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Use of silver nitrate for the assessment of sperm measurements in selected farm and free-living animal species

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Abstract

The study was conducted on spermatozoa of selected farm and free-living animal species, isolated *post mortem* from the tail of the epididymis, and stained with silver nitrate – $AgNO_3$. The material was collected from pigs, goats, wild boar, and European roe deer. Twenty morphologically normal spermatozoa randomly selected from each animal and well visible under the microscope, were analysed. The following measurements were considered: head length, width, perimeter and area, acrosome area, mid-piece length, tail length, and overall sperm length. $AgNO_3$ staining differentiated the acrosomal (light hue) and distal (dark hue) part of the sperm head, and a light-hued mid-piece was visible within the sperm tail. Silver nitrate staining revealed species and variety-related differences, particularly in reference to the sperm head. Clear-cut differentiation within the head and tail area made it possible to perform detailed morphometric measurements of the spermatozoa.

Keywords: farm animals, free-living animals, sperm, acrosome, midpiece, silver nitrate.

Introduction

Mammalian spermatozoa have small dimensions and compact structure, which make it difficult to analyse details in their morphology using a light microscope (1, 3). The use of various techniques of aided reproduction has created a need for supplementing semen analyses with more advanced methods enabling to determine the reasons for reduced sperm quality and fertilisation capacity. One such biotechnique is semen cryopreservation, which requires assessment of the effects of this process on the basis of morphology, DNA structure, and physiological functions of spermatozoa. The development of *in vitro* fertilisation techniques has also underscored the need for a more exact exploration of sperm morphology and ultrastructure changes. Another important issue is to explain the reasons for infertility of farm animal males (3).

The main routine assessment of male infertility, both in the case of man and animals, is based on analysing sperm concentration, motility, and morphology. The morphometric analysis of spermatozoa is considered to be an objective semen assessment method. Nevertheless, its interpretations still require discussion. This is because standard staining techniques applied for the identification of semen quality, and assessment of spermatozoa are in the majority of cases limited to detection of morphological abnormality only (17, 18, 24), while sperm chromatin structure is of a crucial importance for fertilisation and embryo development. Routine semen assessments are the first step in the evaluation of male infertility factors, but this analysis is not considered to be sufficient for determining infertility in *in vivo* and *in vitro* conditions (17). Consequently, the ultimate determination of infertility often cannot be performed on the basis of the

routine semen assessment (24). The lack of conventional sperm parameters that would enable to predict fertilisation efficacy suggests that there might be latent abnormalities precisely at the level of sperm chromatin (17). Over the last decade there have been significant advances made in studies on the role of nuclear sperm DNA integrity in the analysis of male infertility factors. It has been suggested that sperm DNA integrity can be a better gauge in this respect than the parameters of the standard assessment (7, 24). Molina *et al.* (21) have concluded that evaluating the state of chromatin is very important in the assessment of the fertilisation capacity of spermatozoa. The combination of morphological and molecular techniques, as well as optic, electron, and flow cytophotometry methods seems to be optimal (10, 25). An expanding area of interest in the field of sperm chromatin condensation involves explaining whether, and in what way protamination disturbances affect the fertilisation capacity of a spermatozoon (5, 16). Hence, the application of indirect methods of assessing protamin numbers and chromatin structure (DNA or chromatin integrity) measurements on the basis of various staining procedures has become increasingly more common (15, 17).

Silver nitrate is a strongly alkaline dye. It is predominantly used for identification of acidic chromatin proteins and the chromatin of nucleoli and nucleolar organisers (2, 14). The use of this technique helps to reveal more details in the morphological structure of spermatozoa than the methods using the most commonly applied acidic dyes (1). Proteins contained within the sperm head are alkaline. Therefore, after using silver nitrate, the acrosomal part of the sperm head is stained less prominently than the distal part. Silver nitrate staining shows that sperm nucleus chromatin has a different composition in the acrosomal region than in the rear cap, which contains remnants of acidic proteins and nucleoli, positively reacting with silver salts (1). Analyses of semen stained with this method are performed under a light microscope. It is an easy and convenient method. It may be applied for the assessment of fresh spermatozoa from a smear and fixed material. This type of staining is primarily applied to analyze nucleolar organiser regions within chromosomes, but it has not been widely applied for the analysis of spermatozoa, as evidenced by small number of publications describing the use of this method in analyses of spermatozoa.

