BF-1672
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RESEARCH ARTICLE Indian Journal of Animal Research

Thiol Cathepsins and Oxidative Modification of Stallion's Seminal Plasma Proteins with Normal and Low Percentage of Live Spermatozoa Post Cryopreservation

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10.18805/IJAR.BF-1672

ABSTRACT

Background: Stallion sperms are very sensitive to cryopreservation. Free radicals generated during cryopreservation lead to spermatozoa membrane damage, decline in their viability and change the activity of cysteine cathepsins. On the other hand, cathepsins may involve in the utilization of oxidatively modified proteins. The present study was conducted to assess cysteine cathepsins activity and level of oxidatively modified proteins in seminal plasma of stallions with a low (<30%) and normal (>30%) percentage of live spermatozoa post cryopreservation

Methods: A total of 33 breeding stallions comprising 6 stallions of the Budennovskaya breed, 22 Arabian breed, 4 Soviet Heavy-Draft and 1 Bashkir breed. We assessed standard quality parameters in fresh and cryopreserved sperm, as well as cysteine cathepsins B,L,H activity, the level of oxidatively modified proteins in stallions seminal plasma with a low (<30%) and normal (>30%) percentage of live spermatozoa post cryopreservation.

Result: The group with a low percentage of live spermatozoa was characterized by decreased sperm quality, increased level of aldehyde dinitrophenylhydrazones (p=0.045) and ketone dinitrophenylhydrazones (p=0.049) of a neutral nature, as well as increased activity of cathepsin L (p=0.029). Evaluation of activity of cathepsin L and the concentration of oxidatively modified proteins in the seminal plasma can serve as a marker in the selection of stallions for sperm cryopreservation.

Key words: Cathepsins B, Cathepsins H, Cathepsins L, Oxidative modification of proteins, Seminal plasma, Stallions.

INTRODUCTION

Sperm cyopreservation is the most effective method of preserving animal genetic material, while stallions sperm cryopreservation has not reached a high level of efficiency compared to other farm animals yet (Alvarenga *et al*., 2016, Ghallab *et al*., 2019, Kumar *et al*., 2011). This is mainly due to the greater sensitivity of stallion sperm to the freezing process, the higher severity of oxidative stress and damage to plasma membranes that activate cell damage resulting in DNA fragmentation and cell death (Contreras *et al*., 2020). Freezing and subsequent thawing of sperm reduces the qualitative characteristics of biological material (Atroshchenko *et al*., 2019). The technique is quite time-consuming and economically costly, animals selection semen cryopreservation is quite necessary. New diagnostic markers that could help to predict the quality of native and thawed sperm are required (Atroschenko *et al*., 2022). Seminal plasma is not just an inert medium delivering sperm to the female reproductive tract but also provides sperm with proper nutrition and protection (McGraw *et al*., 2015). Such components of seminal plasma like glucose, Ca, ALP and LDH and seminal parameters play a key role in capacitation and onward movement of the spermatozoa in Marwari stallions and Poitou jacks (Talluri *et al*., 2017). The consideration of components of seminal plasma as prognostic markers of the success of cryopreservation is relevant. One of such components may be lysosomal cysteine proteases.

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How to cite this article: Shitikova, A.M., Atroshchenko, M.M. and Zvyagina, V.I. (2023). Thiol Cathepsins and Oxidative Modification of Stallion's Seminal Plasma Proteins with Normal and Low Percentage of Live Spermatozoa Post Cryopreservation. Indian Journal of Animal Research. DOI: 10.18805/IJAR.BF-1672.

Submitted: 14-06-2023 **Accepted:** 31-08-2023 **Online:** 28-09-2023

Cysteine proteinases are a group of enzymes of the papain family, namely, cathepsins B, C, L, F, H, K, O, S, V, X and W. These enzymes differ in their structure, cellular and tissue localization, regulation and expression and biochemical properties (Turk *et al*., 2012, Fomina *et al*., 2016, Fomina *et al*., 2017). Cathepsins B, H, L and C are the most common among lysosomal cysteine proteinases. Previously it was believed that the function of cathepsins is limited by their participation in the breakdown of proteins during necrosis or autophagy. Later many specific functions of lysosomal cysteine proteases were discovered, such as antigen presentation, angiogenesis, bone remodeling,

keratinocyte differentiation, activation of prohormones (Turk *et al*., 2012). In addition, thiol proteinases are involved in processes associated with cancer progression, such as apoptosis, invasion and metastasis, angiogenesis, hyperproliferation of tumor cells (Rudziñska *et al*., 2019).

