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Evaluation of sperm chromatin structure in boar semen

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Abstract

This study was an attempt to evaluate sperm chromatin structure in the semen of insemination boars. Preparations of semen were stained with acridine orange, aniline blue, and chromomycin A3. Abnormal protamination occurred more frequently in young individuals whose sexual development was not yet complete, but may also be an individual trait. This possibility is important to factor into the decision regarding further exploitation of insemination boars. Thus a precise assessment of abnormalities in the protamination process would seem to be expedient as a tool supplementing morphological and molecular evaluation of semen. Disruptions in nucleoprotein structure can be treated as indicators of the biological value of sperm cells.

Keywords: boar, sperm, protamine, histone.

Introduction

Evaluation of semen is a basic tool in the diagnosis of male fertility. However, routinely performed tests of basic semen parameters appear to be insufficient to determine fertility. This takes on greater importance in the case of breeders used at insemination centres. The costs of purchasing and keeping selected males are not always commensurate with the quality of the semen obtained from them and its fertilisation capacity. Hence it would be worth expanding preliminary semen diagnostics to include additional tests, at least at the start of a breeder's exploitation. A lack of success in fertilisation may have deeper causes (23). Some authors suggest that in addition to standard semen analysis, functional tests should be used as well, as the fertilisation rate depends to a large extent on the quality of the semen used for insemination (10, 26). Research on the integrity of boar sperm DNA has been conducted by Fraser and Strzeżek (16), Perez-Llano (29), and Hu *et al.* (20), who used the comet assay to assess the degree of DNA defragmentation due to storage of semen at different temperatures and in different diluents. Evaluation of chromatin structure is one of the means of assessing semen quality. A growing focus of interest in the area of sperm chromatin condensation is clarification of how protamination and retained histones affect the epigenetic state of the mature sperm cell (8, 22). For this reason, the use of indirect methods based on various staining techniques for assessing the quantity of protamines and measuring chromatin structure (DNA or chromatin integrity) is currently becoming widespread (21, 23). The available literature describes studies on human semen, in which chromatin structure is assessed in order to predict fertilisation in patients undergoing intracytoplasmic sperm injection (ICSI) or *in vitro* fertilisation (IVF) (25, 28). It would be advisable to conduct similar pilot research on animals, especially those used for insemination, and in particular young individuals at the start of their exploitation, in order to eliminate breeders with low fertilisation capacity. Hence, the aim of this study was an evaluation of sperm chromatin structure in the semen of insemination boars.

Material and Methods

The material for the study consisted of semen collected from 34 insemination boars used at insemination stations in the Masovian Province. 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.

The animals were 12-56 months old. From each boar 10 ejaculates were collected and evaluated. A total of 340 ejaculates were used. Immediately after collection, the ejaculates were analysed to determine their basic physical characteristics: volume after removal of the gel fraction (mL), concentration of sperm cells determined by photometry (AccuRead photometer, IMV Technologies, France) (1000/mm^3), and percentage of sperm cells exhibiting progressive movement, using a light microscope. Microscope slides were stained using 3 techniques.

One technique was chromomycin (CMA3) staining according to Lolis *et al.* (25). The slides were initially incubated in Carnoy's solution at 40°C for 5 min. Each slide was treated for 20 min with 100 µL of CMA3 stain solution (0.25 mg/mL in McIlvane's buffer, pH 7.0, containing 10 mM $MgCl_2$). The slides were rinsed in the buffer and mounted with buffered glycerol. The percentages of sperm cells with normal chromatin packaging (dull green fluorescence) and abnormal chromatin packaging (bright green fluorescence) were determined.

Aniline blue (AB) staining according to Franken *et al.* (15) was also used. The slides were initially incubated in 3% buffered glutaraldehyde at room temperature. After thorough rinsing with distilled water they were stained with 5% AB in 4% acetic acid (pH 3.5) for 5 min. Next, they were rinsed again and lightly stained with 0.5% eosin for 1 min. This was a modification suggested by Wong *et al.* (36) to augment staining of the sperm cells. The presence of cells with normal histone content (pale blue staining) and histone content that was too high (bright blue staining) were analysed.

