Correlation between mitochondrial dysfunction of spermatozoa and their biological adequacy

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Abstract. One of the most significant indicators affecting male fertility is the sperm nuclear and mitochondrial DNA fragmentation index (DFI). DNA damage depends on biotic and abiotic factors, leading to oxidative stress (O.S.). This research aimed to investigate the relationship between mitochondrial dysfunction of spermatozoa and their biological adequacy. The research material was frozen-thawed sperm samples from the Ayrshire, Russian Black Pied Holstein, Russian Red Pied Holstein, Limousin, and Polled Russian breeding bulls. Assessments of mobility, morphology, and fragmentation index were performed using computer-assisted sperm analysis (CASA). It was found that there is a negative correlation between sperm activity and mitochondrial dysfunction with the correlation coefficient r = -0.24. The incidence of abnormal spermatozoa correlated with sperm dysfunction r = 0.77. The nDNA fragmentation index in chromatin varied from 0 to 25%.

1 Introduction

The sustainability and economics of bovine husbandry depend on obtaining high conception rates through artificial insemination (A.I.). The success of A.I. and optimal use of genetically superior bulls is determined by the bull's fertility, which in turn depends on sperm quality in frozen-thawed semen doses [1]. Sperm fertilizing potential is determined by their ability to reach the oocyte, complete fertilization, and sustain embryogenesis, partly determined by the quality of sperm DNA [1,2]. The accurate prediction of bull fertility is of primary economic importance in the Russian breeding industry.

According to the research data, 20% of breeding bulls are infertile, and about 40% have reduced fertility [3]. Consequently, the assessment of the biological adequacy of sperm is of great economic and biological importance.

With the help of diagnostic tests using computer technology (CASA, DNA technology), it is possible to predict fertility with sufficient reliability [1,2]. Biotic and abiotic factors influence male fertility.

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Spermatogenesis is a collection of numerous complex biological processes. The chromosomal DNA of spermatozoa is compressed into a less than one-nanometer space in the cell nucleus due to protamines and histones' actions, which results in a too dense, almost crystalline structure [4]. DNA fragmentation in chromatin occurs due to the influence of various factors, which leads to idiopathic infertility. There is a high negative correlation between sperm DNA fragmentation and fertility (DFI over 25%) [5]. The index of DNA fragmentation in breeding bulls corresponds to a moderate degree (DFI 11.9%), but it is more than 80% in some individuals.

The reason for oxidative stress (O.S.) is an increase in the production of molecules containing oxygen in an unreduced form - ROS, exceeding the body's antioxidant defense [6]. The disparity between the endogenous antioxidants concentration and free radicals in spermatozoa results in reactive oxygen species (ROS) generation.

The antioxidant sperm system is formed by specialized antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase), non-specialized antioxidant enzymes (glutathione, pantothenic acid, coenzyme Q10, or ubiquinone, steroid hormones, flavonoids, vitamins A, E, K, C, D and chelator proteins). Numerous cellular antioxidant system components, inhibiting excess free radicals (F.R.), maintain O.S. at a physiologically adequate level [6,7,8]. Violation of oxidative balance (overproduction of F.R.s, insufficient activation, depletion of factors of the antioxidant system) leads to oxidative stress. Oxidative stress affects motility, DNA fragmentation, and acrosome response [9,10].

Mammalian sperm comprises different types of cells, such as mature and immature sperm, at various stages of spermatogenesis, leukocytes, and epithelial cells. Of these, leukocytes and immature spermatozoa are the primary sources of ROS [8]. An increased level of ROS in semen (plasma) is the reason for a decrease in the fertilizing ability of the ejaculate, sperm pathology (oligospermia, asthenozoospermia, teratozoospermia, leucospermia), damage to mitochondria in germ cells (mitochondrial dysfunction and mutations of mitochondrial DNA) and genetic apparatus [6, 9 -11].

Research confirms the effect of mitochondria and mDNA status on fertility. The movement of spermatozoa requires a large amount of energy, and mitochondria produce it in the form of ATP; therefore, the bioenergetic function of mitochondria is of decisive importance for the motive activity of sperm, which is one of the leading indicators characterizing male fertility [5, 7, 10].

