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Original study

Comparison of different chromatin staining techniques for bull sperm

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Abstract

Morphological analysis of semen is a very important step in fertility assessment, but many semen defects are not detectable at the morphological level. These include pathological changes in sperm chromatin structure. During mammalian spermiogenesis, histone proteins associated with DNA structure are replaced by specific protamines, with which chromatin does not form nucleosomal complexes. In the fully developed, mature sperm, the histones are replaced with protamines. Disruptions of nucleoprotein structure can be treated as possible indicators of the biological value of spermatozoa. The experimental material consisted of sperm from one-and-a-half-year-old bulls, isolated post mortem from the tail of the epididymis. The smears were stained with silver nitrate ($AgNO_3$), acridine orange (AO), aniline blue (AB) and chromomycin A3 (CMA3). Sperm dimensions largely depend on individual variability among the bulls. In most cases, differences in sperm dimensions were identified between individuals, which was confirmed in the statistics. Sperm with elevated, abnormal histone levels were proportionally quite scarce (1.4 %). Studies of nuclear proteins in the context of infertility demonstrate the important influence of normal chromatin structure on sperm functions.

Keywords: bull, spermatogenesis, sperm, protamine, histone

Abbreviations: AB: aniline blue, $AgNO_3$: silver nitrate, AO: acridine orange, CMA3: chromomycin A3

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Introduction

Animal breeding and related problems have in recent years become one of the principal subjects of study for researchers and scientists. Growing population numbers worldwide have led to an increase in the consumption not only of plant foodstuffs, but also, perhaps to an even greater degree, of animal products. For this reason the need has arisen to enhance the efficiency of farm animal reproduction.

Reproduction is closely linked to semen quality and normal sperm structure. The most important element of sperm ultrastructure is the cell nucleus, as fertilisation efficacy depends on its having a normal structure. The sperm cell nucleus is largely filled with DNA, which accounts for about 40 % of its dry matter mass. A bull spermatozoon contains 3.44 pg DNA/nucleus (Bielańska-Osuchowska & Sysa 1998, Bochenek *et al.* 2001). Spermatozoa are haploid cells, and the chromosomes contained in them are monochromatid structures. Owing to differences in mass between cells that contain X or Y sex chromosomes, male gametes can be separated in order to select the sex of the offspring (Johnson *et al.* 1987, Gledhill 1988)

Semen analysis is a multi-staged process. The first step is evaluation of motility, the number of sperm and the proportions of live and dead sperm (Guzick *et al.* 2001, Hidalgo *et al.* 2006). The next step is morphological evaluation, which is increasingly expanded to include detailed morphometry of the spermatozoon (Oral *et al.* 2002). There are situations, however, in which a motile and morphologically normal spermatozoon is incapable of fertilisation, e.g. due to acrosomal defects or abnormalities at the chromatin level (Bianchi *et al.* 1996, Iranpour *et al.* 2000). Many semen defects are not detectable at the morphological level. For this reason basic semen analysis should be expanded to include cytogenetic and molecular techniques, mainly to determine in vivo and in vitro fertility. Particularly in the case of assisted reproduction techniques and cryoconservation of sperm, their quality is of immense importance (De Vos *et al.* 2003, Nagvenkar *et al.* 2005).

In mammalian spermiogenesis, histone proteins associated with DNA structure are replaced by specific protamines with which chromatin does not form nucleosomal complexes (Bielańska-Osuchowska & Sysa 1998, Zhang *et al.* 2006). Histones are proteins containing considerable numbers of positively charged amino acids (lysine and arginine) that enable strong bonding between these proteins and the negatively charged double DNA helix (Mudrak 2005, Oliva & Castillo 2011). In a fully developed, mature spermatozoon, the histones are replaced with protamines. The elimination of the histones gradually progresses throughout spermatogenesis. Histone replacement with protamines, which are half their size, results in a complete change in chromatin organisation between the spermatocyte and the spermatozoon (Balhorn 2011, Oliva & Castillo 2011). It has been observed in several species that a small fraction of the semen genome is compacted by histones. This histone fraction, currently believed to be present in the sperm of all mammals, is relatively small, constituting no more than 1 % of the genome. The fraction is much larger in humans, probably within the range of 10-15 % (Arpanahi *et al.* 2009). In bulls, on the other hand, the histone fraction in the sperm nucleus is less than 1 % (Tovich & Oko 2003). There is much evidence to suggest that such distribution of genes in regions organised by protamines and those organised by histones is not accidental. Such location of sperm genes has recently been presented by two independent teams, using micromatrices and deep genome sequencing technology, respectively (Balhorn 2011, Oliva & Castillo 2011).

