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Short Communication

PRESERVATION OF BULL SEMEN USING POLYSACCHARIDES OF BASIDIOMYCETES *HERICIUM ERINACEUS* BP 16

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ABSTRACT: For the first time, a study was conducted to determine the effectiveness of the use of *Hericium erinaceus* BP 16 polysaccharides mixed with AndroMed® to preserve the motility of bull spermatozoa after freezing and storage at -5 °C. *H. erinaceus* polysaccharides have been shown to increase the osmolarity of AndroMed® solution, which lowers its freezing point. It has been established that the motility of progressive spermatozoa after 7 days of storage at - 5°C is preserved to the greatest extent in the presence of *H. erinaceus* polysaccharides in the biological medium. At the same time, in the group with polysaccharides, there was a decrease in such indicators of mobility as the speed along the average trajectory, wobble, and beat frequency. The data obtained indicate the promise of using *H. erinaceus* polysaccharides as part of a cryoprotective solution to preserve the functional usefulness of gametes during their freezing.

Keywords: Freezing, Hericium erinaceus, Polysaccharides, Spermatozoa, Storage.

Toxicity, fertility disorders, and other disadvantages of the used protectors are the reasons for the search for new protective substances [1, 2]. Along with the search and testing of various cryoprotectants, research is being carried out to develop universal media for diluting and freezing sperm. Minitube's GMP-certified bovine and other ruminant semen extender AndroMed® provides high sperm safety at liquid nitrogen or vapor temperatures, does not contain ingredients of animal origin, and does not carry the risk of microbiological contamination [3]. The question of the preservation of gametes at other temperatures remains open; scientists from all over the world are developing it. Studies are also being carried out to expand the boundaries of the practical use of polysaccharides of basidiomycetes. *Hericium erinaceus* (Bull.) Pers. [Family: Hericiaceae], is a source of polysaccharides with a wide range of biological effects. Preparations based on the *H. erinaceus* polysaccharide complex have antioxidant [4], hepatoprotective [5], hypolipidemic [6], antimicrobial [7], antitumor [8], immunomodulatory [9], gastroprotective [10], neuroprotective [11] and other effects. We have previously shown that the polysaccharides of the basidial fungus *H. erinaceus* BP16 (PsHE) are non-toxic even in high concentrations, slow down the rate of ice crystallization in cell suspensions during their freezing, and also have an antioxidant effect under conditions of hypothermic 6°C storage of spermatozoa [12, 13]. The purpose of this study is to determine the effectiveness of the use of PsHE mixed with AndroMed® to preserve sperm motility after freezing and storage at -5°C.

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Study pattern

Bulls-producers of the Holstein breed (n=7; age: 5-6 years), constant donors of sperm, were kept by bulls breeding farm Public Corporation "KirovPlem" (Russia).

Growing and obtaining fruiting bodies of the fungus *H. erinaceus* BP16, obtaining polysaccharide fractions (PsHE), and determining their monosaccharide composition were done in the laboratory of biotechnology of plants and microorganisms of the Federal Agricultural Research Center of the North-East named N.V. Rudnitsky [12].

Determination of cryosmotic properties

Osmolar concentrations (mOsm/ L) and freezing points of aqueous solutions of substances (25% AndroMed® with 0.4% PsHE) were determined using an OSKR-1 cryoscopic osmometer (NPP Burevestnik, St. Petersburg).

Freezing and warming up

Semen was collected from each donor bull twice a week. After collection, each semen ejaculate was immediately placed in a 37°C. water bath for initial evaluation (volume, percent motility, and concentration). The motility and concentration of spermatozoa were measured using a phase contrast microscope (CX43RF Olympus, Japan). Analysis of the biological parameters of sperm was performed using the Argus-CASA system. A drop of sperm was examined at the following stages: after taking, after mixing with experimental and control solutions, and after warming up. Sperm motility, velocity distribution and kinematics were analyzed using computer analysis of sperm movement (frame rate: 60 Hz/sec, number of frames: 30, minimum cell contrast: 15, minimum cell size (pixel): 8, cell intensity, threshold straightness: 80, velocity along the average path (VAP): 25 μm/s, low VAP level: 5.0, velocity along the straight-line path (VSL): 0.05 μm/s). The whole set of fixed cells was divided according to the nature of movement into three populations - progressive, non-progressive, and immobile. In the work, only progressively motile spermatozoa were considered as the main object of further conservation. In a population of progressively motile spermatozoa, the following indicators were evaluated: velocity along the curvilinear path (VCL, μm/s), VSL (μm/s), VAP (μm/s), the amplitude of the lateral displacement of the head (ALH, μm), mean angular displacement (MAD, degrees), linearity (LIN, %), wobble (WOB, %), straightness (STR, %), beat-cross frequency (BCF, Hz). At least 200 spermatozoa were evaluated for each motility parameter from the Argus-CASA study protocol.