The study aimed to present the possibilities of using $AgNO_3$ staining in morphological and morphometric analyses of spermatozoa of selected farm and free-living animal species.

Material and Methods

The study was conducted with the consent of the III Ethical Committee in Warsaw. The study concerned spermatozoa of selected farm and free-living animal species, isolated *post mortem* from the tail of the epididymis. The material was collected from boars (*Sus scrofa f. domestica*) of Large White Polish breed; goats (*Capra hircus*) of Polish White Improved breed; wild boar (*Sus scrofa*), and European roe deer (*Capreolus capreolus*). Ten animals from each species were selected for analyses. The spermatozoa were isolated according to the method described by Evans *et al.* (9) and fixed in Carnoy's fluid (a 3:1 proportion of methanol to acetic acid). Fixed cells were suspended in small amount of fresh Carnoy's fluid, spread over degreased and refrigerated slides, and dried at room temperature. The obtained preparations were stained with silver nitrate – $AgNO_3$ (14). Modifications were made both in the case of sperm isolation and staining (1). The sperm samples were evaluated using an Olympus BX50 fluorescence microscope, Multiscan image analysis, and measurement software from Computer Scanning Systems. Fifty morphologically normal spermatozoa (without defects) randomly selected from each animal and well visible under the microscope were analysed. Altogether 2000 spermatozoa (500 spermatozoa from each species) were evaluated. The following measurements were taken: head length, width, perimeter and area, acrosome area, mid-piece length, tail length, and overall sperm length. 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 $P \leq 0.01$.

Results

The $AgNO_3$ staining differentiated the acrosomal (light hue) and distal (dark hue) part of the sperm head. A light-hued mid-piece was, in turn, highlighted within the sperm tail. Clear-cut differentiation within the head and tail areas enabled detailed morphometric measurements of the spermatozoa.

1. Profiles of spermatozoa of farm animals. Table 1 contains data referring to the morphometric traits of spermatozoa isolated from the tail of the epididymis of ten boars. The data in the Table 1 reveals that fully developed spermatozoa obtained from the epididymis were characterised by substantial individual variability. The analysed animals include both individuals with spermatozoa that have smaller dimensions (boars 1 and 8) and males with larger spermatozoa (boars 2 and 10). Despite considerable individual variability, the data contained in the Table makes it possible to determine mean pig sperm dimensions. The mean head length of the spermatozoa was 10.39 μm; the head width – 5.59 μm; head circumference – 27.48 μm, and sperm head area – 43.24 μm^2. The acrosome area was 32.21 μm^2. Mid-piece and tail lengths were 16.2 μm and 65.4 μm respectively. Mean overall sperm length of the spermatozoa amounted to 75.79 μm. The highest variability of sperm dimensions was identified in the case of the acrosome area and sperm head area (7.15%

and 7.12%). The sperm mid-piece length was the most similar, with the lowest variability (3.52%). A sample of pig spermatozoon stained with silver nitrate is presented in Fig. 1.

Individual variability of sperm dimensions was not only observed in the case of pig spermatozoa but also in goat semen. The analysis of the particular goat sperm dimensions revealed a similar correlation in the case of variability coefficients as in pig sperm dimensions (Table 2). Variability was the highest in the area of the head and acrosome, in excess of 7.84%, and the lowest in the length of the mid-piece. The isolated goat spermatozoa had slightly different dimensions from those of pig. Slightly smaller sperm head dimensions were identified with slightly longer mid-pieces and tails in comparison with pig spermatozoa. The mean head length of goat spermatozoa was 10.04 μm; the width – 5.22 μm; the circumference – 27.18 μm, and the area – 38.69 μm^2. The acrosome area was 23.07 μm^2. Mid-piece and tail length was 17.03 μm and 77.97 μm respectively. The mean overall length of goat spermatozoa amounted to 88.0 μm. A sample of a goat spermatozoon stained with silver nitrate is presented in Fig. 2.

2. Profiles of spermatozoa of free-living animals. Although the wild boar is related to the domestic pig, the spermatozoa of both species differ in dimensions. Wild boar spermatozoa have slightly smaller heads but longer mid-pieces and tails in comparison with boar sperms (Table 3). In the case of the wild boar, the greatest variability was observed in the acrosome area and the lowest variability in the mid-piece length. The mean head length of wild boar spermatozoa was 10.05 μm; the width – 5.27 μm; the circumference – 27.83 μm, and the area – 39.15 μm^2. The acrosome area was 28.63 μm^2. Mid-piece and tail length were 16.8 μm and 73.57μm respectively. The mean overall sperm length of wild boar spermatozoa amounted to 83.61 μm. A sample of a wild boar spermatozoon stained with silver nitrate is presented in Fig. 3.