Protamines are relatively small proteins. Their molecules are composed of 27-65 amino acids rich in arginine and cysteine, with a markedly alkaline pH (Balhorn 2011). Protamines present in mammalian sperm are of two types: P1 and P2. The former are smaller proteins present in the sperm of all mammals. In eutherian mammals they consist of a single-chain peptide containing only 50 amino acids. A well-known exception in this group is the stallion, which has 51 amino acids (Balhorn *et al.* 1988, Balhorn 2007). P2 is slightly larger than P1. It contains 63 amino acids in mice. It is only present in eutherian mammal spermatids that are in the process of differentiation (Corzett *et al.* 2002). In contrast to P1, P2 is synthetised as a larger precursor protein, binds with DNA, and is truncated over the next several days. P2 also contains a large number of cysteine residues that participate in the formation of bisulphide bonds (Corzett *et al.* 2002, Balhorn 2011).

Abnormal protamine levels in infertile patients were described over 20 years ago (De Yebra & Oliva 1993, De Yebra *et al.* 1993). Subsequent studies only confirmed the relationship between an abnormal protamine level and anomalous semen parameters and infertility in men (Evenson *et al.* 2000, Aoki *et al.* 2005). The types of protamine anomalies identified involve lower levels of protamines with respect to other proteins, and the P1-P2 transformation index. One possible reason for the excessive P1-P2 ratio observed in some infertile men was found with the identification of P2 transformation and elevated protamine precursor levels in the subgroup of infertile individuals. Moreover, protamine expression may be affected by thermal stress (Balhorn *et al.* 1988, Aoki *et al.* 2005, Oliva & Castillo 2011). A temperature affecting stallion testes may lead to a reduction in the number of bisulphide bonds inserted in the protamines (Love & Kenney 1999).

The importance of basic proteins stabilising DNA structure has been particularly stressed in recent years. Using cytochemical methods it is possible to identify the change of histone-like proteins present in spermatids into stable protamine proteins in sperm. In certain cases, the direct cause of infertility is disorders in the protein transformation processes during spermiogenesis. Hence, the changes are epigenetic rather than molecular and are associated with DNA structure. Furthermore, nucleoprotein structure disruptions can be treated as possible indicators of the biological value of sperm (Meistrich *et al.* 2003, Martianov *et al.* 2005, Enciso *et al.* 2011). The assessment of chromatin compaction within a spermatozoon not only indicates whether spermatogenesis is normal, but can also provide information about the condition of the paternal genome and epigenome.

The aim of the present study was to identify proteins that stabilise sperm structure and to provide an in-depth analysis of bull sperm morphology using different staining methods.

Material and methods

The experimental material consisted of sperm from one-and-a-half-year-old bulls, isolated post mortem from the tail of the epididymis. The material was collected from 20 Polish Holstein-Friesians of the Black-and-White variety. The material for the study was obtained during slaughter at a facility where each stage of processing was subject to veterinary control and met European Union requirements, as well as the requirements of the National Ethics Committee for Animal Experiments of the European Union (authorisation number 37/2011).The spermatozoa were isolated according to a method described by Evans *et al.* (1964), routinely

used for isolating meiotic chromosomes, modified by Andraszek & Smalec (2011). Fixed cells were suspended in a small volume of fresh Carnoy's solution, spread over degreased and refrigerated slides and dried at room temperature. The smears were stained with silver nitrate ($AgNO_3$), acridine orange (AO), aniline blue (AB) and chromomycin A3 (CMA3). All chemicals for sample preparation were of the highest commercially available purity and were obtained from Sigma-Aldrich Co. LLC. (Poland). The sperm cells were analysed with an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan). The MultiScan image analysis system and measurement software from Computer Scanning Systems (Warsaw, Poland) were used for computer analysis of the smears.