Freshly obtained sperm was diluted in a ratio of 1:1: in the control group - with 25% aqueous solution of AndroMed®, in the experimental group - with 25% aqueous solution of AndroMed® with the addition of PsHE at a concentration of 0.4%. Therefore, the final concentration of substances in semen was 12.5% and 0.2%, respectively. The mixture was poured into polymer conical microtubes, 0.5 mL each, and placed in a thermostat with cooling (TVL-K 050B, Russia) for storage at -5°C for 7 days. Heating was carried out in a water bath at 37°C for 2-3 seconds.

Statistical analysis

All data were analyzed for normality using the Shapiro-Wilk test. The results of the study are presented as median, 25th, and 75th centiles (Me, Q1-Q3). To assess differences, nonparametric Mann-Whitney tests were used, considering the differences to be significant at $p<0.05$. Statistical analysis was performed using the software "StatTech ver. 3.1.1".

Results and discussion

It has been established by the cryoscopic method that 0.4% PsHE increases the osmolarity of AndroMed® 25% solution from 1292 mOsm/L to 1531 mOsm/L. This contributes to a change in freezing point from - 2.44°C to - 2.87°C. Perhaps this is due to the formation of complexes between PsHE monosaccharides galactose and glucose [12, 13] and cryoprotectant glycerol, which is present in AndroMed®. These complexes, in our opinion, bind more water molecules than each component separately. This is indicated by an increase in the osmolarity of the AndroMed® solution mixed with PsHE. We believe that in the presence of glycerol-PsHE complexes at the initial stages of freezing, the temperature of water crystallization in cells will shift towards lower temperatures. This will gradually slow down cellular metabolism and promote the appearance of a fine ice structure, as well as ensure the survival of spermatozoa by reducing the risk of damage during freezing.

It was found that in the control group before freezing, the number (Me, Q1-Q3) of progressively motile spermatozoa decreased ($p<0.05$) from 48% (43-53) to 40.5% (32-49), and in the experimental group - from 48% (43-53) to 31.5% (23-40). At the same time, in the experimental group with PsHE, a decrease ($p<0.05$) in such mobility indicators as VAP, WOB, and BCF was revealed (Table 1). Polysaccharides are known

Table 1. Motility indicators in the population of progressive spermatozoa before freezing in the control and experimental groups (Me, Q1-Q3).

Motility indicators	Control group		Experimental group	
	Me	Q1-Q3	Me	Q1-Q3
VAP	47.94	35.49-57.98	40.40 *	25.12-53.20
VSL	33.31	20.36-44.71	30.78	18.74-39.73
VCL	74.65	56.79-90.39	69.27	56.19-83.82
ALH	2.13	1.73-2.99	2.27	1.88-3.02
MAD	50.09	43.69-59.44	53.94	46.89-59.41
LIN	46.34	28.73-57.28	44.13	30.67-52.38
WOB	65.16	56.52-72.78	56.46 *	39.58-68.07
STR	75.28	55.95-86.74	75.71	57.38-92.63
BCF	7.99	3.51-12.44	6.00 *	4.26-8.66

*Difference from the indicators of the control group is statistically significant $p<0.05$.