The data in Table 4 represents measurements of roe deer spermatozoa. The mean head length of the spermatozoa was 10.1 μm; the head width – 5.8 μm; head circumference – 27.8 μm, and sperm head area – 42.7 μm^2. The acrosome area was 26.7 μm^2. Mid-piece and tail length was 14.9 μm and 71.8 μm respectively. The mean overall length of roe deer spermatozoa amounted to 81.9 μm. The lowest variability was observed in the case of mid-piece length, and the highest in the case of acrosome area. A sample of a roe deer spermatozoon stained with silver nitrate is presented in Fig. 4.

3. Comparison of farm and free-living animal spermatozoa. Table 5 contains a juxtaposition of morphometric measurements of the spermatozoa obtained from the analysed animal species. The comparison of the measurements revealed the lowest mean head length in goat sperms and the greatest in pig spermatozoa. The lowest variability of this parameter was identified in the case of the pig and the highest in the roe deer. The mean sperm head width was the lowest in the case of goat spermatozoa and the highest in roe deer. The lowest variability of this parameter was identified in the case of the pig and the highest in roe deer. The smallest mean head perimeter was observed in goat spermatozoa, and the largest in wild boars. The lowest variability of this parameter was identified in pigs and the highest in wild boars. The head and acrosome areas were the smallest in goat spermatozoa and the largest in pigs. In the case of both these parameters the lowest variability was identified in pigs and the highest in wild boars. Roe deer spermatozoa were found to have the shortest mid-pieces, whereas goat sperms had the longest ones. Mid-piece length variability was the lowest in goats and the highest in roe deer. The shortest tails and the smallest overall sperm length were observed in pig spermatozoa. These parameters were the highest in goats. The lowest variability of these two parameters was identified in pig and the highest in roe deer.

Table 1. Morphometric traits of boar sperms

No.	Head length (μm) $\bar{x}$ ± SD	Head width (μm) $\bar{x}$ ± SD	Head circumference (μm) $\bar{x}$ ± SD	Head area (μm^2) $\bar{x}$ ± SD	Acrosome area (μm^2) $\bar{x}$ ± SD	Mid-piece length (μm) $\bar{x}$ ± SD	Tail length (μm) $\bar{x}$ ± SD	Sperm length (μm) $\bar{x}$ ± SD
1	10.12 ± 0.45	5.41 ± 0.41	26.18 ± 1.42	41.31 ± 3.48	30.78 ± 2.56	15.37 ± 0.68	63.83 ± 3.53	73.94 ± 3.82
2	10.49 ± 0.39	5.78 ± 0.41	27.88 ± 1.13	44.73 ± 2.87	33.11 ± 2.19	16.28 ± 0.35	66.23 ± 2.10	76.72 ± 2.18
3	10.55 ± 0.37	5.77 ± 0.34	28.11 ± 0.89	44.90 ± 2.50	33.30 ± 2.08	16.35 ± 0.33	66.59 ± 2.49	77.14 ± 2.65
4	10.46 ± 0.35	5.63 ± 0.38	27.81 ± 0.95	43.58 ± 2.15	32.34 ± 1.56	16.33 ± 0.37	65.91 ± 2.16	76.38 ± 2.17
5	10.50 ± 0.36	5.70 ± 0.32	27.96 ± 0.94	44.15 ± 2.49	32.80 ± 1.94	16.39 ± 0.41	66.02 ± 3.63	76.52 ± 3.77
6	10.31 ± 0.34	5.43 ± 0.36	27.29 ± 1.02	42.40 ± 3.06	31.78 ± 2.25	16.41 ± 0.38	65.53 ± 2.44	75.84 ± 2.55
7	10.37 ± 0.39	5.47 ± 0.30	27.50 ± 1.16	42.09 ± 2.91	31.48 ± 2.27	16.35 ± 0.40	64.05 ± 2.68	74.41 ± 2.80
8	10.22 ± 0.43	5.44 ± 0.38	26.65 ± 1.35	41.73 ± 2.65	31.20 ± 2.09	15.77 ± 0.80	63.77 ± 2.71	73.98 ± 2.89
9	10.42 ± 0.36	5.70 ± 0.38	27.73 ± 1.07	44.17 ± 3.39	32.87 ± 2.53	16.34 ± 0.37	65.93 ± 2.42	76.35 ± 2.56
10	10.42 ± 0.34	5.53 ± 0.35	27.66 ± 1.02	43.30 ± 2.74	32.47 ± 2.10	16.41 ± 0.35	66.22 ± 2.61	76.64 ± 2.79
Total	10.39 ± 0.40	5.59 ± 0.39	27.48 ± 1.24	43.24 ± 3.08	32.21 ± 2.30	16.20 ± 0.57	65.40 ± 2.89	75.79 ± 3.07
V%	3.83	6.92	4.52	7.12	7.15	3.52	4.42	4.05