The third technique was acridine orange (AO) staining according to Tejda *et al.* (35). The stock solution was prepared by dissolving 1 mg of AO in 1000 mL of distilled water. Storage was in the dark at 4°C. The staining solution was prepared from 10 mL of stock solution, 40 mL of 0.1 M citric acid, and 2.5 mL of 0.3 M Na_2HPO_4 and its final concentration was 0.19 mg/mL, with pH 2.5. The slides were then stained with 2-3 mL of staining solution for 10 min. The slides were rinsed with distilled water and the wet slides were covered with a cover slip, which was sealed with rubber cement. Microscope analysis of the slides involved identification of sperm cells with normal DNA structure (green fluorescence) and damaged single-stranded DNA (orange fluorescence). All chemicals for sample preparation were obtained in the highest commercially available purity from Sigma-Aldrich Co. LLC (Poland).

The microscope analysis of the slides was performed using an Olympus BX50 fluorescence microscope (Olympus, Japan). In each slide 500 sperm cells were analysed, for a total of 17 000 sperm cells.

The material collected was divided into three groups according to the age of the boar. The first group contained data on ejaculates collected from boars aged up to 18 months, the second from boars aged 18-24 months, and the third from boars older than 24 months (the age given relevance was the age of the boars on the day the ejaculates were collected). Statistical differences between the samples were tested using Tukey's test and one-way analysis of variance (ANOVA) (STATISTICA version 10.0, StatSoft Inc., USA). The level of significance was set at $P \leq 0.05$ or $P \leq 0.01$.

Results

Table 1 presents data concerning the basic physical characteristics of boar ejaculates and evaluation of sperm chromatin structure performed comparatively using 3 staining techniques. The data show that quantitative and qualitative semen characteristics depend on the individual predisposition of a given boar. The semen characteristics of most boars were within the norms adopted for insemination boars. In boar 1, the mean percentage of sperm exhibiting progressive motion was low (60%), which is borderline normal for a breeder. However, no significant percentage of sperm with abnormal protamination was observed in the semen of this individual. Markedly reduced sperm motility was found in boars 7 and 17. These were young males assigned to the first age group. Most of the ejaculates collected from boar 7 exhibited asthenozoospermia. Another breeder whose sperm had abnormal protamination was boar 24. This boar was between 1.5 and 2 years of age when the semen was collected and was thus assigned to the second age group. Its ejaculates were of fairly high volume and sperm concentration, and the sperm cells had normal motility. However, the percentages of sperm with abnormal chromatin packaging and with excessively high histone content were 14.5% and 7.9% respectively. No sperm cells with fragmented DNA were found in the semen of this boar. Apart from these 3 cases, the ejaculates were within reference values for insemination boars, despite considerable individual variation.

The proportion of normal spermatozoa in the semen of 3 groups of boars after staining by 3 techniques is presented in Fig. 1.

Table 2 shows data pertaining to ejaculate characteristics and the results of staining, taking into account the age of the boar on the day of semen collection. Older individuals were found to have ejaculates of somewhat lower volume, but greater sperm concentration. The percentage of sperm exhibiting progressive movement in the ejaculate is significantly higher in individuals over 1.5 years of age. It should be kept in mind, however, that the results obtained for the 3 boars discussed above unquestionably affected the average for the population. Abnormal protamination was more often observed in young boars (up to 1.5 years) whose sexual development was not yet complete. This was evidenced by the higher percentages of sperm with lower chromatin condensation and abnormal histone retention - 9.88% and 8.41% respectively. The frequency of sperm with abnormal protamination appears to decrease as the boars grow older, as in individuals aged over 2 years the frequency of sperm with chromatin abnormalities did not exceed 0.7%.

Table 3 presents correlation coefficients between the staining techniques. The data show a high positive correlation between semen stained with CMA3 and AB, as the 2 techniques complemented each other, and a smaller but also positive correlation between these 2 techniques and AO, as sperm cells with abnormal protamination did not always have fragmented DNA.