$AgNO_3$ staining

The smears were dyed with an $AgNO_3$ solution following Howell & Black's (1980) method, modified by Andraszek & Smalec (2011). The smears were examined microscopically using immersion lenses with a 100x zoom on an Olympus BX50 light microscope. In each smear, morphometric measurements were performed on 30 randomly selected morphologically well-formed spermatozoa, well visible in the field of vision of the microscope, according to a method devised by Kondracki *et al.* (2006, 2012). A total of 600 spermatozoa were examined. The following morphometric measurements were made: sperm head length, sperm head width, sperm head perimeter, sperm head area, perimeter and area of the acrosomal part of the sperm head, sperm mid-piece length, sperm tail width and total sperm length. Based on these measurements, morphological structure parameters of the spermatozoa were computed as well. The data were arranged with reference to particular bulls in order to compare the morphometric sperm traits of individual animals.

Acridine orange staining

The staining was carried out according to a procedure described by Tejada *et al.* (1984). The microscopic analysis of the smears included identification of spermatozoa with normal DNA structure (green fluorescence) and those with damaged, single-strand DNA (orange fluorescence). 100 spermatozoa from each animal were analysed, for a total of 2 000 spermatozoa.

Aniline blue staining

The staining was carried out following the procedure described by Franken *et al.* (1999), with a modification proposed by Wong *et al.* (2008) to enhance sperm pigmentation. We looked for sperm with histone levels that were normal (light blue colour) and too high (intensive blue colour). 100 spermatozoa from each animal were analysed, for a total of 2000 spermatozoa.

Chromomycin A3 staining

The smears were stained according to Lolis *et al.* (1996). We analysed the percentage of spermatozoa with normally compacted chromatin (subdued green fluorescence) and abnormally packed chromatin (intensive green fluorescence). 100 spermatozoa from each animal were analysed, for a total of 2000 spermatozoa.

Statistical analysis

The results were statistically processed using analysis of variance according to the following mathematical model:

$$Y_{ij} = \mu + a_i + e_{ij} \quad (1)$$

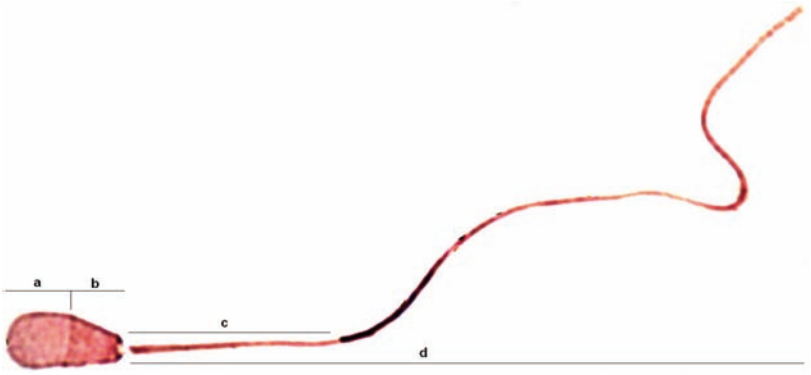
where Y_{ij} is the trait value, μ is the population mean, a_i is the bull effect and e_{ij} is the error. The significance of between-group differences was verified using Tukey's test at $P \leq 0.05$ or $P \leq 0.01$. All statistical analyses were performed in STATISTICA 10.0 (StatSoft, Inc. Tulsa, OK, USA).

Results

The material fixed on the slides was stained using four methods involving chemical agents with different properties. The following subsections present the sperm staining results obtained using the techniques described in »Material and methods«.

$AgNO_3$ staining

The sperm stained with silver nitrate took on different colours, enabling identification of particular structural elements of their morphology (Figure 1). In the sperm head, the light acrosomal region (a) and dark distal cap (b) were clearly differentiated. The light mid-piece (c) of the tail (d) clearly contrasted with the rest of the tail. This pigmentation made it possible to carry out detailed measurements.



a: acrosomal part, b: distal postacrosomal region (cap), c: mid-piece, d: tail

Figure1
Bull spermatozoon stained with $AgNO_3$

Table 1 contains data on the morphometric parameters of each bull analysed. The data show that the sperm dimensions depended largely on individual variation in the bulls. In most cases, differences were identified in sperm dimensions between individual animals, which was confirmed by the statistics. Sperm dimensions in individual bulls were highly variable. The mean sperm head length ranged from 10.00 µm (in bull no. 6) to over 10.50 µm (bulls 1,

