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THE INFLUENCE OF A COMPLEX BIOLOGICALLY ACTIVE PREPARATION ON THE PRESERVATION OF THE REPRODUCTIVE POTENTIAL OF THE SPERM OF STUD RAMS AFTER CRYOPRESERVATION

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Abstract

In sheep farming, the adoption of reproductive technologies remains at a low level. One of the key challenges is the reduced efficiency of using frozen semen from stud rams. This is due to the fact that cryopreservation and subsequent thawing processes result in significant cell losses and structural damage, which greatly affect the quality of the material. To develop a method for long-term storage and preservation of the biological value of spermatozoa during the process of dilution, freezing and thawing of ram sperm, a liquid biologically active microbial preparation (MPSP) containing yeast manoprotein and sulfated cyanobacterial polysaccharides with antioxidant action, introduced into the composition of the synthetic sucrose-citrate-egg yolk (SȚJ) medium in different concentrations, was tested. It was found that the introduction of the drug MPSP into the SȚJ medium at concentrations of 0.4-0.8%/V allowed us to obtain the highest indicators of motility, survival, preservation of acrosome integrity, and speed of sperm movement compared to the control group.

Key words*: cryopreservation, diluents, motility, ram, sperm.*

INTRODUCTION

Semen cryopreservation is one of the key technologies in animal husbandry, enabling the preservation of genetic material for long-term storage and use. However, the process of freezing and thawing spermatozoa is often accompanied by damage to cellular structures, reduced motility, and overall sperm viability. In this regard, increasing attention is being directed toward finding methods that minimize the negative effects of cryopreservation. One promising approach involves the use of biologically active preparations capable of protecting cells from oxidative stress and improving their post-thaw survival.

The cryopreservation process consists of several stages, during which spermatozoa are exposed to low temperatures and chemical agents. The main damage occurs due to ice crystal formation, oxidative stress, and disruptions in cell metabolism. Research indicates that a significant portion of spermatozoa die or lose motility after cryopreservation, which substantially reduces their reproductive potential (Mazur, 1970; Erdem Öztürk et al., 2020).

Biologically active preparations, particularly those with antioxidant properties, can significantly improve semen parameters after cryopreservation. Antioxidants protect cells from damage caused by oxidative stress and help maintain the integrity of sperm cell membranes. Antioxidant - based preparations, such as vitamins C and E, as well as natural substances like plant extracts and enzymes, have shown a positive impact on semen quality (Kurmi et al., 2018; Saha et al., 2022; Sharafi et al., 2022).

Studies have demonstrated that the use of biologically active supplements during the cryopreservation of ram semen helps preserve its quality characteristics. Specifically, the addition of antioxidant agents to cryopreservation media improves sperm motility, enhances cell viability, and reduces

the percentage of spermatozoa with damaged acrosome. For example, the inclusion of vitamin C and E-based preparations, as well as selenium, has shown beneficial effects on maintaining the reproductive potential of ram semen post-thawing (Khalil et al., 2020; Darie et al., 2017).

The primary mechanism of action of biologically active preparations lies in protecting sperm cells from oxidative stress and restoring damaged cell membranes. Antioxidants prevent the formation of free radicals, which can damage membrane lipids and proteins, thereby affecting sperm motility and fertilizing ability (Yeni et al., 2022; Cibotaru et al., 2022).

The use of complex biologically active preparations, such as MPSP, represents a promising approach for improving ram semen parameters after cryopreservation. These preparations help protect spermatozoa from damage occurring during the freezing and thawing process, enhancing their motility and fertilizing capability. Continued research in this area may contribute to the development of more effective cryopreservation methods, improving reproductive outcomes in animals and ensuring the preservation of valuable genetic material.

MATERIALS AND METHODS

As research material, Tsigai rams (wool-meat-milk type) and fresh semen obtained from them were used. The sheep were kept at the sheep farm of the Technological Experimental Station "Maximovca" in the village of Maximovca, Anenii Noi district, Republic of Moldova. The maintenance and feeding conditions met zoohygienic standards. Semen was collected from each ram twice a week, developing a conditioned sexual reflex in breeding rams for an artificial vagina. Ejaculates were obtained in the arena of the artificial insemination station using an artificial vagina from IMV, France.