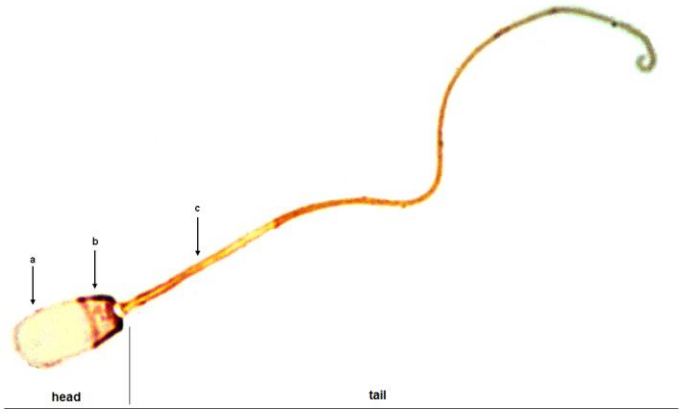

Fig. 1. A pig spermatozoon stained with $AgNO_3$. a – acrosomal part of the head; b – distal region; c – mid-piece

Table 2. Morphometric traits of goat sperms

No.	Head length (µm) $\bar{x} \pm SD$	Head width (µm) $\bar{x} \pm SD$	Head circumference (µm) $\bar{x} \pm SD$	Head area (µm^2) $\bar{x} \pm SD$	Acrosome area (µm^2) $\bar{x} \pm SD$	Mid-piece length (µm) $\bar{x} \pm SD$	Tail length (µm) $\bar{x} \pm SD$	Sperm length (µm) $\bar{x} \pm SD$
1	10.09 ± 0.37	5.20 ± 0.41	26.91 ± 1.27	38.68 ± 3.25	22.93 ± 1.69	16.98 ± 0.34	77.93 ± 4.07	88.02 ± 3.95
2	10.07 ± 0.36	5.15 ± 0.37	27.01 ± 1.34	38.46 ± 2.70	23.50 ± 1.57	16.94 ± 0.38	78.89 ± 4.45	88.95 ± 4.59
3	10.00 ± 0.44	5.28 ± 0.37	27.38 ± 1.92	38.99 ± 3.90	22.71 ± 2.38	17.03 ± 0.38	77.02 ± 5.27	87.01 ± 5.33
4	10.21 ± 0.37	5.36 ± 0.30	28.24 ± 1.28	40.16 ± 2.33	23.53 ± 1.66	17.16 ± 0.45	78.60 ± 4.90	88.81 ± 4.86
5	9.94 ± 0.26	5.15 ± 0.35	26.73 ± 0.96	37.87 ± 2.46	22.92 ± 1.37	16.95 ± 0.38	78.08 ± 3.16	88.02 ± 3.13
6	10.13 ± 0.41	5.38 ± 0.33	27.90 ± 1.66	40.26 ± 3.08	23.15 ± 2.18	17.06 ± 0.38	77.72 ± 5.70	87.85 ± 5.79
7	9.90 ± 0.34	4.98 ± 0.28	26.22 ± 0.95	36.47 ± 2.12	22.61 ± 1.74	17.04 ± 0.34	78.48 ± 3.66	88.38 ± 3.76
8	10.00 ± 0.30	5.17 ± 0.34	27.50 ± 1.58	38.35 ± 2.32	23.34 ± 1.19	17.00 ± 0.50	76.63 ± 3.67	86.63 ± 3.61
9	9.92 ± 0.30	5.19 ± 0.37	26.71 ± 1.06	38.29 ± 3.41	22.77 ± 2.16	17.07 ± 0.39	77.97 ± 5.09	87.89 ± 5.10
10	10.10 ± 0.38	5.31 ± 0.39	27.22 ± 1.47	39.40 ± 2.60	23.22 ± 1.74	17.02 ± 0.37	78.34 ± 4.99	88.44 ± 4.93
Total	10.04 ± 0.37	5.22 ± 0.37	27.18 ± 1.48	38.69 ± 3.03	23.07 ± 1.81	17.03 ± 0.39	77.97 ± 4.54	88.00 ± 4.59
V%	3.64	7.02	5.45	7.84	7.86	2.31	5.86	5.21