Table 1
Morphometric traits of sperm in each of the bulls

Bull	Head length, µm $\bar{x} \pm SD$	Head width, µm $\bar{x} \pm SD$	Head perimeter, µm $\bar{x} \pm SD$	Head area, µm² $\bar{x} \pm SD$	Acrosome perimeter, µm $\bar{x} \pm SD$	Acrosome area, µm² $\bar{x} \pm SD$	Mid-piece length, µm $\bar{x} \pm SD$	Tail length, µm $\bar{x} \pm SD$	Sperm length, µm $\bar{x} \pm SD$
1	10.50±0.36	4.70±0.29	31.53±1.79	45.09±4.36	21.83±1.64	25.65±2.67	18.42±0.59	81.88±6.51	92.38±6.67
2	10.37±0.34	4.58±0.27	29.86±2.29	43.11±2.82	20.02±1.56	23.75±2.27	18.52±0.81	87.19±6.88	97.56±6.97
3	10.34±0.32	4.62±0.34	29.05±3.72	42.34±5.16	19.55±1.53	23.96±2.72	18.66±0.65	87.96±5.78	98.30±5.84
4	10.36±0.30	4.87±0.33	27.93±1.57	44.65±3.27	19.11±1.20	24.21±2.78	17.84±0.72	82.88±5.31	93.25±5.24
5	10.46±0.33	4.84±0.33	27.37±1.95	44.39±3.89	18.90±1.11	23.65±2.24	18.77±0.97	86.58±6.77	97.04±6.66
6	10.00±0.29	4.81±0.22	26.95±1.27	43.75±2.65	18.08±1.49	21.63±2.95	18.37±0.99	83.78±8.46	93.78±8.61
7	10.35±0.32	4.61±0.40	27.73±1.24	44.39±3.55	18.59±1.61	22.96±3.70	18.13±1.11	82.97±8.84	93.32±8.96
8	10.57±0.38	4.57±0.31	27.80±1.51	45.76±3.23	18.85±1.67	23.61±2.87	17.99±0.77	83.02±6.07	93.59±6.11
9	10.39±0.37	4.42±0.30	27.31±1.27	42.59±3.79	17.81±1.26	21.24±3.01	18.02±0.59	81.85±4.66	92.25±4.71
10	10.57±0.38	4.51±0.33	27.58±1.05	44.53±2.76	18.35±1.71	22.46±3.65	18.22±0.88	80.91±5.89	91.48±6.04
11	10.36±0.34	4.64±0.34	30.59±1.76	43.72±3.38	20.96±1.68	24.48±2.29	18.63±0.66	83.83±8.32	94.19±8.42
12	10.40±0.34	4.72±0.30	28.49±1.92	43.98±3.23	19.67±1.60	24.45±2.42	18.33±0.90	84.42±6.39	94.82±6.45
13	10.23±0.33	4.93±0.30	27.34±1.49	44.58±3.42	18.45±1.23	22.65±2.44	18.69±0.89	86.31±8.26	96.55±8.39
14	10.46±0.36	4.56±0.35	27.91±1.53	45.02±3.95	18.73±1.64	23.09±3.73	17.65±0.62	82.04±8.18	92.49±8.17
15	10.44±0.37	4.57±0.30	27.20±1.33	43.19±3.34	17.84±1.14	21.38±2.87	17.94±0.66	81.32±4.86	91.76±4.98
16	10.52±0.36	4.66±0.25	30.73±3.97	43.32±5.84	19.99±1.89	24.15±3.20	18.30±0.80	85.74±6.20	96.27±6.29
17	10.65±0.38	4.49±0.36	29.59±2.52	45.40±2.97	20.29±1.94	24.60±2.83	18.29±0.89	81.54±6.31	92.20±6.47
18	10.36±0.30	4.74±0.37	27.98±1.33	43.91±2.56	19.08±1.21	23.92±3.29	18.10±0.68	87.72±4.53	98.07±4.48
19	10.30±0.36	4.91±0.32	26.93±1.40	43.71±4.05	18.36±1.34	22.55±2.78	18.72±0.94	84.47±6.55	94.77±6.60
20	10.38±0.37	4.65±0.36	27.18±1.12	44.36±3.59	18.66±1.70	22.86±3.58	18.21±1.27	81.86±8.35	92.23±8.42
Total	10.40±0.37	4.67±0.35	28.35±2.36	44.09±3.73	19.16±1.82	23.36±3.12	18.29±0.88	83.91±7.04	94.31±7.09
LSD0,05	0.309	0.286	1.746	3.277	1.360	2.830	0.747	6.044	6.106
LSD0,01	0.349	0.323	1.973	3.703	1.536	2.972	0.844	6.829	6.900