Mitochondrial DNA (mtDNA) is highly unstable and mutable. MtDNA mutations are possible at different stages of spermatogenesis. A distinctive feature of mtDNA is the absence of intron sequences in all genes. It is quickly replicated, and a high content of oxidants in its environment leads to mutations in mtDNA with a frequency 10–20 times higher than in nuclear DNA [12].

This research aimed to study the relationship between mitochondrial dysfunction of spermatozoa and their biological adequacy.

2 Materials and methods

The research was carried out in the laboratory of cell engineering of L.K. Ernst Federal Research Center for Animal Husbandry. The research material was frozen-thawed sperm samples from the Ayrshire, Russian Black Pied Holstein, Russian Red Pied Holstein, Limousin, and Polled Russian breeding bulls (n = 57). An expanded spermogram was performed to study the biological adequacy of spermatozoa.

Sperm was thawed in a biological thermostat (thawer) at a temperature of 38 °C. For this, the straws with sperm were removed with sterile, pre-cooled in nitrogen, tweezers, shaken to remove the remaining nitrogen, and quickly immersed in for 8-10 s. For uniform thawing of the seed, the straw was stirred in a circular motion. The straw was then removed from the

thermostat, thoroughly wiped with a sterile gauze cloth, and shaken to bring the air bubble down to the stopper. Bulls and semen consignments were entered into the database and semen evaluation cards of the breeding bulls. With sharp scissors, the cork from one end of the straw was cut off, and this end was released into a test tube with a 2.9% sodium citrate solution. The volume of sodium citrate solution was 1 cm^3. The ARGUS-CASA software was used to assess the semen parameters (sperm motility and morphology). Slides were prepared to study the assessment of the condition of the acrosome, nDNA, and mDNA.

To prepare smears, a sperm-drop was applied to a defatted glass slide, spread in a thin layer with a plastic spatula, and dried at room temperature. To assess the state of the acrosomes on the slides, they were stained with a Diff-Quick set by the attached protocol.

The acrosome state was assessed using a Nikon Eclipse Ni microscope equipped with a Nikon DS-Qi2 camera with high resolution (4908x3264) (Nikon, Japan) NIS-Elements software. The acridine orange test (A.O. test) was used to determine the nuclear and mitochondrial fragmentation index. For this, slides were fixed in a solution (methanol + acetic acid, ratio 3: 1) for 60 min at + 4 °C (in a refrigerator). Slides were stained in 1% acridine orange solution. The analysis results were visualized using fluorescence microscopy equipped with a cube of GFP-LEX 460-500 DM 505BA510 fluorescent filters with a 15x eyepiece and a 20x, 40x, 100x objective.

Statistical data processing was performed using the M.S. Office Excel software package. Statistical significance was determined by Student's t-test for independent variables.

3 Results and discussion

Motility is commonly believed to be one of the most crucial sperm attributes associated with fertility [13]. A shortage in energy supply causes decreased motive activity. Relatively lesser sperm motility has been associated with lesser fertility rates. However, A.I. stations generally strive to mitigate this effect by producing only semen doses that contain large numbers of motile spermatozoa [14]. These cattle industry strategies have resulted in the impact of sperm motility on fertility having become less evident [15]. The number of spermatozoa with straight-forward movement (SFM) should be at least 40%. In our samples, this indicator averaged $45 \pm 3\%$: inbreeding bulls of dairy breeds (Ayrshire, Russian Black Pied Holstein, Russian Red Pied Holstein) on average $42 \pm 1\%$, in meat bulls (Limousin and Polled Russian) - $43 \pm 2\%$ (Fig. 1).
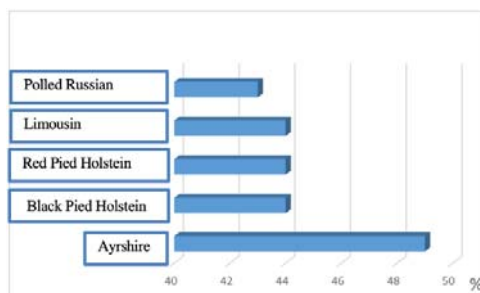

Fig. 1. The content of spermatozoa with SFM in the semen of sire bulls of different breeds.