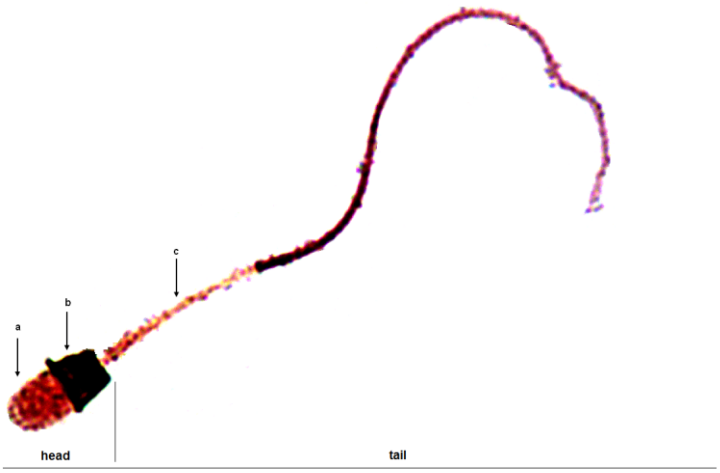

Fig. 2. A goat spermatozoon stained with AgNO3. a – acrosomal part of the head; b – distal region; c – mid-piece

Table 3. Morphometric traits of wild boar sperms

No.	Head length (µm) $\bar{x} \pm SD$	Head width (µm) $\bar{x} \pm SD$	Head circumference (µm) $\bar{x} \pm SD$	Head area (µm^2) $\bar{x} \pm SD$	Acrosome area (µm^2) $\bar{x} \pm SD$	Mid-piece length (µm) $\bar{x} \pm SD$	Tail length (µm) $\bar{x} \pm SD$	Sperm length (µm) $\bar{x} \pm SD$
1	10.14 ± 0.80	5.29 ± 0.33	27.82 ± 2.37	39.94 ± 5.39	29.50 ± 4.68	16.80 ± 0.37	70.69 ± 3.19	80.84 ± 2.78
2	9.88 ± 0.540	5.14 ± 0.40	27.16 ± 1.72	37.49 ± 3.80	27.42 ± 3.18	16.79 ± 0.38	71.14 ± 4.19	81.02 ± 4.20
3	10.26 ± 0.59	5.35 ± 0.40	28.37 ± 2.03	40.74 ± 4.13	29.91 ± 3.51	16.76 ± 0.40	70.27 ± 3.21	80.53 ± 3.10
4	9.92 ± 0.57	5.15 ± 0.39	27.08 ± 1.72	37.82 ± 4.19	27.77 ± 3.48	16.72 ± 0.43	73.81 ± 6.24	83.73 ± 6.22
5	10.16 ± 0.36	5.38 ± 0.35	28.56 ± 1.60	40.41 ± 2.65	29.49 ± 2.29	16.69 ± 0.33	74.19 ± 4.58	84.35 ± 4.47
6	10.02 ± 0.41	5.28 ± 0.36	28.11 ± 1.74	38.96 ± 3.04	28.19 ± 2.55	16.87 ± 0.43	76.59 ± 4.84	86.60 ± 4.69
7	10.00 ± 0.48	5.39 ± 0.36	27.44 ± 1.42	39.67 ± 3.08	28.92 ± 2.85	16.98 ± 0.34	78.07 ± 2.94	88.08 ± 3.02
8	9.88 ± 0.38	5.19 ± 0.41	27.50 ± 1.64	37.57 ± 3.02	27.40 ± 2.37	16.63 ± 0.39	73.96 ± 5.99	83.84 ± 6.11
9	9.87 ± 0.43	5.22 ± 0.36	27.76 ± 1.72	37.51 ± 2.61	26.99 ± 2.17	16.96 ± 0.41	76.30 ± 4.87	86.16 ± 4.97
10	10.34 ± 0.71	5.34 ± 0.34	28.46 ± 2.28	41.41 ± 4.72	30.72 ± 4.10	16.78 ± 0.36	70.64 ± 3.28	80.98 ± 2.99
Total	10.05 ± 0.56	5.27 ± 0.38	27.83 ± 1.90	39.15 ± 3.99	28.63 ± 3.40	16.80 ± 0.40	73.57 ± 5.18	83.61 ± 5.09
V%	5.61	7.17	6.82	10.2	11.88	2.36	7.04	6.09

