads in the semen of these stallions differed only slightly (0.04 μm). This indicates that the sperm heads of these individuals were differently shaped. The sperm of stallion no. 3 had the smallest heads, with a surface area of 18.53 μm^2, of which the acrosome accounted for as much as 12.13 μm^2, i.e. 65.5% of the sperm head area. The largest sperm were observed in stallion no. 1, with the head area exceeding 22 μm^2, of which the acrosome occupied nearly 14 μm^2. Based on analysis of the length of the tail and midpiece of the sperm it cannot be definitively stated that sperm with longer tails also have longer midpieces. In stallion no. 3, whose sperm had the longest tails (84.40 μm) the midpieces were only slightly longer than 14 μm, 0.71 μm shorter than in stallion no. 8 ($P \leq 0.01$). In the sperm of stallion no. 7, which had the shortest tails (76.06 μm), the midpieces were nearly 13.50 μm long, so that the midpiece accounted for 17.8% of the length of the tail. The total length of the sperm was also characterized by substantial individual variability, ranging from about 83.50 μm (stallion no. 7) to nearly 92 μm (no. 3) ($P \leq 0.01$). The sperm head parameters indicate certain differences in the shape of the heads of sperm from different individuals. Sperm from stallions 3 and 10 seem to have more elongated heads than, for example, sperm from stallion no. 1 ($P \leq 0.01$). Moreover, sperm from stallions 3, 9 and 10 have more oval heads, resulting from their greater ellipticity values in comparison with the other stallions.

Functionally the sperm cell consists of three regions: the head, the midpiece and the tail. During spermiogenesis, histones bound with DNA are replaced by protamines. Disulfide bonds formed between protamines stabilize the sperm chromatin (34). This reorganization results in a highly condensed sperm nucleus. Moreover, protamines protect the sperm DNA from enzymatic attack by nucleases and polymerases. Because of the tight compaction of chromatin by protamines, disturbances in the protamination process lead to sperm chromatin anomalies which affect the morphological quality of the sperm and its fertilization capacity. Poor-quality sperm is often observed to have chromatin packed too loosely or damaged DNA (33).