$\bar{x}$: mean value, SD: standard deviation

10, 16 and 17) ($P \leq 0.01$). In some cases bulls whose sperm had shorter heads (no. 6, 13 and 19) at the same time produced sperm with slightly broader heads (over 4.80 µm) than those of the other bulls, which additionally reveals certain differences in sperm head shapes. Differences were also found in sperm head perimeter between individual animals. The largest sperm head perimeter was more than 31.5 µm (bull no. 1), while sperm with smaller perimeters, by as much as 4.60 µm, were also observed (bull no. 19) ($P \leq 0.01$). The area of the sperm head was slightly less variable. In the majority of cases, intergroup differences were small and statistically unconfirmed. High variability was noted in the case of acrosome perimeter and area. Certain correlations were observed in some of the bulls. The spermatozoa that had larger head perimeters and areas (bulls no. 1, 11, 16 and 17) also had larger acrosomal perimeters and areas. This was not, however, uniform for all the bulls, which further supports the hypothesis of variability not only within a species or breed, but also at the level of the individual, or even ejaculate-specific differences. This was also confirmed in the case of the mid-piece and tail dimensions. Some bulls were found to have longer mid-pieces, but also longer tails and greater overall sperm length (bulls no. 2, 3, 4), while others did not exhibit this pattern. An example is bull no. 18, whose sperm had long tails of approximately 87.72 µm, i.e. almost 7 µm longer than in some of the other bulls ($P \leq 0.05$), and greater overall length, but average mid-piece length (18.10 µm). The inverse relationship was also noted, in which sperm had relatively long mid-pieces, e.g. 18.72 µm (bull no. 19), but tail and overall length at an average level with respect to the other bulls.

In accordance with the methodology, proportions were determined between selected morphological parameters and correlation coefficients were calculated for pairs of traits (Table 2). The morphological parameters were found to be slightly positively or (in three cases) negatively correlated. The highest negative correlation was identified between sperm head length and width (−0.037), while the highest positive correlation was determined between acrosome area and sperm head area (0.399).

Table 2
Shape proportions and correlation coefficients of selected semen parameters

Feature		Proportion a/b	Correlation coefficient
a	b	$\bar{x} \pm SD$	r_{ab}
Head length	Head width	2.24±0.19	−0.037
Head length	Mid-piece length	0.57±0.03	0.021
Head length	Tail length	0.12±0.01	0.118
Head length	Sperm length	0.11±0.01	0.169
Mid-piece length	Tail length	0.22±0.02	0.134
Mid-piece length	Sperm length	0.19±0.02	0.134
Acrosome area	Head area	0.53±0.08	0.399
Head area	Mid-piece length	2.42±0.23	0.075
Head area	Tail length	0.53±0.06	−0.006
Head area	Sperm length	0.47±0.05	−0.003

$\bar{x}$: mean value, SD: standard deviation

Analysis of samples stained with acridine orange, aniline blue and chromomycin A3

Acridine orange staining

Acridine orange is used to identify spermatozoa with normal DNA structure and damaged single-strand DNA. When bound with damaged DNA or RNA, or in sites where there is no DNA in the cell (e.g. the sperm tail), the dye emits red or orange fluorescence. Green fluorescence corresponds to a normal double-strand DNA structure. The sperm exhibited green fluorescence in all the smears analysed (Figure 2), indicating that the genetic material of the sperm produced by the bulls was undamaged (Figure 5).

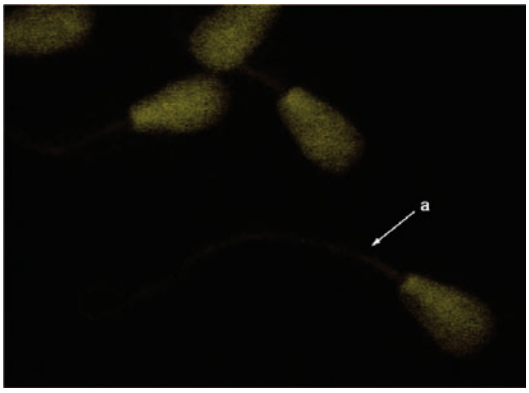


Figure 2
Bull spermatozoa stained with acridine orange; a: normal spermatozoon

Aniline blue staining

Aniline blue enables identification of spermatozoa with normal or abnormal histone retention (Figure 3). Sperm staining revealed mostly sperm with normal histone retention, while sperm with elevated histone levels were proportionally quite scarce (1.4 %) – Figure 5. The highest number of spermatozoa with elevated histone levels was observed in bull 3.