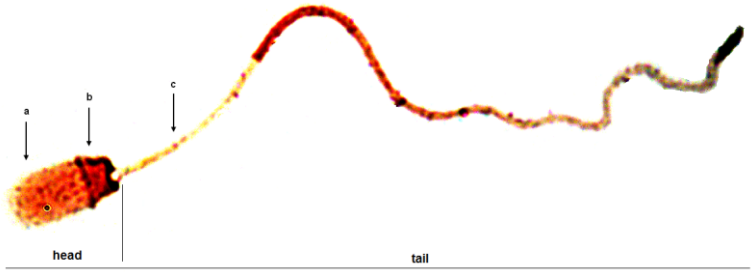

Fig. 3. A wild boar spermatozoon stained with AgNO3, a – acrosomal part of the head; b – distal cap; c – mid-piece

Table 4. Morphometric traits of roe deer sperms

No.	Head length (µm) $\bar{x} \pm SD$	Head width (µm) $\bar{x} \pm SD$	Head circumference (µm) $\bar{x} \pm SD$	Head area (µm^2) $\bar{x} \pm SD$	Acrosome area (µm^2) $\bar{x} \pm SD$	Mid-piece length (µm) $\bar{x} \pm SD$	Tail length (µm) $\bar{x} \pm SD$	Sperm length (µm) $\bar{x} \pm SD$
1	9.97 ± 0.65	5.83 ± 0.43	27.91 ± 1.77	42.03 ± 3.98	26.62 ± 2.42	15.10 ± 0.51	73.95 ± 4.20	83.91 ± 4.17
2	9.68 ± 0.53	5.73 ± 0.35	26.80 ± 1.46	40.55 ± 2.95	25.61 ± 2.23	15.00 ± 0.66	72.76 ± 5.46	82.44 ± 5.48
3	10.07 ± 0.45	5.66 ± 0.37	27.28 ± 0.95	41.96 ± 2.37	26.32 ± 1.90	14.91 ± 0.64	69.11 ± 7.62	79.18 ± 7.55
4	10.11 ± 0.57	5.91 ± 0.45	27.93 ± 1.86	43.99 ± 4.49	27.06 ± 2.82	14.73 ± 0.46	70.75 ± 5.74	80.85 ± 5.76
5	10.19 ± 0.58	5.80 ± 0.51	27.94 ± 1.80	43.70 ± 4.99	26.83 ± 3.11	14.73 ± 0.58	69.83 ± 7.33	80.02 ± 7.35
6	10.18 ± 0.66	5.96 ± 0.46	28.36 ± 2.15	44.58 ± 5.19	27.23 ± 2.88	14.75 ± 0.51	71.20 ± 4.42	81.38 ± 4.24
7	9.96 ± 0.68	5.78 ± 0.39	27.59 ± 1.88	42.10 ± 3.85	26.31 ± 2.62	14.94 ± 0.70	72.15 ± 6.26	82.11 ± 6.34
8	10.17 ± 0.76	5.80 ± 0.42	28.05 ± 1.74	42.74 ± 4.09	27.08 ± 2.50	15.01 ± 0.49	72.69 ± 4.46	82.86 ± 4.43
9	10.19 ± 0.77	5.89 ± 0.38	28.20 ± 1.60	43.32 ± 4.04	27.31 ± 2.69	14.95 ± 0.58	72.87 ± 6.22	83.06 ± 6.17
10	10.06 ± 0.66	5.82 ± 0.42	27.78 ± 1.73	42.72 ± 4.18	26.71 ± 2.62	14.91 ± 0.58	71.83 ± 6.02	81.89 ± 5.98
Total	10.10 ± 0.70	5.80 ± 0.40	27.80 ± 1.70	42.70 ± 4.20	26.70 ± 2.60	14.90 ± 0.60	71.80 ± 6.00	81.90 ± 6.00
V%	6.60	7.20	6.20	9.80	9.80	3.90	8.40	7.30