Table 1. Semen characteristics (average ±SD) and evaluation of sperm chromatin structure for the 3 staining techniques in particular boars

Boar	Ejaculation	Concentration	Progressive	Results of CMA3	Results of AB	Results of AO
	$\bar{X}$ ±SD	$\bar{X}$ ±SD	$\bar{X}$ ±SD	$\bar{X}$ ±SD	$\bar{X}$ ±SD	$\bar{X}$ ±SD
1	385 ± 70.61	552 ± 57.62	60 ± 18.00	1.00 ± 0.53	0.52 ± 0.36	0.00 ± 0.00
2	333 ± 67.42	482 ± 29.50	80 ± 0.00	0.28 ± 0.39	0.32 ± 0.23	0.04 ± 0.09
3	315 ± 35.00	900 ± 112.45	80 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4	456 ± 52.87	386 ± 80.80	80 ± 0.00	0.46 ± 0.41	0.23 ± 0.34	0.09 ± 0.23
5	188 ± 29.00	510 ± 47.49	80 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
6	124 ± 28.00	600 ± 79.20	80 ± 0.00	0.40 ± 0.30	0.00 ± 0.00	0.00 ± 0.00
7	370 ± 108.63	370 ± 57.15	15 ± 17.00	41.25 ± 5.29	35.25 ± 2.58	0.80 ± 0.37
8	237 ± 45.50	708 ± 71.90	80 ± 0.00	1.72 ± 1.67	0.36 ± 0.17	0.00 ± 0.00
9	540 ± 49.50	480 ± 67.45	80 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10	125 ± 27.76	612 ± 212.53	80 ± 0.00	0.60 ± 0.28	0.40 ± 0.32	0.00 ± 0.00
11	326 ± 27.02	540 ± 171.61	80 ± 0.00	0.44 ± 0.38	0.36 ± 0.30	0.04 ± 0.09
12	175 ± 47.70	680 ± 80.00	80 ± 0.00	0.47 ± 0.64	0.00 ± 0.00	0.00 ± 0.00
13	262 ± 38.34	630 ± 168.08	80 ± 0.00	1.88 ± 1.50	0.96 ± 0.50	0.00 ± 0.00
14	271 ± 84.05	617 ± 148.41	80 ± 0.00	0.37 ± 0.34	0.27 ± 0.35	0.07 ± 0.16
15	250 ± 71.00	490 ± 58.30	80 ± 0.00	0.20 ± 0.25	0.00 ± 0.00	0.00 ± 0.00
16	228 ± 30.61	648 ± 178.93	80 ± 0.00	0.97 ± 0.63	0.67 ± 0.27	0.00 ± 0.00
17	254 ± 58.22	475 ± 28.87	38 ± 5.00	11.25 ± 1.65	8.90 ± 1.18	1.25 ± 0.77
18	380 ± 45.90	360 ± 47.09	80 ± 0.00	1.00 ± 0.50	0.00 ± 0.00	0.00 ± 0.00
19	120 ± 32.20	380 ± 76.99	80 ± 0.00	0.60 ± 0.30	0.20 ± 0.40	0.00 ± 0.00
20	243 ± 45.96	615 ± 133.35	80 ± 0.00	0.80 ± 1.13	0.90 ± 0.14	0.10 ± 0.14
21	380 ± 40.20	525 ± 106.07	80 ± 0.00	0.30 ± 0.42	0.20 ± 0.28	0.00 ± 0.00
22	420 ± 34.50	400 ± 53.22	80 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
23	260 ± 42.43	425 ± 17.07	80 ± 0.00	0.10 ± 0.14	0.50 ± 0.71	0.00 ± 0.00
24	470 ± 84.85	515 ± 120.21	80 ± 0.00	14.50 ± 0.71	7.90 ± 0.14	0.00 ± 0.00
25	320 ± 39.50	420 ± 45.33	70 ± 10.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
26	203 ± 111.22	496 ± 71.27	70 ± 18.00	0.32 ± 0.30	0.24 ± 0.22	0.04 ± 0.09
27	280 ± 58.20	430 ± 23.67	80 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
28	247 ± 24.95	763 ± 94.45	80 ± 0.00	0.00 ± 0.00	0.80 ± 0.57	1.40 ± 1.70
29	250 ± 45.30	650 ± 43.50	80 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
30	362 ± 104.02	336 ± 80.81	80 ± 0.00	0.24 ± 0.26	0.28 ± 0.23	0.00 ± 0.00
31	450 ± 74.83	352 ± 52.63	80 ± 0.00	0.80 ± 0.55	0.76 ± 0.84	0.04 ± 0.09
32	390 ± 56.44	520 ± 44.00	80 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
33	308 ± 45.60	468 ± 34.00	80 ± 0.00	0.20 ± 0.20	0.00 ± 0.00	0.00 ± 0.00
34	420 ± 78.40	640 ± 59.80	80 ± 0.00	1.60 ± 1.50	0.00 ± 0.00	0.00 ± 0.00
$LSD_{0.05}$	137.413	249.242	15.218	0.606	1.454	2.824
$LSD_{0.01}$	175.188	317.759	19.402	0.772	1.854	3.600