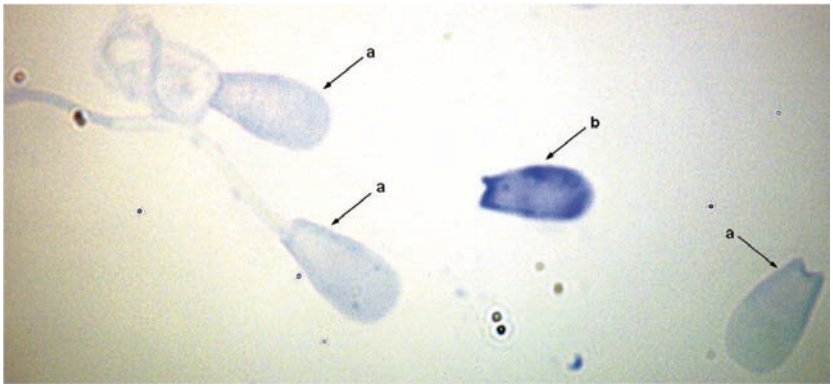


Figure 3
Bull spermatozoa stained with aniline blue; a: normal spermatozoon, b: spermatozoon with high histone retention

Chromomycin A3 staining

Chromomycin A3 staining was used to test chromatin compaction (Figure 4). Five bulls (no. 1, 5, 14, 18 and 19) were found to have normally compacted chromatin in all the sperm, while the remaining bulls exhibited slight deviations in the number of sperm with normally packed chromatin (Figure 5). The highest number of sperm with abnormally packed genetic material was noted in bull no. 12, for which four defective spermatozoa were identified.

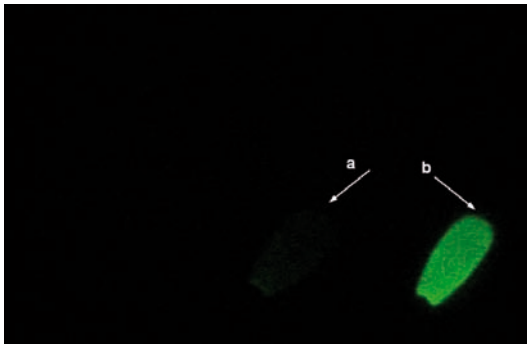


Figure 4
Bull spermatozoa stained with Chromomycin A3; a: normal spermatozoon, b: spermatozoon with anomalous protamination

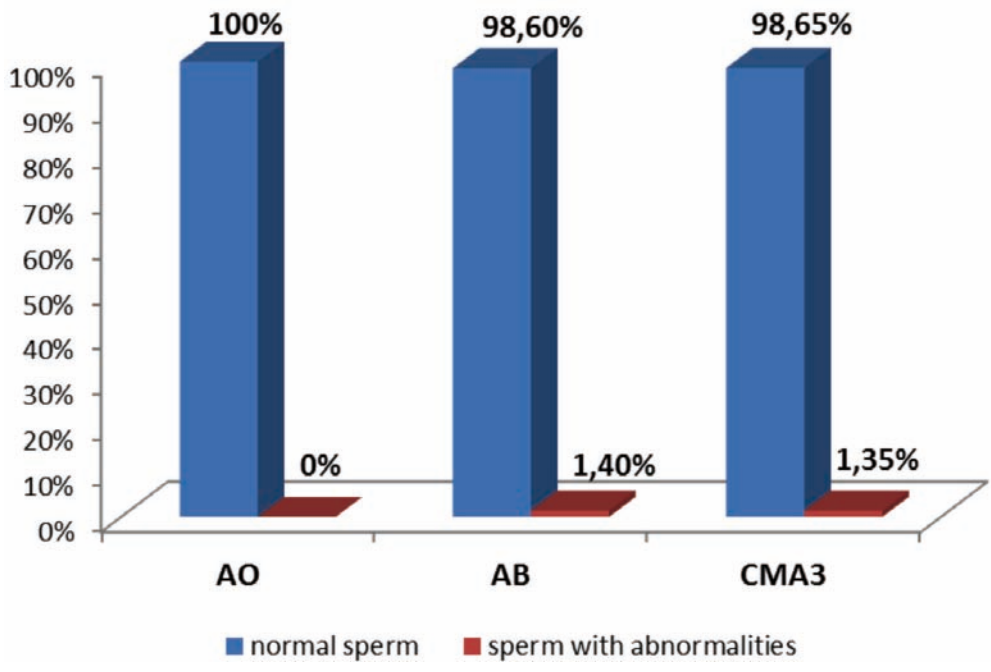


Figure 5
Analysis of smears stained with acridine orange, aniline blue and chromomycin A3