Tab. 1. Morphometric traits of sperms depending on the stallion

Item		Stallion										Total	$LSD_{0,05}$	$LSD_{0,01}$
		1	2	3	4	5	6	7	8	9	10			
Head length (µm)	$\bar{x}$	7.94	7.96	7.57	7.40	7.74	7.78	7.46	7.81	8.14	7.98	7.78	0.415	0.479
	Sd	0.32	0.40	0.57	0.39	0.39	0.46	0.49	0.38	0.45	0.48	0.49		
Head width (µm)	$\bar{x}$	4.03	3.96	3.38	3.55	3.89	3.92	3.73	3.80	3.70	3.38	3.74	0.340	0.393
	Sd	0.38	0.30	0.33	0.23	0.44	0.36	0.41	0.41	0.30	0.37	0.41		
Head perimeter (µm)	$\bar{x}$	20.90	21.03	19.13	19.16	20.67	21.05	20.09	20.65	21.01	19.95	20.36	1.252	1.446
	Sd	1.18	0.96	1.42	0.80	1.41	1.44	1.84	1.34	1.18	1.36	1.47		
Head area (μm^2)	$\bar{x}$	22.33	21.67	18.53	19.14	21.04	21.13	18.93	20.36	20.71	19.51	20.33	1.987	2.295
	Sd	2.22	1.65	2.74	1.55	2.19	2.24	2.40	2.24	1.42	1.94	2.38		
Acrosome area (μm^2)	$\bar{x}$	13.81	13.52	12.13	12.96	14.04	13.44	12.88	12.84	13.38	13.07	13.21	1.422	1.642
	Sd	1.59	1.04	1.92	1.41	1.17	1.30	1.92	1.64	1.44	1.30	1.56		
Acrosome coverage (%)	$\bar{x}$	61.87	62.46	65.51	67.63	66.96	63.78	67.94	63.10	64.51	67.13	65.09	3.934	4.544
	Sd	4.05	3.38	4.44	4.13	4.39	4.14	3.92	4.33	3.98	4.57	4.57		
Mid-piece length (µm)	$\bar{x}$	13.33	13.51	14.04	14.05	13.38	13.57	13.48	14.75	14.32	14.23	13.87	0.531	0.613
	Sd	0.77	0.45	0.62	0.60	0.52	0.41	0.38	0.68	0.52	0.54	0.71		
Mid-piece coverage (%)	$\bar{x}$	16.40	16.59	16.65	16.92	16.77	17.21	17.76	18.02	18.11	18.56	17.30	1.045	1.207
	Sd	1.22	0.86	0.95	0.86	1.00	0.93	0.95	1.27	1.49	1.25	1.29		
Tail length (µm)	$\bar{x}$	81.49	81.59	84.40	83.12	79.89	79.01	76.06	82.11	79.51	76.89	80.41	4.107	4.743
	Sd	5.77	3.75	2.88	2.88	3.75	3.40	3.63	5.27	6.17	4.36	4.92		
Sperm length (µm)	$\bar{x}$	89.44	89.55	91.97	90.52	87.63	86.79	83.52	89.93	87.65	84.86	88.19	4.185	4.834
	Sd	5.77	3.75	3.00	2.90	3.91	3.57	3.60	5.22	6.41	4.54	4.98		
Head ellipticity	$\bar{x}$	1.98	2.02	2.25	2.10	2.01	1.99	2.01	2.08	2.22	2.38	2.10	0.195	0.225
	Sd	0.15	0.17	0.23	0.20	0.21	0.14	0.17	0.22	0.26	0.26	0.24		
Head elongation	$\bar{x}$	0.33	0.34	0.38	0.35	0.33	0.33	0.33	0.35	0.37	0.40	0.35	0.040	0.046
	Sd	0.04	0.04	0.04	0.04	0.05	0.03	0.04	0.05	0.05	0.05	0.05		
Head roughness	$\bar{x}$	0.64	0.62	0.63	0.66	0.62	0.60	0.59	0.60	0.59	0.62	0.62	0.044	0.051
	Sd	0.04	0.04	0.04	0.04	0.04	0.04	0.07	0.05	0.04	0.06	0.05		
Head regularity	$\bar{x}$	1.13	1.14	1.09	1.08	1.13	1.13	1.16	1.15	1.14	1.09	1.12	0.061	0.071
	Sd	0.05	0.05	0.06	0.06	0.07	0.06	0.09	0.08	0.06	0.06	0.07		

During spermiogenesis, with the loss of most typical organelles and the cytoplasm the volume of the cells decreases and the aerodynamic properties of the sperm increase, improving the fertilization potential of the semen (31). Most changes in sperm cells that reduce their fertilization capacity are due to abnormalities in spermatogenesis. These are molecular and cytogenetic changes associated with abnormal chromatin structure in the sperm or anomalies within the sperm tail. Numerous sperm defects are associated with abnormalities within the midpiece, which often lead to reduced motility. Disturbances in mitochondrial function can be of molecular or genetic origin, and in many cases can lead to apoptosis of sex cells. These organelles are thus structures determining the potential of sperm to fertilize an egg cell.

Sperm morphology evaluated according to Tygerberg's strict criteria can be an excellent biomarker of sperm dysfunction, which is one of the main causes of fertility disorders in the male, and can find application in predicting the results of assisted reproduction. Morphological evaluation can also to some extent indicate the functional capacity of sperm as it relates to the role of the acrosome. Semen containing sperm with a high percentage of abnormally formed or damaged acrosomes can be the cause of fertilization problems. In human sperm the normal area of the acrosome is 40-70% of the sperm head, and if the acrosome occupies less than 40% of the head the sperm are considered abnormal (38). There have been few studies characterizing this parameter in stallion sperm. In the present study, the area of the acrosome area ranged from nearly 62% to almost 68% of the head area, which indicates normal acrosome structure in the stallions analysed. Acrosome area as a percentage of the area of the sperm head is a valuable parameter in predicting fertility (22) because it indicates the ability of the sperm cells to undergo a normal acrosome reaction, and thus to penetrate the zona pellucida of the oocyte during fertilization (8). An accurate estimate of this parameter requires a suitable staining technique that clearly differentiates regions of the sperm head (20). Silver nitrate staining allows for a precise estimate of the acrosome percentage of the head area, because the reagents clearly differentiate the head into the acrosome and the post-acrosomal cap, due to the somewhat different chemical composition of the acrosome. Many studies have compared sperm stained using different techniques (4). These studies have found differences in sperm dimensions that may result from varying ability of sperm structure components to bind with fixatives or stains. The cytoskeleton of the sperm head

consists of resistant structural proteins of the nucleus and perinuclear theca (9). Observations by Fouguet and Kann indicate that the distribution of actin fibres in the sperm head can vary depending on the osmolality of the solution. Reagents and water, by penetrating the sperm membrane in order to achieve osmotic balance, cause the cell to swell or shrink (20), so certain differences in sperm dimensions may occur depending on what staining technique is used.