$\bar{X}$ mean value, SD - standard deviation; LSD - least significant difference; CMA3 - chromomycin; AB - aniline blue; AO- acridine orange

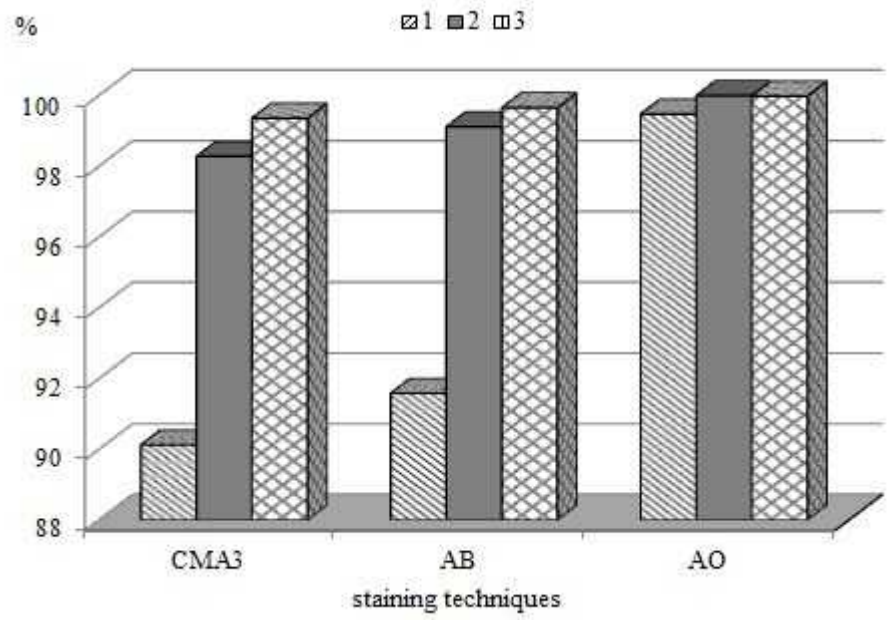

Fig. 1. The proportion of normal sperm resulting from staining with different techniques in three age groups (1 - boars aged up to 18 months, 2 - boars aged 18-24 months, 3 - boars older than 24 months). Abbreviations as in Table 1

Table 2. Semen characteristics (average ±SD) and evaluation of sperm chromatin structure for the 3 staining techniques compared with spermatozoa depending on boar age

Boar	Ejaculation volume (mL)	Concentration ($x10^6$/mL)	Progressive motility (%)	Results of CMA3	Results of AB	Results of AO
	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$
1	330.14 ± 101.63	468.41 ± 153.91	60.45 ± 28.20	9.88 ± 15.83	8.41 ± 13.39	0.53 ± 0.75
2	348.20 ± 91.36	518.72 ± 149.17	80.00 ± 0.00	1.73 ± 3.97	0.89 ± 2.13	0.01 ± 0.04
3	272.29 ± 117.94	555.96 ± 158.65	76.92 ± 9.61	0.65 ± 0.75	0.35 ± 0.40	0.02 ± 0.10
$LSD_{0.05}$	50.957	73.044	7.023	0.170	2.991	3.615
$LSD_{0.01}$	63.370	90.837	8.734	0.211	3.720	4.495