The role of cathepsins in reproduction is of greater interest. Cathepsins are involved in a complex relationship of events during maturation and remodeling of the seminal epithelium in rodent testes (Mathur *et al*., 1997). Cathepsins B, D, L are involved in the growth and maturation of ovarian follicles (Liu *et al*., 2021). It was found that the activity of cathepsins B, L, S in the human seminal plasma is ten times higher than blood serum. Seminal plasma cathepsins are associated with prostasomes and may bind to spermatozoa and migrate to the egg, affecting not only the male but also the female reproductive tract (Inayat *et al*., 2012). In seminal plasma of men with oligozoospermia and azoospermia, the activity of asparagine cathepsin D is lower than that in healthy men (Dandekar and Harikumar, 1997). In stallions, cathepsin D is synthesized in the head and appendage of the testis (Fouchécourt *et al*., 2000) and likely participates in the proteolytic remodeling of sperm membrane components during epididymal transit.

To date, there is little information about the role of cathepsins in sperm and seminal plasma, the question of the origin of cathepsins also raises doubts. Immunoelectronic microscopic examination conducted on differentiating rat spermatids showed that cathepsin H was detected in the acrosome at all stages of differentiation, while cathepsins B, D, L and lysosomal membrane protein LGP107 were not detected in the acrosome (Haraguchi *et al*., 2003). McDonald and Emerick (1995) discovered a molecule of cathepsin L in the acrosome of epididymal sperm cells of guinea pigs (McDonald and Emerick, 1995). Cathepsin L was found in the fluid of the accessory sex glands in Holstein bulls along with other proteins involved in the remodeling of the extracellular matrix, such as TIMP-2, clusterin. These proteins may play an important role in the acrosomal response, participating in the penetration of sperm through the Zona pellucida of the egg (Moura *et al*., 2007). In humans, apparently, cathepsin B enters the spermoplasm from the prostate gland and cathepsin L from the seminal vesicles (Inayat *et al*., 2012).

Of particular interest is the study of the relationship of cathepsins with oxidative stress. Oxidative stress is understood as the toxic effect on biological targets of reactive oxygen species (hydroxyl, peroxide and superoxide radicals) with a decrease in the functions of antioxidant protection. Reactive molecules are capable of modifying various biomolecules, such as carbohydrates, unsaturated lipids, nucleic acids, but due to the high content of proteins in biological objects, they are the most common target for the action of free radicals and oxidative modification of proteins is currently considered the most biologically significant manifestation of oxidative stress (Lone *et al*., 2019). Of all the oxidized protein derivatives, carbonyl derivatives are most often found - they are formed at a rate 10 times higher than the rate of formation of any other protein modification (Jung *et al*., 2014). In carbonyl stress, reactive aldehydes are formed, which can oxidize carbohydrates or lipids, forming carbonyl-containing intermediates, which, in turn, can lead to the carbonylation of neighboring proteins. Both reactive aldehydes and carbonyl-containing proteins inhibit papain and cathepsins B and L by glycation of the Cys25 active center (Zeng *et al*., 2006). On the other hand, oxidative modification of proteins is considered as a regulatory mechanism of cell signaling (Lennicke and Cochemé 2021) and reversible modifications of cysteine (for example, S-nitrosylation and S-thiolation) may represent additional regulatory pathways that outnumber mechanisms controlled by phosphorylation (Lalmanach *et al*., 2020).

Even more interesting in this context is the study of cathepsins, since it has become known that they are not just a target for oxidative modification, but are themselves capable of attacking oxidatively modified proteins. Thus, cathepsin G participated in the degradation of proteins oxidatively modified by 4-hydroxynonenal (Ohta *et al*., 2014) and cathepsin D and to a lesser extent cathepsins B and K in an in vitro experiment showed activity in the destruction of albumin oxidatively modified during glycation (Grimm *et al*., 2010). In the seminal plasma of men with a high level of lipid peroxidation, increased expression of cathepsin B was observed (Intasqui *et al*., 2015), which could serve as a regulatory mechanism that allows eliminating the effects of oxidative stress.