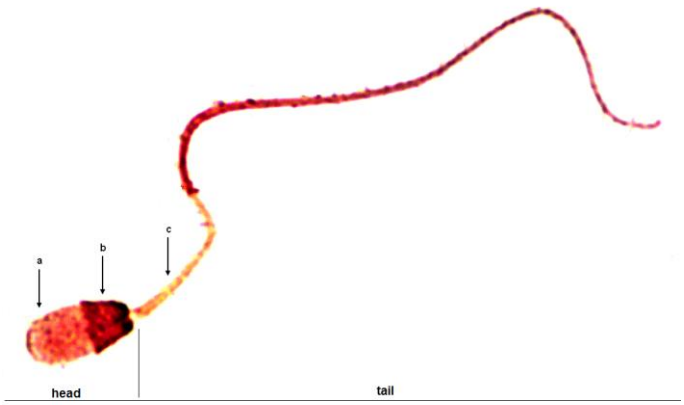

Fig. 4. A roe deer spermatozoon stained with AgNO3, a – acrosomal part of the head; b – distal cap; c – mid-piece

Table 5. Juxtaposition of morphometric parameter values for the spermatozoa of the analysed animal species

Species	Head length (µm) $\bar{x} \pm SD$	Head width (µm) $\bar{x} \pm SD$	Head circumference (µm) $\bar{x} \pm SD$	Head area (µm^2) $\bar{x} \pm SD$	Acrosome area (µm^2) $\bar{x} \pm SD$	Mid-piece length (µm) $\bar{x} \pm SD$	Tail length (µm) $\bar{x} \pm SD$	Sperm length (µm) $\bar{x} \pm SD$
pig	10.39 ± 0.40	5.59 ± 0.39	27.48 ± 1.24	43.24 ± 3.08	32.21 ± 2.30	16.20 ± 0.57	65.40 ± 2.89	75.79 ± 3.07
V%	3.83	6.92	4.52	7.12	7.15	3.52	4.42	4.05
goat	10.04 ± 0.37	5.22 ± 0.37	27.18 ± 1.48	38.69 ± 3.03	23.07 ± 1.81	17.03 ± 0.39	77.97 ± 4.54	88.00 ± 4.59
V%	3.64	7.02	5.45	7.84	7.86	2.31	5.86	5.21
wild boar	10.05 ± 0.56	5.27 ± 0.38	27.83 ± 1.90	39.15 ± 3.99	28.63 ± 3.40	16.80 ± 0.40	73.57 ± 5.18	83.61 ± 5.09
V%	5.61	7.17	6.82	10.2	11.88	2.36	7.04	6.09
roe	10.10 ± 0.70	5.80 ± 0.40	27.80 ± 1.70	42.70 ± 4.20	26.70 ± 2.60	14.90 ± 0.60	71.80 ± 6.00	81.90 ± 6.00
V%	6.60	7.20	6.20	9.80	9.80	3.90	8.40	7.30

Discussion

The standard semen analysis is the crucial tool in diagnosis and treatment of human and animal infertility (22). The most frequently assessed, and the most important semen parameters include concentration, motility, and morphology of the sperm (13). These parameters are considered to be the most useful since they have been correlated with the fertility potential (14, 17, 18). For example, low sperm concentration, which is connected with the number of spermatozoa present in one millimetre of semen, indicates male infertility (11). After passing through the epididymal duct, spermatozoa acquire motion capacity. Motility is a particularly important function as it enables spermatozoa to reach the oocyte. Moreover, the capacity of these cells to move is crucial at each moment of fertilisation since it indirectly facilitates the passage of the spermatozoon through the zona pellucida of the oocyte (6). Of the three parameters, sperm morphology is the most objective index of *in vivo* and *in vitro* fertility (22). Abnormal morphological structure of spermatozoa can be indicative of pathology underlying spermatogenesis disorders. It is the most stable parameter with a direct influence on fertility levels. Therefore techniques of sperm morphology assessment are constantly being improved and optimised.