To evaluate the effectiveness of the complex biologically active microbial preparation (MPSP), it was added as an additional component to the synthetic sucrose-citrate-egg yolk (SȚJ) medium with the following composition: 6.4 g of sucrose, 0.8 g of sodium citrate, 10 ml of egg yolk, 5 ml of glycerin, and 100 ml of bidistilled water. Semen was frozen in pellet form. This preparation was specifically developed and produced by the Institute of Microbiology and Biotechnology of the Technical University of Moldova.

The MPSP preparation contains 200 mg ml^{-1} of manoprotein derived from brewer's yeast and 200 mg ml^{-1} of sulfated polysaccharides extracted from the biomass of *Arthrospira* (*Spirulina*) *platensis*. The method of production, biochemical composition, antioxidant, and enzymatic activity of the manoprotein complex obtained from the sedimentary biomass of brewer's yeast used in the creation of MPSP were studied and published by the authors (Chiseliţa et al., 2022). According to the literature, sulfated cyanobacterial polysaccharides also exhibit antioxidant and antimicrobial activity (Periyannan et al., 2019). To study the protective effect of the MPSP preparation during deep freezing of ram semen at –196°C, ejaculates with motility ranging from 70 to 100% and a sperm concentration of no less than 2 billion/ml were used. The qualitative analysis of semen samples was conducted using the "CEROS" computer program. Leja slides were used for semen analysis, providing the precision necessary for reliable sperm evaluation, ensuring that spermatozoa are evenly distributed across the slide.

The following parameters were assessed: motility and the percentage of spermatozoa with progressive movement, sperm motility speed (average velocity - VAP, straight-line velocity - VSL, and curvilinear velocity - VCL) after dilution, after equilibration, and after the freezing-thawing process. Additionally, sperm abnormalities and the percentage of spermatozoa with damaged acrosomes were analyzed after dilution and post-freezing-thawing.

The qualitative parameters of semen were evaluated using the "CEROS" computer software. This program allows for a rapid analysis of semen samples and provides a comprehensive set of qualitative indicators.

The experimental data obtained were statistically processed using the "Excel" program for Windows on a computer.

RESULTS AND DISCUSSIONS

One of the main methods for improving the breeding and productive characteristics of sheep is the rational and effective use of breeding rams.

Cryopreservation of semen plays a crucial role in this process, allowing for the accumulation and preservation of significant genetic material reserves for future use.

The success of cryopreservation largely depends on the composition of the synthetic medium used, which provides protection for spermatozoa and maintains their functional integrity throughout the freezing and storage process.

In our experiments, the protective effect of the MPSP preparation was studied. MPSP exhibits protective properties during the cryopreservation of spermatozoa and stabilizes cellular and lysosomal membranes.

The preparation was introduced as an additional component into the synthetic SȚJ medium at different concentrations. The most optimal results were observed when the percentage of the preparation in the medium was 0.4-0.8%/v.

The experimental data on the effect of the biologically active MPSP preparation at concentrations of 0.4-0.8%/v on the motility indicators of spermatozoa during cryopreservation at –196°C are presented in Table 1.

Table 1. Motility indicators of ram spermatozoa

Specification	Indices		Control SȚJ	Experienced SȚJ+MPSP(%)		
				0.4	0.6	0.8
after dilution	Motility, %		81.6±3.0	80.2±1.1	82.0±1.9	81.6±1.8
	Progressive, %		38.6±4.6	42.6±2.2	41.8±1.9	41.4±3.7
	Sperm motility speed, μm/s	VAP	122.9±4.6	152.0±1.9	151.1±3.0	144.6±6.9
		VSL	97.2±4.7	123.1±1.8	119.3±2.1	119.0±5.0
		VCL	191.8±10.4	233.1±5.0	239.4±3.0	198.5±18.2
after equilibration	Motility, %		81.0±2.4	80.8±1.3	83.2±1.9	81.2±3.1
	Progressive, %		36.8±2.1	42.2±4.1	42.0±1.9	38.8±3.5
	Sperm motility speed, μm/s	VAP	115.9±4.8	115.1±5.5	122.8±6.6	112.3±1.8
		VSL	92.0±5.3	101.7±4.8	99.9±3.4	92.1±3.5
		VCL	189.1±4.7	195.6±9.0	199.6±13.4	190.7±4.4
after freezing-thawing	Motility, %		39.8±1.8	48.2±2.7*	49.2±1.7**	44.0±2.1
	Progressive, %		18.0±1.7	25.4±2.5*	28.0±2.4**	19.8±0.7
	Sperm motility speed, μm/s	VAP	92.1±3.5	101.2±1.7*	105.5±2.7*	104.6±5.7
		VSL	73.6±3.1	86.9±3.1*	91.5±4.5*	89.6±3.9*
		VCL	145.4±8.3	165.0±4.3	170.7±8.5	154.5±5.7

Note: *P≤0.05;**P≤0.01; VAP - average velocity, VSL - straight-line velocity, and VCL - curvilinear velocity; SȚJ - synthetic medium for ram sperm; MPSP - complex biologically active microbial preparation.