Discussion

Standard assessment of the sperm of farm animals consists in detecting morphological anomalies. However, most sperm defects that reduce fertilisation potential are the result of faulty spermatogenesis. These are molecular, cytogenetic and epigenetic changes connected with abnormal chromatin organisation.

Routine assessments of sperm morphology in cattle and pigs predominantly use the eosin-gentian or eosin-nigrosin staining proposed by Hancock (1951). Eosin is particularly recommended for quick evaluation of bull sperm (Chenoweth *et al.* 1992, Chenoweth 2005, Enciso *et al.* 2011, Kondracki *et al.* 2006, 2012). Studies by Kondracki *et al.* (2006) and Banaszewska *et al.* (2011) confirm that eosin staining is the standard technique for assessment of sperm morphology in farm animals, commonly applied at animal breeding and insemination centres. Eosin-nigrosin staining is also recommended by WHO as a basic method for evaluating human sperm morphology (Björndahl *et al.* 2003, 2004). Apart from sperm morphology assessments, this method also enables the identification of living and dead sperm (Sprecher & Coe 1996, Zambelli & Cunto 2006, Freneau *et al.* 2010). Nevertheless, both staining methods allow only for observation of the spermatozoon as a uniform structure and enable measurements of only the sperm head and tail.

Eosin is an acidic dye used to identify alkaline molecules, whereas silver nitrate is a basic pigment applied to detect acidic chromatin proteins, chromatin, nucleolar organisers and nucleoli (Howell & Black 1980, Andraszek *et al.* 2009, Andraszek & Smalec 2011). The use of silver nitrate in sperm morphology assessment has made it possible to identify sperm structures not revealed by eosin. Sperm stained with $AgNO_3$ have differential pigmentation within the head and tail areas and a prominent mid-piece. Sperm head proteins are alkaline, which means that silver nitrate stains the acrosomal part of the head less intensively than the distal part. Silver nitrate staining shows that sperm nucleus chromatin has a different composition in the acrosomal part than in the distal region, which contains acidic proteins and the nucleolus, which reacts positively with silver salts.

Among the tests devised for identification of DNA damage, the most frequently used is acridine orange staining, which enables determination of sperm chromatin stability in an acidic environment. The dye has metachromatic properties. Bound with double-strand DNA, it emits fluorescence in the green band, while bound with RNA and single-strand DNA, it emits red fluorescence. Evenson *et al.* (1980) found that spermatozoa containing denatured single-strand DNA reduce fertilisation efficacy and the quality of embryos obtained in vitro. Despite the practice of using acridine orange in analyses of sperm of various species, the level of pathological DNA fragmentation has only been defined in humans. A level of up to 15 % spermatozoa with damaged DNA is considered to be a normal state, while a level of 15-25 % reduces fertility and more than 25 % is regarded as representing a high risk of infertility (Evenson *et al.* 1999, 2000). Similar limits have not been determined for other species. There have been reports of bulls observed to have reduced fertility with over 10 % damaged sperm (Bochenek *et al.* 2001).

One of the critical moments in spermatogenesis is the replacement of standard chromatinic proteins (histones) with protamines. This reorganisation results in a highly condensed sperm nucleus. Moreover, protamines protect sperm DNA from enzymatic attack by nucleases and

polymerases. Owing to tight compaction of chromatin by protamines, any changes in or lack of protamines leads to sperm nucleus anomalies and impinges on the morphological quality of semen and its fertilisation capacity. Poor-quality semen is often observed to have chromatin packed too loosely or damaged DNA (Saxena *et al.* 2008).