Abbreviations as in Table 1

Table 3. Correlation between sperm chromatin structure for the 3 staining techniques

Traits	Results of CMA3	Results of AB	Results of AO
Results of CMA3	1	0.98*	0.45*
Results of AB	0.98*	1	0.44*
Results of AO	0.45*	0.44*	1

*$P \leq 0.05$
Abbreviations as in Table 1

Discussion

The functional effectiveness of germ cells, called fertility, is determined by sperm chromatin and the integrity of sperm DNA. If there are no abnormalities at this level, there is a strong probability of a high fertilisation rate and normal embryonic and foetal development (4). DNA integrity can be a useful indicator of fertility in humans and animals (4). Research on animals which were experimentally administered chemotherapeutic agents causing DNA damage showed that damage to the paternal genome affected the development of the embryo, and even had a negative effect on subsequent generations (9, 14). Factors within sperm are also thought to influence pregnancy losses (27, 31). Some authors indicate that defects of the sperm nucleus or chromatin may reduce the fertilisation rate and affect the development of the foetus (21). Sperm chromatin abnormalities may have several causes: disturbances during histone-to-protamine exchange, a lack of protamines, disturbances at the level of sperm maturation in the epididymis, or maintenance of chromatin stability during ejaculation (23), which is dependent on zinc ions (4, 11).

CMA3 is a fluorochrome that can be used to detect cells with poor chromatin condensation due to abnormal protamination (15). Some authors suggest that sperm stained with CMA3 can be used to differentiate morphologically distinct populations from an entire group of morphologically normal sperm (25). Other studies have shown that an increased percentage of sperm cells in the semen reacting positively to CMA3 is linked to reduced fertility and increased frequency of recurrent pregnancy loss in humans (23). The role of proteins is not limited to protamines, which are the most abundant chromatin proteins in the sperm cell (22). Histones play an equally important role. The use of AB staining makes it possible to detect the presence of histones in the sperm nucleus, which is commensurate with a smaller quantity of protamines in these sperm cells (27). AB selectively stains histones due to their high lysine content (22). No threshold value has yet been established beyond which cells take on the stain and which would indicate functional disturbances in the sperm cell, because the nucleus of each gamete contains a certain small quantity of histones, which is the physiological norm. Thus the determination of such values in particular species continues to be the subject of experiments by numerous researchers, as histones are presumed to be involved in the sperm maturation process and perhaps also in fertilisation and early embryonic development (22). However, the proper proportions must be maintained, as abnormal histone retention leaves chromatin with substantially lower condensation and thus greater susceptibility to DNA damage (22). Studies show that histones associated with DNA are associated with genes that have an important role in cell differentiation in the early stages of embryonic development (17). In the spermatids, histones are replaced by protamines leading to an intense chromatin condensation (18). This process is also aimed at protecting sperm DNA from enzymatic attack by nucleases and polymerases (23). Sperm DNA is so well protected that, in contrast to chromatin contained in somatic cells, it is resistant to sonication (34) and nucleases (33). The reorganisation of chromatin during spermatogenesis and its concentrated structure in mature sperm cells is believed to protect against DNA damage (31), to silence transcription, and to facilitate movement of the germ cell so that it can safely reach the oocyte (7). Critical chromatin structures are thought to occur in sperm cells before they undergo meiotic division (1). The absence of protamines or abnormal protamine structure leads to abnormalities in the chromatin condensation process in the sperm nucleus, which affects the quality and morphology of the semen, and thus its fertilisation capacity (32, 37). Kazerooni *et al.* (23) showed that the percentage of sperm cells exhibiting progressive movement and the morphology of sperm are significantly positively correlated with the percentage of sperm cells with damaged chromatin detected by CMA3 staining, and negatively correlated in the case of AB staining. Moreover, a correlation was found between abnormal semen parameters (motility disorders and morphology) and a deficiency of protamines detected by CMA3 and AB (23). The present study showed a high positive correlation (0.98) between CMA3 and AB staining, as increased frequency of sperm stained with CMA3 was often accompanied by an increased percentage of sperm stained with AB. These 2 staining methods complement one another. There was also a positive but considerably lower correlation (0.44 - 0.45) found between staining with AO and staining with AB and CMA3. This may be explained by the fact that sperm cells that do not take on the AO stain may have normal DNA structure while epigenetically having abnormal protamination, which is only detected by the CMA3 and AB staining. Furthermore, normal chromatin condensation has been shown to enable the formation of a smaller sperm head with a more hydrodynamic shape, which indirectly contributes to the final shape of the sperm head (3). This is confirmed by the observations of other authors, which showed that sperm cells with abnormalities in chromatin packaging often had enlarged or atypically shaped heads (6).