A drop in sperm viability may occur due to membrane damage during the action of free radicals formed during cryopreservation (Upadhyay *et al*., 2021). The aim of this study was to assess cysteine cathepsins activity and level of oxidatively modified proteins in seminal plasma of stallions with a low (<30%) and normal (>30%) percentage of live spermatozoa post cryopreservation.

MATERIALS AND METHODS

Animals and semen collection

The study was carried out at All-Russian Research Institute for Horse Breeding (ARRIH, Ryazan Region, Russia) and the Tersk Stud Farm N169 (Stavropol Region, Russia), Perevozsky and Pochinkovsky studs (Nizhny Novgorod region, Russia). All procedures were at par with the "European Convention for the protection of vertebrates used for experimental and other scientific purposes" ETS No. 123 (18 March 1986) and the Law of the Russia Federation on Veterinary Medicine No. 4979-1 (14 May 1993). The protocol was approved by the Local Ethics Committee of the All-Russian Research Institute for Horse Breeding (ARRIH), Ryazan Region, Russia.

There were 33 breeding stallions with an average age 11.2±6.2 years comprising 6 stallions of the Budennovskaya, 22 stallions of the Arabian, 4 of the Soviet Heavy-Draft and 1 of the Bashkir.

Clinically healthy stallions were kept in individual stalls. The stallions received hay, oats and granulated compound

feed with added minerals in accordance with the established standards and were exercised for at least 1 h daily.

Ejaculates were obtained during the breeding season of the 2022 year (February- April) with an interval of 48 hours for an artificial vagina (ARRIH model, Ryazan, Russia) for a mare in heat. After a long period of sexual rest (10 days or more), five ejaculates were collected from each stallion at 48-hour intervals.

Sperm examination

The gel was removed immediately from ejaculates. Then the sperm was filtered using a sterile gauze filter (150*60 mm). Thereafter the volume of the ejaculate, concentration of spermatozoa in 1 ml sperm, total and progressive motility were recorded. The ejaculate volume after filtration was determined using measuring cylinder. Sperm concentration was measured using the SDM1 photometer (Minitube GmbH, Tiefenbach, Germany). A computer-assisted sperm analysis (CASA) was used to evaluate the spermatozoa total (TM) and progressive motility (PM). The assessment of PM was implemented using an Argus CASA system (ArgusSoft LTD., St. Petersburg, Russia) and a Motic BA 410 microscope (Motic, Hong Kong, China) in a Mackler chamber at 37°C.

Sperm freezing and thawing

20 mL of sperm diluted at a ratio of 1:3 (v/v) with lactose-chelate-citrate-yolk (LCCY) medium containing 3.5% glycerin in aluminum tubes were frozen as per standard operating procedure (SOP) of the All-Russian Research Institute for Horse Breeding in liquid nitrogen vapor and the distance to the surface of liquid nitrogen was 20 mm (Naumenkov and Roman'kova 1971). Briefly, the polyurethane rack with sperm containing tubes was cooled from +4°C down to -127°C for 420 s at freezing rate of 3.2°C per second. The frozen sperm samples were stored in liquid nitrogen.

Another part of the ejaculate was centrifuged at 2000 g using an ELMI CM-6M centrifuge (SIA ELMI, Riga, Latvia) for 20 minutes. After examination of the supernatant by microscopy, aliquots of seminal plasma that did not contain spermatozoa were frozen at a temperature of -18°C prior to analysis.

The frozen sperm was quickly thawed in a water bath at +40°C for 90 sec. The viability of spermatozoa was assessed. Sperm smears were eosin stained and live: dead-ratio was examined using an Olympus BX41 phase contrast microscope (Olympus Corporation, Japan). Then stallions were divided into 2 groups: with a low (<30%, n=12) and normal (>30%, n=21) percentage of live spermatozoa after cryopreservation. The morphology of spermatozoa, were determined in the same smears. At least 300 spermatozoa were evaluated in each smear.

Biochemical analysis of seminal plasma

Determination of activity of cysteine cathepsins

The activity of cathepsins B, L and H was studied by the spectrofluorometric method (System 3 Scanning Spectrofluorometry, Optical technology devices, inc. Elmstord, NewYork, 10523) by Barrett and Kirschke (1981) (Barrett and Kirschke 1981). The method is based on the quantitative determination of a fluorescent compound - 7-amino-4-methylcoumarin, the product of an enzymatic hydrolysis reaction of a peptide bond in a substrate specific to each defined proteinase. The specific activity of cathepsins was expressed in ncat/g of protein. The protein content was determined by the Lowry method using a commercial kit of the Eco-Service Scientific and Practical Center (St. Petersburg).