Recent years have seen increased interest in the role of sperm histones in semen maturation, fertilisation and early embryonic development. Histones in fertile individuals are linked to micro-RNA promoters and the expression of genes active during embryogenesis (Miller *et al.* 2010). The histone structure of chromatin in spermatocytes and early spermatogonia enables active transcription. The transcriptional activity of chromatin in mature sperm is inhibited by its protamination. Anomalies in the proportion of histones and protamines within a spermatozoon are correlated with DNA instability, and thus with vulnerability to damage. Higher histone levels have been observed in infertile individuals or those with reduced fertility that have certain anomalies: oligospermia – reduced numbers of mature spermatozoa, teratospermia – spermatozoa with abnormal structure, and asthenozoospermia – anomalous sperm motility (Zhang *et al.* 2006, Zini *et al.* 2008). These unique proteins and their possible role in early embryogenesis must be investigated in order to understand the epigenetic factors in male infertility.

Protamines are arginine- and cysteine-saturated proteins. They form an exceptionally condensed and transcriptionally suppressed chromatin conformation in the spermatozoon (Vilfan *et al.* 2004). The incorporation of protamines P1 and P2 in the mammalian genome is strictly regulated during spermatogenesis. As it turns out, not only anomalous protamination but also the proportions between P1 and P2 affect fertility. Individuals found to have low sperm motility or concentration in morphological analysis exhibit anomalies both in protamination and in P1-P2 proportions. Protamination is indispensable to ensure normal condensation of sperm chromatin, which in turn affects the normal functioning of the sperm (Aoki *et al.* 2006).

Staining of sperm with AB and CMA3 is a rapid assay to evaluate the quality of chromatin in the sperm. Aniline blue is a dye specific to proteins with high lysine content. Histones, which are replaced with protamines during spermiogenesis, are such proteins. Aniline staining reveals abnormal, excessive histone content in the spermatozoon. Such sperm are more vulnerable to DNA damage and chromatin stability anomalies. Research has shown that early miscarriages and embryonic death are correlated with elevated histone levels in the sperm (Hammadeh *et al.* 2001, Kazerooni *et al.* 2009). Chromomycin is a fluorescence dye that specifically binds with guanine-cysteine dinucleotides. Owing to its unique properties, chromomycin can be applied to detect spermatozoa with low chromatin condensation resulting from faulty protamination. However, scientists are cautious about confirming the efficacy of this technique because it becomes targeted at any available G-C dinucleotides. Increased chromomycin availability for these complexes can result from a number of factors. One of them may be DNA damage caused by protamine deficiency, which results in enhanced fluorescence. Although this may be true, researchers still call into question data obtained using this staining method (Hammadeh *et al.* 2001, Kazerooni *et al.* 2009).

Some studies have shown that protamine deficiency measured by means of CMA3 staining independently affects fertilisation (Iranpour *et al.* 2000). Nevertheless, chromatin anomalies within sperm can be present at several levels: histone replacement by protamines, lack of

protamines, maturation disorders within epididymides, and chromatin stability. Kazerooni *et al.* (2009) observed that the number of spermatozoa positively stained with CMA3 and AB is greater in individuals with reduced fertility. Since these two assays reveal the quality of the chromatin contained within sperm cells, it can be concluded that the semen of individuals whose parents suffered from spontaneously recurring abortion has low-quality chromatin, specifically with concurrent protamine deficiency. Moreover, sperm vitality and abnormal morphology have been shown to be correlated with the percentage of spermatozoa positively stained with CMA3 and AB. This indicates that abnormal chromatin compaction has a destructive effect on semen parameters, which is consistent with the observations of other researchers (Coetzee *et al.* 1998, Hammadeh *et al.* 1998).

Analysis of protamination disorders is increasingly used to supplement morphological and molecular assessments of semen. Protamination is regarded as an important parameter in the assessment of male fertility (Aoki *et al.* 2006, Zini *et al.* 2007). Unfortunately, evaluations of protamination are mainly conducted on human material; this type of analysis of animal sperm is rare and generally limited to laboratory experiments, without practical application. Studies of nuclear proteins in the context of infertility demonstrate the importance of normal chromatin structure for sperm functions. More in-depth understanding of the complex chromatin structure is essential for the development of new, increasingly comprehensive and precise diagnostic tools.

In conclusion, the significance of basic proteins that stabilise the DNA structure of spermatozoa has received particular recognition in recent years. Cytochemical methods make it possible to characterise the change of histone-like proteins present in spermatids into stable protamine proteins in spermatozoa. Certain cases of bull infertility are directly caused by disorders in spermiogenetic protein transformations, not by higher or lower DNA content. Additionally, nucleoproteinic structure anomalies can be regarded as indicative of the biological value of spermatozoa.

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