More sperm cells with abnormal morphology are observed in stallion semen than in the semen of other farm animals, partly due to the differing classification of changes in sperm. In infertile stallions or those with reduced fertility, the percentage of sperm cells with abnormal morphological structure has been observed to increase (13, 29). However, even a level of about 40% normally formed sperm has been found to enable satisfactory fertilization results (6).

Many studies indicate a link between sperm dimensions and fertility in horses (7), pigs (16, 28) and canines (25). Moreover, a correlation has been determined between the dimensions of the sperm head alone and fertility in many animal species (7, 14, 16, 25, 28), as well as in humans (8). An evaluation by Gravance et al. (13) of the semen of fertile stallions and stallions with reduced fertility found that the dimensions of the sperm head varied between the two groups. Larger heads were noted in the semen of stallions with fertility disorders, as well as a higher percentage of morphologically abnormal heads. There are also studies describing differences in the size and shape of sperm heads not only in stallions (15, 30), but also in bulls (3, 18) and boars (2, 32). Such variation occurs not only between species and breeds, but also in different ejaculates from the same individual.

The morphology and dimensions of sperm vary strongly between and within species and breeds (24). The present study showed fairly substantial variation in sperm dimensions within one species. Results obtained by Morato-Morales et al. (23) indicate substantial differences in sperm head dimensions in rams from herds of different ancestry. In the present study, the sperm of the stallions analysed had larger dimensions than were noted in stallion sperm evaluated by other authors (13, 30).

Moreover, the genotype of the stallions evaluated in different studies should be taken into account. It has been suggested that differences in sperm morphology are shaped during spermatogenesis (35), while the sperm phenotype is under the control of genes in the premeiotic phase of development (24). Thus the assumption seems justified that differences in sperm dimensions in stallions could be the result of different ancestry.

According to some authors, the mitochondria are the cell structures most sensitive to cryopreservation processes (27, 28). Evaluation of the mitochondria and the midpiece during semen analysis can thus be useful as a marker of sperm fertility (12).

Besides the standard dimensions of the sperm head, the present study also analysed other parameters, characterizing its shape. These provide a picture of the relative shape of the sperm head, which not only enables morphometric classification but also characterizes normal sperm head morphology. Roughness means irregular or amorphous heads, while regularity indicates pyriform heads (21). Higher regularity values for the sperm head indicate a more symmetrical shape. Ellipticity and elongation define to what degree the head is oval, narrow, round or conical. Ellipticity is expressed as the ratio of the length of the head to its width. Greater width of the head causes a decrease in ellipticity. Higher ellipticity values indicate the occurrence of oval sperm heads, while lower values indicate narrower sperm heads (21). This correlation is confirmed in the present study, in which both narrower sperm heads (stallions 1 and 6) and more oval ones (stallions 3, 9 and 10) were observed. A comparative study by Phetudomsinsuk et al. (30) shows differences in sperm head dimensions and shape in stallions of different genotypes. Sperm heads in Thai native cross-breeds were larger and rounder than in purebred stallions. Gage (11) explains that the shape of the sperm head is an important factor affecting the hydrodynamics of the sperm cell, and presumes that sperm cells with narrower and more oval heads are characterized by more effective movement. Thus we can look for a link between the shape of the head and motility by observing whether sperm with more oval heads have longer midpieces, whose organelles undoubtedly affect sperm movement. In the present study, in the stallions that produced sperm with more oval-shaped heads (stallions 9 and 10), the length of the midpiece as a percentage of the tail length was greatest as well, which may indicate more effective movement in these sperm and thus greater fertilization capacity.

Despite the vast body of knowledge on semen, the subject of the male gamete with high fertilization potential remains open. The differences described in this paper in the dimensions and shape of sperm within different species, breeds and individuals, and the fact that in the same ejaculate sperm are always heterogeneous and the semen contains both functionally normal and damaged sperm are the main obstacles to accurate analysis of the ejaculate. According to studies by some authors, the staining method and the method of evaluating the smears can significantly affect the results of morphometric measurements (4). The silver nitrate staining used in the present study enables staining of sperm structures which are not visible when other methods of smear preparation are used. In further research this method will be compared with other others routinely used in laboratories in order to determine the extent to which the staining method affects sperm morphology and artefact formation.