Table 1 provides a comprehensive overview of the motility indicators of ram spermatozoa assessed under three key conditions: after dilution, after equilibration, and after freezing-thawing.

An analysis of the experimental data presented in Table 1 shows that the biologically active microbial preparation MPSP, introduced as an additional component into the SȚJ medium at concentrations of 0.4-0.8% v/v, did not negatively affect the quality of ram sperm after dilution.

Sperm motility after dilution in the control group was 81.6±3.0%, while in the experimental groups, the values ranged from 80.2±1.1% to 82.0±1.9%. Progressive motility was also higher in the experimental groups, reaching a maximum value of 42.6±2.2% where the MPSP concentration was 0.4%.

The sperm motility speed indicators (VAP, VSL, VCL) were notably higher in the experimental groups, particularly where the MPSP concentration in the diluent was 0.4% and 0.6%, indicating a beneficial effect of the supplement on maintaining sperm quality during the initial stages of processing.

Significant differences were identified after the freezing-thawing process. Sperm motility in the control group was 39.8±1.8%, whereas in the experimental groups with MPSP concentrations of 0.4% and 0.6%, sperm motility reached 48.2±2.7% (P<0.05) and 49.2±1.7% (P<0.01), respectively, indicating the efficacy of the tested preparation. The percentage of

spermatozoa with progressive motility in the experimental groups with 0.4% and 0.6% MPSP concentrations was also significantly higher ($P<0.05$; $P<0.01$) than in the control group.

Outstanding results were observed for sperm motility speed parameters (VAP, VSL, VCL) in the experimental groups compared to the control. These findings emphasize the effectiveness of MPSP in maintaining the viability of spermatozoa, as evidenced by statistically significant improvements in motility and kinetic parameters.

Protective media used for the dilution and storage of sperm during cryopreservation may include various agents with protective properties. However, it is important to consider that despite their protective functions, these agents can also exert negative effects, potentially altering the morphology of spermatozoa. In this context, the analysis of morphological parameters of spermatozoa when using such agents becomes a crucial aspect of sperm preservation technology.

To address this, a study was conducted to determine the number of spermatozoa with abnormalities in the semen samples. The experimental data are presented in Figure 1.

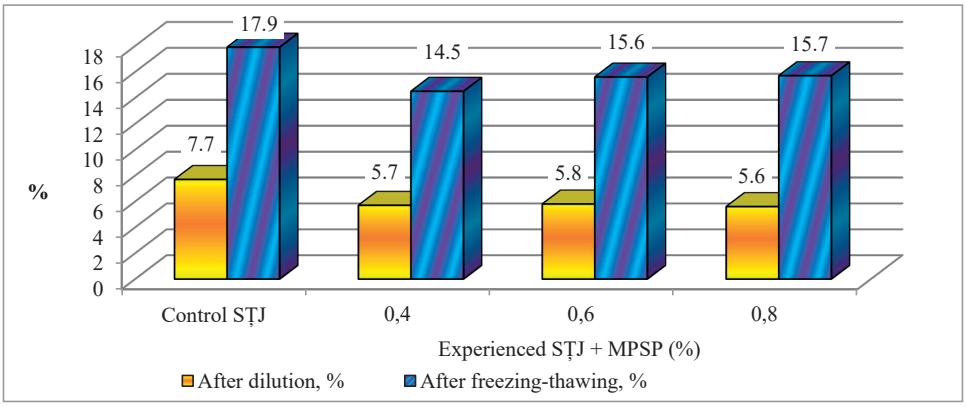

Figure 1. Percentage of Abnormal Spermatozoa, %

The experimental data presented in Figure 1 indicate that the percentage of spermatozoa with morphological abnormalities after dilution in the control group averaged 7.7%. In contrast, the experimental groups showed lower percentages, suggesting a reduction in abnormalities with the addition of the MPSP preparation. The 0.4% concentration of MPSP in the diluent demonstrated a decrease to 5.7% of spermatozoa with abnormalities, while the 0.6% concentration reduced this to 5.8%, and the 0.8% concentration exhibited the lowest percentage of abnormalities at 5.6%.