Determination of the level of protein oxidative modification

The oxidative modification of proteins (OMP) was evaluated as per method of Dubinina *et al*., (1995). The principle of the spectrophotometric method is based on the reaction of carbonyl derivatives of oxidized amino acid residues of proteins with 2,4-dinitrophenylhydrazine (2,4-DNPH) to form 2,4-dinitrophenylhydrazones. Carbonyl derivatives of neutral amino acids were recorded at wavelengths 230, 254, 270, 280, 356 nm - neutral aldehyde-dinitrophenylhydrazones (ÀDNPH neutral) and at 363 nm and 370 nm - neutral ketone-dinitrophenylhydrazones (KDNPH neutral). Carbonyl derivatives of basic amino acids were recorded at wavelengths of 428 and 430 nm - basic aldehyde-dinitrophenylhydrazones (ÀDNPH basic) and at 434, 524, 530, 535 nm - basic ketone-dinitrophenylhydrazones (KDNPH basic). The obtained extinction values were used to plot the absorption spectrum of OMP products and further calculate the area under the absorption spectrum curve of OMP in conventional units per gram of protein (c.u/g of protein), which consisted of the sum of the areas under the absorption spectrum curve of aldehyde and ketone carbonyl derivatives (Dubinina *et al*., 1995). The calculation was carried out according to the formulas as described in the author's patent (Fomina *et al*., 2014).

Statistical analysis

Statistical analysis was carried out using the program Statistica 10 and "Microsoft Office Excel 2016" (StatSoft Inc., USA). The median value and the upper and lower quartiles were determined for each sample. The results were presented in the format Me [Q1; Q3]. The nonparametric Mann-Whitney U-test was used to check the statistical significance of the differences in the values in the studied groups. The differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

A comparative analysis of the sperm quality indicators of stallions in the studied groups showed that the group with a low percentage of live sperm after thawing is characterized by reduced progressive ($p= 0.0008$) and total sperm motility in thawed sperm ($p= 0.0006$), as well as reduced progressive motility in fresh sperm ($p= 0.033$) compared with the group

with normal percentage of live spermatozoa after cryopreservation (Table 1). In addition, statistically significant differences were found in studied groups in the percentage of spermatozoa with normal morphology (p=0.024) and live spermatozoa in native sperm (p= 0.036) (Table 1).

Analysis of the absorption spectrum of the protein oxidative modification products showed that the level of carbonyl derivatives was higher in the group with a low percentage of live spermatozoa after thawing (Fig 1). Statistically significant differences were observed with respect to aldehyde dinitrophenylhydrazones and ketone dinitrophenylhydrazones of a neutral nature (Table 2). It can be assumed that in the group with a low % of live spermatozoa, oxidative modification of sperm plasma proteins occurred due to the oxidation of neutral amino acids.

An increased level of oxidatively modified proteins is observed in the seminal plasma of stallions with a low percentage of live spermatozoa after freezing-thawing. This group of stallions is also characterized by reduced progressive motility in fresh and cryopreserved sperm, viability in fresh sperm and a low percentage of spermatozoa with normal morphology (Table 1). It is known that the production of ROS increases significantly in the presence of non-viable or morphologically abnormal spermatozoa, especially spermatozoa characterized by the presence of proximal cytoplasmic droplets or anomalies of the middle part (Ball *et al*., 2001). Under these conditions, the generation of more ROS is mainly due to the leakage of electrons from the mitochondrial electron transfer chain, followed by the reduction of molecular oxygen to form a superoxide anion (Sabeur and Ball 2006, Aitken *et al*., 2015). These events could lead to hyperproduction of free radicals, followed by the formation of oxidatively modified proteins in the spermoplasm.

Regarding the activity of lysosomal cysteine proteinases in the seminal plasma of stallions, it was found that the activity of cathepsin L in the group with a low percentage of live spermatozoa was statistically significantly higher than in the group with a normal percentage of live spermatozoa after freezing-thawing (p=0.029) (Fig 2). No significant differences were found with respect to cathepsins B and H. Apparently, an increase in the activity of only cathepsin L

Table 1: Qualitative and quantitative indicators of fresh and cryopreserved sperm of stallions.