References

1. *Andraszek K., Smalec E.*: The use of silver nitrate for the identification of spermatozoon structure in selected mammals. Can. J. Anim. Sci. 2011, 91, 239-246.
2. *Banaszewska D., Kondracki S., Wysokińska A.*: Effect of age on the dimensions and shape of spermatozoa of Large White Polish boars. Arch. Tierz. 2011, 54, 504-514.
3. *Boersma A. A., Braun J., Stolla R.*: Influence of random factors and two different staining procedures on computer assisted sperm head morphometry in bulls. Reprod. Domest. Anim. 1999, 34, 77-82.
4. *Brito L. F., Kelleman A., Greene L. M., Raz T., Barth A. D.*: Semen characteristics in a sub-fertile Arabian Stallion with idiopathic teratospermia. Reprod. Domest. Anim. 2010, 45, 354-358.
5. *Brito L. F., Lauren M. G., Kelleman A., Knobbe M., Turner R.*: Effect of method and clinician on stallion sperm morphology evaluation. Theriogenology 2011, 76, 745-750.
6. *Card C.*: Cellular associations and the differential spermiogram: making sense of stallion sperml morphology. Theriogenology 2005, 64, 558-567.
7. *Casey P. J., Gravance C. G., Davis R. O., Chabot D. D., Liu I. K.*: Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology 1997, 47, 575-582.
8. *Chan P. J., Corselli J. U., Jacobson J. D., Patton W. C., King A.*: Spermac stain analysis of human sperm acrosomes. Fertil. Steril. 1999, 72, 124-128.
9. *Dvorakova K., Moore H. D., Sebkova N., Palecek J.*: Cytoskeleton localization in the sperm head prior to fertilization. Reproduction 2005, 130, 61-69.
10. *Evans E. P., Breckon G., Ford C. E.*: An air-drying method for meiotic preparation from mammalian testes. Cytogenetics 1964, 3, 295-298.
11. *Gage M. J.*: Mammalian sperm morphometry. Proc. Biol. Sci. 1998, 265, 97-103.
12. *Gallon F., Marchetti C., Jouy N., Marchetti P.*: The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil. Steril. 2006, 86, 1526-1530.
13. *Gravance C. G., Liu I. K. M., Davis R. O., Hughes J. P., Casey P. J.*: Quantification of normal head morphometry of stallion spermatozoa. J. Reprod. Fertil. 1996, 108, 41-46.
14. *Hidalgo M., Rodriguez I., Dorado J.*: Influence of staining and sampling procedures on goat sperm morphometry using the Sperm Class Analyzer. Theriogenology 2006, 66, 996-1003.
15. *Hidalgo M., Rodríguez I., Dorado J., Soler C.*: Morphometric classification of Spanish thoroughbred stallion sperm heads. Anim. Reprod. Sci. 2008, 103, 374-378.
16. *Hirai M., Boersma A., Hoeflich A., Wolf E., Foll J., Aumüller T. R., Braun J.*: Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors. J. Androl. 2001, 22, 104-110.
17. *Howell W. M., Black D. A.*: Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer, a 1-step method. Experentia 1980, 36, 1014-1015.
18. *Kondracki S., Banaszewska D., Wysokińska A., Iwanina M.*: The Effect of Sperm Concentration in the Ejaculate on Morphological Traits of Bull Spermatozoa. Folia Biol. (Krakow) 2012, 60, 85-91.
19. *Kruger T. F., Van der Merwe F., Van Waart J.*: The Tygerberg strict criteria: what are the clinical thresholds for in vitro fertilization, intrauterine insemination, and in vivo fertilization? [in:] Kruger T. F., Franken D. R. (ed.): Atlas of Human Sperm Morphology Evaluation, Taylor and Francis, London 2004, p. 13-18.
20. *Maree L., du Plessis S. S., Menkveld R., van der Horst G.*: Morphometric dimensions of the human sperm head depend on the staining method used. Hum. Reprod. 2010, 25, 1369-1382.
21. *McAlister D. A.*: A comparison of motility and head morphology of sperm using different semen processing methods and three different staining techniques. Praca hab. Medical Physiology, Stellenbosch University 2010.