These results indicate that the addition of MPSP helps maintain the structural and functional integrity of spermatozoa during the dilution phase.

The percentage of abnormalities increased after the freezing-thawing process, which is typical due to the stress of cryopreservation. The control group showed a significant rise in abnormal spermatozoa to 17.9%, indicating the negative impact of freezing-thawing on sperm quality. However, the experimental groups with the complex biologically active preparation MPSP, introduced as an additional component into the SȚJ medium at various concentrations, demonstrated lower percentages of abnormalities compared to the control group, showcasing the protective effect of the experimental preparation. The group with 0.4% MPSP added to the main diluent recorded 14.5%, the 0.6% group showed 15.6%, and the 0.8% group had 15.7%. The reduction in abnormalities in these groups underscores the effectiveness of MPSP in mitigating cryodamage.

Scientific studies indicate that various factors, including exposure to toxic substances, environmental stress conditions, and genetic

anomalies, can affect the acrosome. Specifically, the process of cryopreservation can lead to acrosomal damage, thereby reducing the fertilizing potential of spermatozoa after thawing (Watson, 2000). Consequently, new cryoprotective methods and media containing antioxidants and other protective agents are being developed and implemented to help maintain acrosomal integrity during the freezing and storage of sperm (Holt & North, 1994).

To preserve acrosomal integrity during the freezing and storage process, a complex biologically active preparation, MPSP, was developed, and its effect on the percentage of spermatozoa with damaged acrosomes was studied. The experimental data are presented in Table 2.

Table 2. Spermatozoa with Damaged Acrosomes, %

Specification	Control SȚJ	Experienced SȚJ + MPSP(%)		
		0.4	0.6	0.8
After dilution, %	5.9±0.8	4.0±0.5	4.0±0.6	4.2±0.4
After freezing-thawing, %	26.9±0.8	24.4±0.4*	24.5±0.6	24.9±1.1

Note: * P≤0.05;
SȚJ - synthetic medium for ram sperm; MPSP - complex biologically active microbial preparation.

The experimental data presented in Table 2 show that the tested preparation did not have a negative impact on this parameter. The percentage of spermatozoa with damaged acrosomes in the control group after dilution was 5.9±0.8%.

The experimental groups demonstrated lower percentages of damaged acrosomes, indicating a positive effect of MPSP on maintaining the structural integrity of spermatozoa. Both MPSP concentrations of 0.4% and 0.6% recorded 4.0 ± 0.5% and 4.0 ± 0.6%, respectively, showing a notable reduction in acrosomal damage compared to the control. The 0.8% concentration resulted in a slightly higher but still improved percentage of 4.2 ± 0.4%. These results suggest that the inclusion of MPSP helps protect the acrosome during the initial stages of sperm processing.

After thawing, the percentage of damaged acrosomes in the control group increased significantly to 26.9±0.8%, emphasizing the stress induced by the cryopreservation process. The addition of MPSP to the diluent medium demonstrated a protective effect, with all experimental groups showing lower percentages of acrosomal damage compared to the control. The 0.4% MPSP group recorded 24.4±0.4%, which was statistically significant (*P≤0.05) compared to the control. The 0.6% and 0.8% concentrations had slightly higher percentages of 24.5±0.6% and 24.9±1.1%, respectively, but they were still lower than the control group. This indicates that MPSP provided a degree of protection against cryodamage at all tested concentrations.

CONCLUSIONS

The addition of the complex biologically active microbial preparation MPSP to the diluent medium for ram semen has a positive effect on sperm motility, progressive movement, and motility speed, particularly after the freezing-thawing process. Values marked with P≤0.05 and P≤0.01 indicate statistically significant differences compared to the control group.

The use of the experimental preparation as an additive in the diluent medium helps reduce morphological and functional anomalies in ram spermatozoa both after dilution and after the freezing-thawing process.

The protective role of the experimental preparation in maintaining acrosomal integrity in ram spermatozoa during the cryopreservation process is confirmed by values marked with P≤0.05. Although other experimental groups did not reach statistical significance, they still demonstrated a reduced level of damage compared to the control, showcasing the overall beneficial effect of MPSP.

Statistically significant improvements in the experimental groups indicate that the complex biologically active microbial preparation MPSP can be an effective additive for maintaining the viability and fertilizing potential of ram spermatozoa during cryopreservation.

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