Indicator	Group with a low percentage of live spermatozoa in thawed sperm	Group with a normal percentage of live spermatozoa in thawed sperm
Ejaculate volume, ml	29.50 [25.25;38.75]	30.00 [27.00;40.00]
Sperm concentration, million/ml	194.50 [180.00;279.50]	223.00 [189.00;294.00]
Progressive motility (FS), %	46.00 [40.25;56.50]*	58.00 [50.00;62.00]
Progressive motility (TS), %	15.00 [10.75;25.00]**	34.00 [27.00;38.00]
Total motility (FS), %	62.50 [52.75;72.00]	81.00 [63.00;90.00]
Total motility (TS), %	24.50 [15.50;36.00]**	49.00 [39.00;65.00]
Spermatozoa with normal morphology,%	69.50 [56.75;72.63]*	76.00 [70.00;86.00]
Live spermatozoa (FS),%	59.50 [47.75;67.25]*	67.00 [62.00;71.00]

Abbreviations: TS-thawed sperm; FS-fresh sperm *p<0.05 **p<0.01.

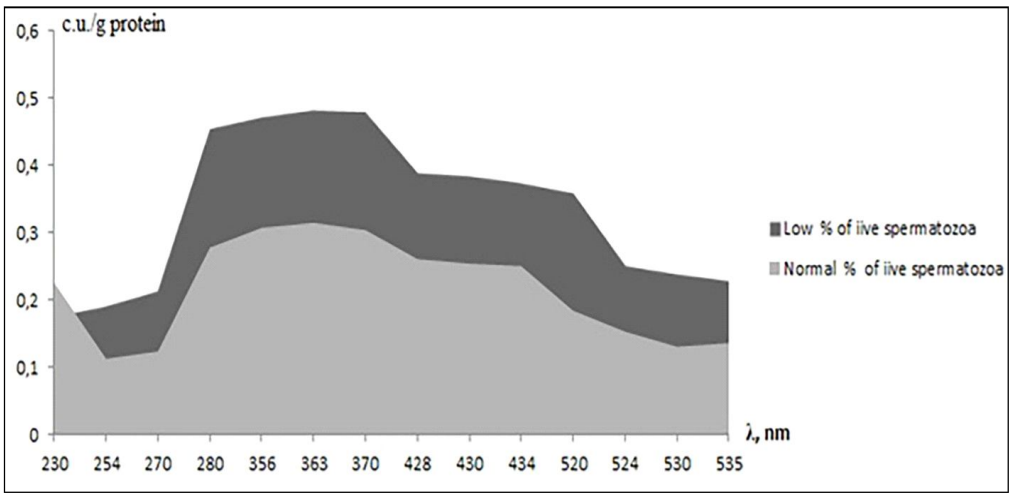


Fig 1: Absorption spectrum of oxidative modification products of proteins in groups with normal and low % of live spermatozoa after freezing-thawing (c.u./g protein), Me.

under these conditions is associated with its greater resistance to the action of carbonyl derivatives, as shown in the article of Lalmanach *et al*. (Lalmanach *et al*., 2020).