22. *Menkveld R., El-Garem Y., Schill W. B., Henkel R.*: Relationship between human sperm morphology and acrosomal function. J. Assist. Reprod. Genet. 2003, 20, 432-438.
23. *Morato-Morales A., Ramón M., García-Álvarez O., Soler A. J., Esteso M. C., Martínez-Pastor F., Pérez-Guzmán M. D., Garde J. J.*: Characterization of ram (Ovis aries) sperm head morphometry using the Sperm-Class Analyzer. Theriogenology 2010, 73, 437-448.
24. *Morrow E. H., Gage M. J. G.*: Consistent significant variation between individual males in spermatozoa morphometry. J. Zool. Lond. 2001, 254, 147-153.
25. *Núñez-Martínez I., Moran J. M., Peña F. J.*: Sperm indexes obtained using computer-assisted morphometry provide a forecast of the freezability of canine sperm. Int. J. Androl. 2007, 30, 182-189.
26. *Oral E., Yetis O., Elibol F., Senol H., Irez T., Aksu F. M.*: Assessment of human sperm morphology by strict criteria: comparison of wet preparation versus stained with the modified Diff-Quick method. Arch. Androl. 2002, 48, 307--314.
27. *Ortega-Ferrusola C., Garcia B. M., Gallardo-Bolanos J. M., Gonzales-Fernandez L., Rodriguez-Martinez H., Tapia J. A.*: Apoptotic markers can be used to forecast the freezeability of stallion spermatozoa. Anim. Reprod. Sci. 2009, 114, 393-403.
28. *Peña F. J., Saravia F., García-Herreros M., Núñez-Martínez I., Tapia J. A., Johannisson A., Wallgren M., Rodríguez-Martínez H.*: Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to posthaw quality. J. Androl. 2005, 26, 716-723.
29. *Pesch S., Bostedt H., Failing K., Bergmann M.*: Advanced fertility diagnosis in stallion semen using transmission electron microscopy. Anim. Reprod. Sci. 2006, 91, 285-298.
30. *Phetudomsinsuk K., Sirinarumitr K., Laikul A., Pinyopummin A.*: Morphology and head morphometric characters of sperm in Thai native crossbred stallions. Acta. Vet. Scand. 2008, 50, 1-9.
31. *Ramalho-Santos J., Amaral A., Sousa A. P., Rodrigues A. S., Martins L., Baptista M., Mota P. C., Tavares R., Amaral S., Gamboa S.*: Probing the Structure and Function of Mammalian Sperm using optical and Fluorescence Microscopy. Modern Research and Educational Topics in Microscopy. FORMATEX 2007, 394-402.
32. *Saravia F., Núñez-Martínez I., Morán J. M., Soler C., Muriel A., Rodríguez-Martínez H., Peña F. J.*: Differences in boar sperm head shape and dimensions recorded by computer-assisted sperm morphometry are not related to chromatin integrity. Theriogenology 2007, 68, 196-203.
33. *Saxena P., Misro M. M., Chaki S. P., Chopra K., Roy S., Nandan D.*: Is abnormal sperm function an indicator among couples with recurrent pregnancy loss? Fertil. Steril. 2008, 90, 1854-1858.
34. *Sutovsky P., Manandhar G.*: Mammalian spermatogenesis and sperm structure, anatomical and compartmental analysis, [in:] The sperm cell. Production, maturation, fertilization, regeneration. Cambridge University Press, Cambridge, UK 2006, p. 1-31.
35. *Thurston L. M., Watson P. F., Mileham A. J., Holt W. V.*: Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J. Androl. 2001, 22, 382-394.
36. *Turner R. M.*: Tales from the tail: what do we really know about sperm motility? J. Androl. 2003, 24, 790-803.
37. *Vlasiu T. I., Groza I., Morar R.*: Cătană the effect of different freezing procedures on sperm head morphometry in stallions. Bulletin UASVM, Veterinary Medicine 2008, 65, 146-151.
38. WHO: WHO laboratory manual for the examination of human semen and sperm-cerivical mucu interaction. Cambridge, UK: Published on behalf of the World Health Organization by Cambridge University Press 1999 .

Corresponding author: Dr engineer Dorota Banaszewska, ul. B. Prusa 14, 08-110 Siedlce, Poland; e-mail: banaszd@op.pl