The AO staining additionally used in this study determines the percentage of sperm cells with DNA fragmentation. Despite the use of AO in research on the sperm of various species, the level of pathological DNA fragmentation has been determined only for humans. A percentage of sperm cells with damaged DNA of up to 15% is considered normal, 15%-25% is thought to reduce fertility, and a level above 25% is considered to constitute a high risk of infertility (12, 13). In the present study, a small percentage of sperm cells with fragmented DNA exceeding 1% was observed in only 2 individuals. These were young boars less than 1.5 years of age.

Staining techniques are often labour-intensive, but may be a source of important and precise diagnostic information on the sperm population in the ejaculate (22). This is of particular importance in the case of young animals used for reproduction and expected to ensure a high selection response within the species. An earlier diagnosis based on additional tests would enable stricter selection and elimination of males with fertility disorders, which could be replaced with high-quality breeders. Boars intended for reproduction have already undergone preliminary selection, in terms of both breeding value and an assessment of their libido and semen. These, however, are routine tests that do not include an evaluation of chromatin structure, which has a key role in male fertility. Tests evaluating chromatin structure are worth implementing during the breeder's exploitation, as abnormal protamination may be an individual trait, but may also result from other factors, such as age or environmental factors. Even when morphological evaluation of spermatazoa shows no abnormalities, the cells may be deficient in protamines. In the present study, on the basis of staining with

CMA3 and AB, this phenomenon was clearly visible in 3 boars, whose semen had a high percentage of sperm with chromatin abnormalities. These were young animals aged less than 1.5 years. In one of them asthenozoospermia was observed. The highest percentage of sperm cells with abnormal protamination was also noted in this individual: over 41% in the case of CMA3 staining and over 35% with AB staining. In this case there were also isolated sperm cells with DNA fragmentation. Another individual whose semen contained sperm with low motility was boar 17, whose ejaculates had a volume of about 250 mL and a sperm concentration of over 470 000/mm^3. The percentage of sperm cells exhibiting progressive movement in the ejaculate did not exceed 40, which is below the reference values for the species. Sperm cells with abnormal protamination were also observed in the semen of this individual - 11.25% with CMA3 staining and 8.90% with AB, as well as DNA fragmentation at a level of 1.25% detected by AO staining.

Asthenozoospermia may have been the effect of severe disruptions in sperm morphogenesis leading to the lack of formation of a mitochondrial sheath or to the simultaneous appearance of defects in the midpiece and non-specific anomalies in the sperm tail. It should be mentioned that impairment or absence of sperm motility (asthenozoospermia) is a typical example of male infertility. Such a boar should always be eliminated from the population used at the insemination station. Nevertheless, it is an interesting subject for clinical research.

Research conducted on young (1.5-year-old) bulls, from which sperm was isolated post mortem from the tail of the epididymis, also showed a slight deviation in the number of sperm cells with abnormally packaged genetic material (2). The present study shows that the quality of boar semen improves as sexual development progresses. This is evidenced by the decreasing percentage of sperm cells with abnormal protamination and DNA fragmentation in older animals. Results obtained by Banaszewska and Kondracki (5) clearly demonstrate that sexual development in boars continues to progress during their exploitation for reproduction beyond the age of 2 years, which may also influence the improvement in sperm quality in older individuals.