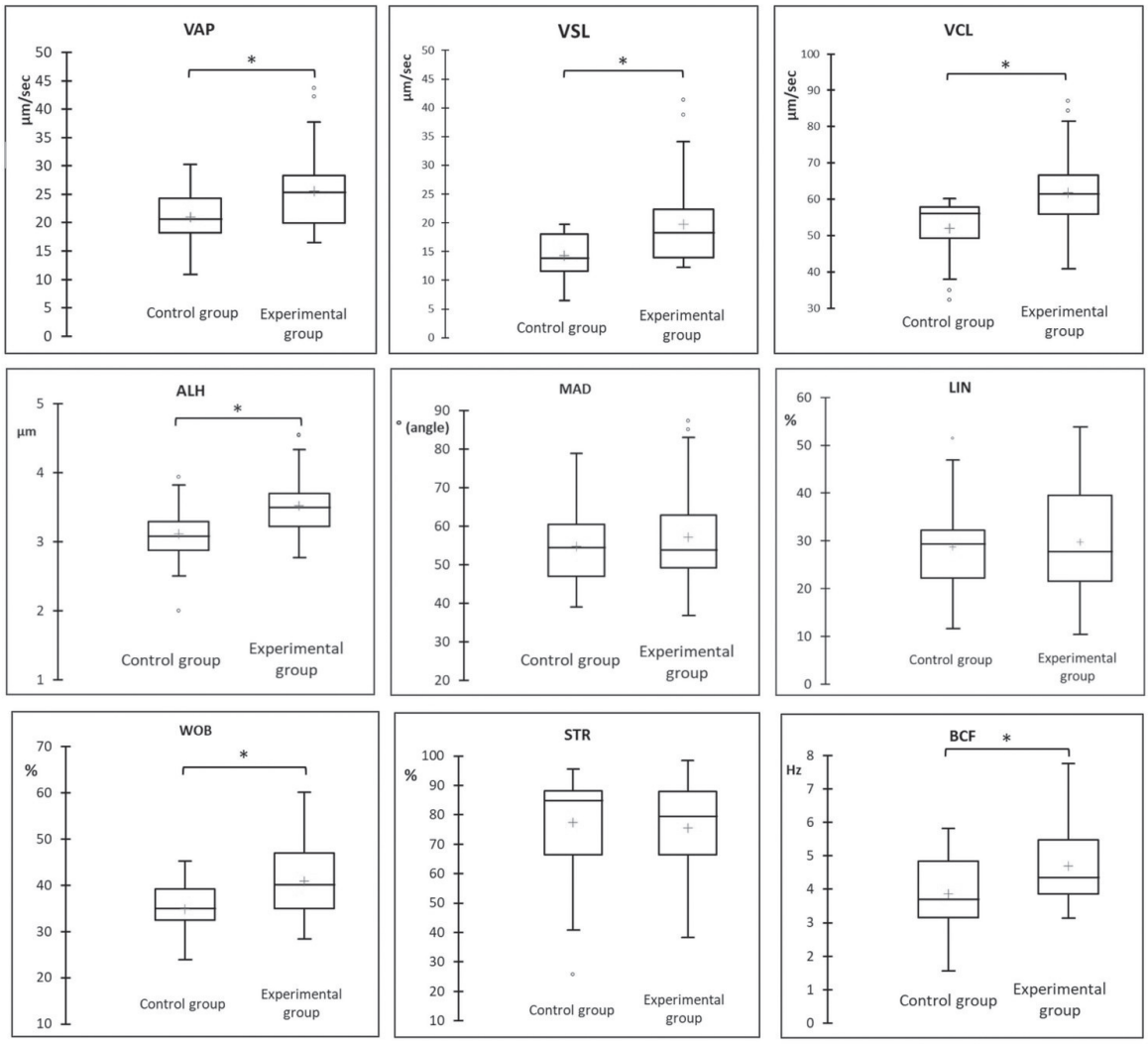

Fig. 1. Diagrams of comparison of motility indices in the population of progressive spermatozoa after 7 days of storage at -5°C in the control and experimental groups. [*difference from the indicators of the control group is statistically significant $p<0.05$].

to increase the viscosity of the medium [14]. Due to this property, in our study, the presence of PsHE in the environment of spermatozoa, even at a low concentration of 0.2%, contributes to a decrease in the VAP index, as well as the WOB and BCF indices. It was found that after 7 days of storage of the material at -5°C and subsequent warming, the ability for the translational movement was retained in 13.0% (10 - 16) of spermatozoa in the control group and 18.5% (15-19) in the experimental group with PsHE. At the same time, VCL, VSL, VAP, ALH, BCF, and WOB were statistically significantly higher ($p<0.05$) in the experimental group with PsHE (Fig. 1).

The structural and functional stability of sperm membranes plays a key role in their viability. We believe that PsHE forms bonds not only with molecules of the cryoprotectant glycerol in the extracellular medium but also with membrane molecules. The formation of biocomplexes between the components of membranes and synthetic media increases the probability of stabilization of intermolecular interactions in the membrane-medium system and can serve as an additional protective mechanism. In addition, PsHE shows no signs of cytotoxicity when mixing the medium with sperm and shows an antioxidant effect [4]; PsHE is able not only to interrupt the chain reaction of lipid peroxidation but also to bind toxic products of the radical process. We showed this feature earlier during hypothermic storage of bull semen on a medium with PsHE [13]. Even though there is an SOP/MSP standard for preserving bull sperm at liquid nitrogen temperature, the question of preserving gametes at other temperatures remains open, since the standardized freezing procedure is available only to a narrow circle of highly qualified specialists. In our study, we showed that the procedure for freezing bull sperm at a subcooling temperature (-1°C to -10°C) can be accessible to a wide range of researchers since it is cost-effective and easy to perform. In addition, from a scientific point of view, our study allows us to determine the mechanisms of sperm preservation at subcooling temperatures (-1°C to -10°C), at which intracellular water does not freeze and cell metabolism slows down only slightly.

Conclusion

The results of this study show for the first time that the presence of PsHE in AndroMed® improves sperm motility after 7 days of storage at -5°C. This gives grounds to propose PsHE as a component of a diluent for freezing bull semen based on glycerol. However, further studies of the mechanisms of the cryoprotective action of polysaccharides at other negative temperatures are needed.

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