Determination of motility gives an indirect estimate of the viability of the sperm. For a more accurate characterization and further use of the sperm, it is necessary to conduct a morphological study of spermatozoa and the condition of their acrosomes [6-11]. In terms of localization, sperm defects can be divided into groups: anomalies of the head, neck, middle part, and tail, and their combination. In our samples, defects in the flagellum structure and the central part of the spermatozoa were the most frequent (Fig. 2). Simultaneously,

inbreeding bulls of beef breeds (Limousin 4.8%, Polled Russian 4.5%), the content of abnormal spermatozoa was less common than in dairy breeds (Ayrshire 5.1%, Russian Black Pied Holstein 6.8%, Russian Red Pied Holstein 7%).
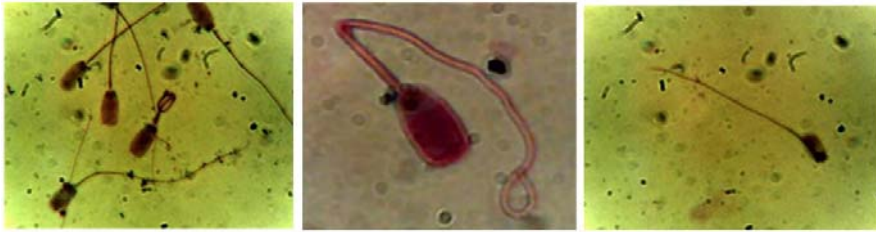

Fig. 2. Sperm with abnormal morphology.

Morphometric analysis showed that spermatozoa's content with abnormal morphology averaged $4.48 \pm 0.41\%$ and $5.95 \pm 0.50\%$ with damaged acrosome.

The study of chromatin's condition showed that the fragmentation index of nuclear DNA is up to 25%.

This indicator has a wide range of variations and depends on the breed, direction of productivity, and individual characteristics. The coefficient of variation in the studied biological samples was 77%. The frequency of sperm with abnormal morphology ranged from 4.34 to 100%. (Fig. 3).
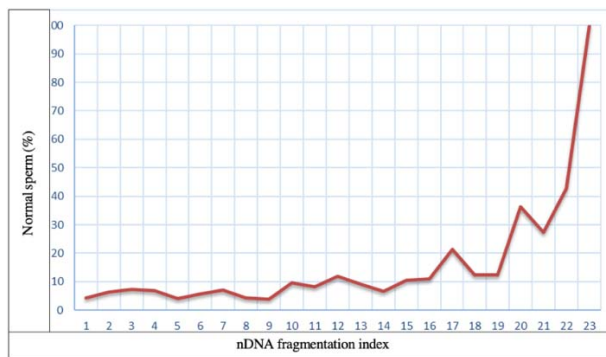

Fig. 3. The frequency of sperm with abnormal morphology.

Mitochondria have a crucial involvement in sustaining standard functionality and energy homeostasis, yielding in the activation of the motility apparatus [10]. However, during replication, a three-stranded mtDNA (intermediate) is formed, which is very susceptible to deletions [4]. Point mutations and deletion of mtDNA fragments lead to the pathology of mitochondrial respiratory dysfunction, one of the causes of pathospermia [4,15]. Studies of sperm ultrastructures show that mtDNA mutation and other damage to sperm mitochondria are accompanied by a decrease in their motor activity and the formation of morphological abnormalities [1, 9-11]. Some authors believe that an excessive amount of morphologically abnormal spermatozoa forms is an indicator of violations of chromatin packing and DNA integrity [4]. Spermatozoa with impaired motility have a more significant number of mtDNA copies per cell than progressively motile spermatozoa [11].

The results of the correlation relationship analysis show that there is a negative correlation between sperm and mitochondrial dysfunction. The correlation coefficient between these indicators was $r = -0.24$. According to our study results, in samples with a high frequency of mitochondrial dysfunction, the proportion of spermatozoa with abnormal morphology was higher, the correlation coefficient was $r = 0.77$.

4 Conclusion

In conclusion, the study of frozen-thawed semen samples from breeding bulls by the method of detailed spermogram and analysis of the correlation dependence between individual parameters confirms the influence of the functional state of mitochondria (mtDNA and nDNA) on the biological adequacy of spermatozoa. Disturbances in mtDNA structure negatively correlate with sperm activity (r = -0.24) and the number of sperm with abnormal morphology (r = 0.77). Abnormal morphology was recorded more often in the flagellum structure and the middle part of the spermatozoa. The nDNA fragmentation index in chromatin varied from 0 to 25%.

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