To sum up, semen staining with the methods used in this study may be an effective diagnostic tool for evaluating sperm quality with respect to chromatin abnormalities in the sperm nucleus. Staining with AO identifies abnormal, single-strand DNA structure in the sperm cell. The use of AB enables identification of abnormal histone retention, while CMA3 identifies sperm cells with protamination disorders. Examination of nuclear proteins with regard to infertility shows the importance of normal chromatin structure on the functioning of sperm cells. An advantage of the techniques presented is that they do not require costly computer-assisted sperm analysis systems, which are not at the disposal of most insemination stations.

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References

1. Aitken R.J., Irvine D.S.: Fertilization without sperm. Nature 1996, 379, 493–495.
2. Andraszek K., Banaszewska D., Czubaszek M., Wójcik E., Szostek M.: Comparison of different chromatin staining protamine family of sperm nuclear techniques for bull sperm. Arch Tierz 2014, 57, 1–15.
3. Balhorn R.: The protamine family of sperm nuclear proteins. Genome Biol 2007, 8, 227-234.
4. Barrat Ch.L.R., Aitken R.J., Björndahl L., Carrell T., de Boer P., Kvist U., Lewis S.E.M., Perreault S.D., Perry M.J., Ramos L., Robaire B., Ward S., Zini A.: Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications a position report. Hum Reprod 2010, 25, 824–838.
5. Banaszewska D., Kondracki S.: An assessment of the breeding maturity of insemination boars based on ejaculate quality changes. Folia Biol (Krakow) 2012, 60, 151–162.
6. Belokopytova I.A., Kostyleva E.I., Tomilin A.N., Vorob'ev V.I.: Human male infertility may be due to a decrease of the protamine P2 content in sperm. Mol. Reprod Dev 1993, 34, 53–57.
7. Carrell D.T., Emery B.R., Hammoud S.: Altered protamine expression and diminished spermatogenesis: what is the link? Hum Reprod Update 2007, 13, 313–327.
8. Carrell D.T., Hammoud S.S.: The human sperm epigenome and its potential role in embryonic development. Mol Hum Reprod 2010, 16, 37–37.
9. Delbès G., Hales B.F., Robaire B.: Toxicants and human sperm chromatin integrity. Mol Hum Reprod 2010, 16, 14–22.
10. De Vos A., Van De Velde H., Joris H., Verheyen G., Devroey P., Van Steirteghem A.: Influence of individual sperm morphology on fertilization, embryo morphology and pregnancy outcome of intracytoplasmic sperm injection. Fertil Steril 2003, 79, 42–48.
11. Dias G.M., Retamal C.A., Tobella L., Arnholdt A.C., Lopez M.L.: Nuclear status of immature and mature stallion spermatozoa. Theriogenology 2006, 66, 354–365.
12. Evenson D.P., Jost L.K., Marshall D., Zinaman M.J., Clegg E., Purvis K., de Angelis P., Claussen O.P.: Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 1999, 14, 1039–1049.
13. Evenson D.P., Jost L.K., Corzett M., Balhorn R.: Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. J Androl 2000, 21, 739–746.
14. Fernandez-Gonzalez R., Moreira P.N., Perez-Crespo M., Sanchez-Martin M., Ramirez M.A., Pericuesta E., Bilbao A., Bermejo-Alvarez P., De Dios Hourcade J., De Fonseca F.R., Gutiérrez-Adán A.: Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol Reprod 2008, 78, 761–772.
15. Franken D.R., Franken C.J., de la Guerre H., de Villiers A.: Normal sperm morphology and chromatin packaging:

comparison between aniline blue and chromomycin A3 staining. Andrologia 1999, 31, 361–366.
16. Fraser L., Strzeżek J.: The use of comet assay to assess DNA integrity of boar spermatozoa following liquid preservation at 5°C and 16°C. Folia Histochem Cytobiol 2004, 42, 49–55.
17. Hammoud S.S., Nix D.A., Zhang H., Purwar J., Carrell D.T., Cairns B.R.: Distinctive chromatin in human sperm packages genes for embryo development. Nature 2009, 460, 473–478.
18. Hecht N.B.: Regulation of 'haploid expressed genes' in male germ cells. J Reprod Fertil 1990, 88, 679–693.
19. Hofmann N., Hilscher B.: Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. Hum Reprod 1991, 6, 979–982.
20. Hu J.H., Li Q.W., Jiang Z.L., Li W.Y.: Effects of different extenders and DNA integrity of boar spermatozoa following freezing-thawing. Cryobiology 2008, 57, 257–262.
21. Iranpour F.G., Nasr-Esfahani M.H., Valojerdi M.R., al-Taraihi T.M.: Chromomycin A3 staining as a useful tool for evaluation of male fertility. J Assist Reprod Genet 2000, 17, 60–66.
22. Jenkins T.G., Emery B.R., Carrell D.T.: Assays used in the study of sperm nuclear proteins. In: *Sperm Chromatin – Biological and Clinical Applications in Male Infertility and Assisted Reproduction*. Springer Science+Business Media, London, 2011, pp. 233–241.
23. Kazerooni T., Asadi N., Jadid L., Kazerooni M., Ghanadi A., Ghaffarpasand F., Kazerooni Y., Zolghadr J.: Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. J Assist Reprod Genet 2009, 26, 591–596.
24. Lewis J.D., Song Y., de Jong M.E., Bagha S.M., Ausio J.: A walk through vertebrate and invertebrate protamines. Chromosoma 2003, 111, 473–482.
25. Lolis D., Georgiou I., Syrrou M., Zikopoulos K., Konstantelli M., Messinis I.: Chromomycin A3 staining as an indicator of protamine deficiency and fertilization. Int J Androl 1996, 19, 23–27.
26. Nagvenkar P., Zaveri K., Hinduja I.: Comparison of the sperm aneuploidy rate in severe oligozoospermic and oligozoospermic men and its relation to intracytoplasmic sperm injection outcome. Fertil Steril 2005, 84, 925–931.
27. Nanassy L., Carrell D.T.: Paternal effects on early embryogenesis. J Exp Clin Assist Reprod 2008, 5, 2.
28. Nasr-Esfahani M.H., Salehi M., Razavi S., Anjomshoa M., Rozbahani S., Moulavi F., Mardani M.: Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. Reprod Biomed Online 2005, 11, 198–205.
29. Pérez-Llano B., Enciso M., Garcia-Casado P., Sala R., Gosálvez J.: Sperm DNA fragmentation in boars is delayed or abolished by using sperm extenders. Theriogenology 2006, 66, 2137–2143.
30. Puscheck E.E., Jeyendran R.S.: The impact of male factor on recurrent pregnancy loss. Curr Opin Obstet Gynecol 2007, 19, 222–228.
31. Rousseaux S., Caron C., Govin J., Lestrat C., Faure A.K., Khochbin S.: Establishment of male-specific epigenetic information. Gene 2005, 345, 139–153.
32. 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.
33. Sotolongo B., Lino E., Ward W.S.: Ability of hamster spermatozoa to digest their own DNA. Biol Reprod 2003, 69, 2029–2035.
34. Tateno H., Kimura Y., Yanagimachi R.: Sonication *per se* is not as deleterious to sperm chromosomes as previously inferred. Biol Reprod 2000, 63, 341–346.
35. Tejda D.P., Cameron J.C., Normal A.: A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril 1984, 42, 87–91.
36. Wong A., Chuan S.S., Patton W.C., Jacobson J.D., Corselli J., Chan P.J.: Addition of eosin to the aniline blue assay to enhance detection of immature sperm histones. Fertil Steril 2008, 90, 1999–2002.
37. Wu T.F., Chut D.S.: Sperm chromatin. Fertile grounds for proteomic discovery of clinical tools. Mol Cell Proteomics 2008, 7, 1876–1886.