Apart from precise identification of the sperm head and tail, the optimisation of histological staining techniques more often emphasises the aspect of acrosome and mid-piece identification and assessment. Additionally, there are attempts to supplement standard semen diagnostics with these two structures (12, 19). It is a well-known fact that the functional capacity of spermatozoa is associated with correct acrosome functioning. Semen containing a low percentage of undamaged acrosomes affects fertilisation capacity (20). Another structure that does not receive particular attention in routine assessments of sires is the mid-piece. It contains the mitochondrial spiral (constituting up to 80% of the mid-piece). Its function is to supply an optimal amount of energy that spermatozoa need for motion (26). If mitochondrial semen defects are relatively frequent, it may lead to reduced fertility or complete infertility (23). Considering the above fact, an

exact morphological analysis of the mid-piece, supplemented with functional diagnostics of the mitochondria, should be applied in a routine semen assessment.

In the present study, the use of $AgNO_3$ staining enabled precise differentiation of the sperm head and tail, including their specific components. A clearly visible light-hued acrosomal region and a much darker distal cap within the head were observed. The tail, on the other hand, contained a light-hued mid-piece, the remainder of the tail featuring dark pigmentation. Silver nitrate staining has been predominantly used for the identification of nucleolus and NOR chromatin so far. It is a relatively new and rarely used method in sperm analyses, as evidenced by a low number of publications describing the application of this method in such studies. Silver nitrate staining is a technique which allows numerous details to be observed in the morphological structure of sperms (head and its components, mid-piece, and tail), as well as differences between various species in this respect. The staining reveals species- and variety-related differences, particularly in reference to the sperm head. Proteins of the sperm head are alkaline. Thus, following silver nitrate application, the part of the head that contains the acrosome stains less prominently (is lighter) than the distal part (rear cap). This shows that sperm nucleus chromatin has a different composition in these two regions, as the rear cap contains remnants of acidic proteins and nucleoli, which positively react with silver salts (1). The biochemical conditioning of $AgNO_3$ staining is also ascribed to the presence of sulhydryl (thiolic) proteins and disulphide bond-rich proteins of sperm chromatin (4). Dyes routinely used in assessments of farm animal semen morphology are usually acidic, whereas silver nitrate is an alkaline pigment. The use of this technique helps to reveal more details in the morphological structure of spermatozoa than the methods using acidic dyes (1). Additionally, this method makes it possible to clearly observe differences in the integrity of the acrosome (thanks to the possibility of observing numerous details in its structure), which can result from damage, ageing processes, or various kinds of semen anomalies (4). As regards the standard semen assessment, there is a perceptible lack of clearly formulated conventional semen parameters that would allow an effective evaluation of fertilisation. This suggests that there might be latent anomalies at the chromatin level in the sperm. It has been shown that sperm chromatin structure is of a critical importance for fertilisation and subsequent embryo development (17). While there are many studies and descriptions of the rudimentary morphological analysis of animal semen, there are not many publications on the details of morphometric measurements of particular farm animal sperm structures. Human studies on the effect of cryopreservation on the morphometric dimensions of the sperm head, and morphometric measurements of sperm heads of fertile and infertile individuals indicate that this type of measurement can be a valuable tool in determining semen fitness for refrigeration and semen capacity for fertilisation (8).

At a time, when aided reproduction techniques are commonly applied, there are attempts made at establishing a standard method to be recommended and applicable at all laboratories that perform sperm assessments for the seminological evaluation of human and animal sperm. Standardisation of the method would ensure repeatability and comparability of results obtained at different research and diagnostic institutions. Silver nitrate staining of spermatozoa is a relatively inexpensive, fast, and repeatable technique. It can be used for the assessment of fresh spermatozoa from a smear and fixed sample. Various authors have concluded that it can be used in biological studies and assessments of semen for artificial insemination (1, 4). It can be assumed that the use of the technique in semen analyses can provide detailed information concerning the morphological structure and morphometric dimensions of spermatozoa, as these aspects are gaining more importance in studies on fertility and preservation techniques for semen to be used for artificial insemination. This work confirms the accuracy of using silver nitrate for staining spermatozoa. The method can be successfully applied on a broader scale. While defects of morphological sperm structure have already been quite well explored, a better understanding of sperm ultrastructure and the changes provided by different ways of dealing with semen remain a great challenge. Silver nitrate staining is one of many options that enable such understanding. Its advantage primarily concerns its low cost and ease of application. Moreover, the possibilities of modifying the method also deserve a great attention. Its prototype was devised by Howell and Black (14). Its modification by Andraszek and Smalec (1) was used in this study. There is a need to continue analyses using this method and further explore its modifications in order to obtain even a better differentiation of sperm structures and the possibility to perform more exact measurements.

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