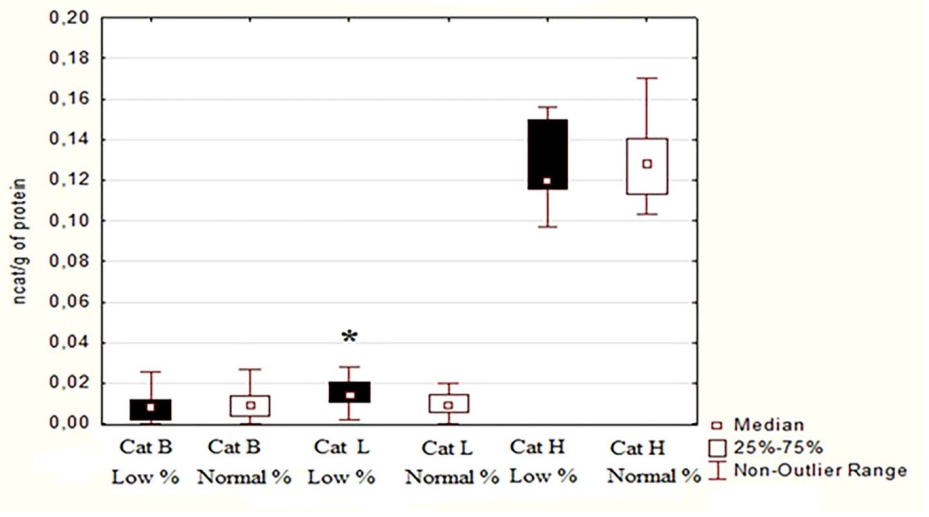
Negative correlation of cathepsin B and the resistance of sheep spermatozoa to cryopreservation was reported (Rickard *et al*., 2015). De Lazari *et al*. found that the high expression of cathepsin B in the seminal plasma of boar is associated with a decrease in the total and progressive motility of spermatozoa, as well as with poor morphology and this protease can be used as a biomarker of sperm quality. The authors of the study suggested that the results obtained may be explained by the regulation of the TLR7 and TLR9 signaling pathways by cathepsin B (De Lazari *et al*., 2019), since it became known that TLR activation aimed at combating pathogenic microorganisms may also be accompanied by a decrease in mitochondrial potential in spermatozoa and a decrease in their mobility (Zhu *et al*., 2016). In our study, the activity of cathepsin L was higher in the spermoplasm of stallions with poor semen quality. It is known that cathepsin L may also play a role in the CpG-B-dependent conformation change of TLR9 (Matsumoto *et al*., 2008). However, in stallions, TLR9 expression was detected in the spleen, lymph nodes and peripheral blood leukocytes (Zhang *et al*., 2008), while TLR8 expression was detected in semen (Swegen *et al*., 2015). In connection with the above, the question of the regulation of the TLR signaling pathway by cathepsin L in the sperm of stallions remains open.

In addition, one of the possible mechanisms explaining the connection of cathepsin L with the poor quality of cryopreserved sperm may be its participation in apoptosis (Wang *et al*., 2022). It is known that cathepsin L is able to activate Bid, which leads to conformational changes in the Bax and Bak proteins from the Bcl-2 family, then they cause classical mitochondrial apoptosis, including permeabilization of the outer mitochondrial membrane, release of cytochrome c and activation of caspases (Cirman 2004). These events may be accompanied by electron leakage from mitochondria, the occurrence of oxidative stress (Sabeur and Ball, 2006, Aitken 2015) and, possibly, an increase in the concentration of oxidatively modified proteins, which we observe in the seminal plasma of stallions with a low percentage of viable spermatozoa after thawing.

Table 2: Comparative analysis of the absorption spectrum of oxidative modification products of proteins in groups with normal and low % of live spermatozoa after freezing-thawing (c.u./g protein), Me [Q1;Q3].

Indicator	Group with a low percentage of live spermatozoa in thawed sperm	Group with a normal percentage of live spermatozoa in thawed sperm	P-value
S ADNPH neutral	34.02 [12.17;55.13]*	49.53 [34.98;76.57]	0.045
S KDNPH neutral	19.15 [12.41;24.00]*	29.99 [18.46;41.30]	0.049
S ADNPH basic	21.25 [11.94;27.33]	34.32 [12.02;57.82]	0.139
S KDNPH basic	3.67 [1.86;4.51]	5.90 [1.51;10.52]	0.160
S total	75.50 [47.84;107.89]	123.73 [70.20;183.34]	0.069

* p<0.05.



*-Statistically significant differences in groups with normal and low percentage of live spermatozoa after freezing-thawing (p<0.05).

Fig 2: The activity of lysosomal cysteine proteases in the seminal plasma of stallions with normal and low percentage of live spermatozoa after freezing-thawing, Me [Q1;Q3], ncat/g of protein.

CONCLUSION

An increase in cathepsin L activity and the concentration of oxidatively modified proteins in stallions seminal plasma with low percentage of live spermatozoa after cryopreservation in comparison with stallions with normal percentage of live spermatozoa post cryopreservation. Assessment of cathepsin L activity and the concentration of oxidatively modified proteins in the seminal plasma can serve as a potential marker for the selection of stallions for sperm cryopreservation and improvement of fertility indicators.

ACKNOWLEDGMENT

The research was carried out at the expense of the grant of the Russian Science Foundation No. 20-16-00101.

Conflict of interest

The Authors declare that there is no conflict of interest.

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