



Research on the Influence of Dilution Media on Ram Sperm Cryopreservation

Elena CIBOTARU^{1*}, Grigore DARIE², Irina DJENJERA², Oleg CHISALITA³

¹ Faculty of Agricultural, Forestry and Environmental Sciences, Republic of Moldova

² Practical Scientific Institute of Biotechnologies in Animal Husbandry and Veterinary Medicine, Maximovca village, Anenii Noi District

³ Institute of Mycobiology and Biotechnology, Technical University of Moldova

*Corresponding author: E. Ciubotaru e-mail: cibotaruelena7@gmail.com

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RESEARCH ARTICLE

Abstract

In the study, ram sperm from the Moldovan Karakul breed bred in the experimental section of the Scientific Institute of Biotechnology Practices in Animal Husbandry and Veterinary Medicine. Ejaculates with a mobility of no less than 70% and a concentration of 2.0 billion/ml were admitted for processing. For dilution, GTJ, STJ and STJ+ dilution media were used, in which the biologically active preparation extracted from cyanobacteria at the Institute of Microbiology and Biotechnology of the Technical University of Moldova was introduced as an additional component. After resuscitation, the mobility, rectilinear movements of the spermatozoa, acrosome integrity were studied. The experimental results showed that the STJ+ dilution medium in which the biologically active preparation extracted from the cyanobacteria was introduced gave the possibility to obtain after thawing the mobility of 49.2+1.7, spermatozoa with rectilinear movements VAP – 105.5+2.7, VSL-91.5+4.5, VCL-170.7+8.5, sperm abnormalities -14.5%, acrosome integrity 24.4+0.4 compared to the other GTS and STJ dilution media, which gave us the possibility to recommend for the cryopreservation of ram sperm the STJ+ medium in which the biologically active preparation was introduced as an additional component in a concentration of 0.4-0.7% for the cryopreservation of ram sperm.

Keywords: Ram; diluent; motility; freezing; thawing.

INTRODUCTION

Artificial insemination represents one of the most effective methods of genetic improvement used in sheep breeding, offering the possibility to efficiently exploit rams with high genetic potential (Miclea et al., 2010; Zăhan, 2017). However, compared to cattle or goats, the fertility outcomes obtained with cryopreserved semen in sheep are often lower, mainly due to the increased sensitivity of ram spermatozoa to the freeze-thaw process (Aibazov, 2003). The membrane of ram sperm cells is rich in polyunsaturated fatty acids, which makes them highly vulnerable to oxidative stress and lipid peroxidation during exposure to extreme temperature variations (Milovanov & Sokolovskaia, 1980; Milovanov et al., 1989). These characteristics lead to a heightened susceptibility to structural damage typically associated with cryogenic procedures.

Recent international literature emphasizes that oxidative stress is one of the main factors responsible for reducing the post-thaw viability of spermatozoa, and that the inclusion of antioxidant compounds can significantly improve cell survival (Leahy & Gadella, 2015). The extender used during the cryopreservation process plays a crucial role in protecting germ cells. Several authors have demonstrated that the presence of

biologically active additives with antioxidant and membrane-stabilizing potential can reduce the cellular damage induced by freezing (Aibazov & Malahova, 2002). Within modern sheep reproduction programs, optimizing freezing procedures is considered essential for improving fertility rates (Paulenz et al., 2018).

The biologically active preparation PD, extracted from cyanobacteria and protected by patent MD-4804, contains polysaccharides, amino acids, vitamins, and other natural compounds with potential antioxidant and membrane-stabilizing properties. Recent studies confirm that biologically active extracts can reduce acrosomal damage and enhance post-thaw sperm motility (Yimer et al., 2022).

Although the STJ extender is widely used in the Republic of Moldova, information regarding the efficiency of its supplementation with PD remains limited. In this context, the aim of the present study was to evaluate the influence of the biologically active preparation PD, introduced at different concentrations into the STJ base extender, on the functional, morphological, and acrosomal parameters of ram semen before and after cryopreservation, with the purpose of identifying the optimal concentration for maintaining semen quality.

MATERIALS AND METHODS

The research was carried out on breeding rams of the Moldovan Karakul breed (3 rams of the same breed), maintained under identical conditions at the experimental farm of STE "Maximovca." Semen was collected during the reproductive season (autumn). A total of eight ejaculates were included in the study (n = 8 ejaculates). Semen was collected manually using an artificial vagina. Only ejaculates meeting the following criteria were admitted into the experiment: initial motility $\geq 70\%$; concentration $\geq 2 \times 10^9$ spermatozoa/mL; and normal morphological and macroscopic appearance.

The base extender STJ is commonly used in the Republic of Moldova for ram semen cryopreservation. It contains components with osmoprotective, antioxidant, and membrane-stabilizing properties. The biologically active preparation PD is a bioproduct extracted from cyanobacteria, rich in polysaccharides, amino acids, and bioactive metabolites with antioxidant potential. The product is protected by patent MD-4804 and was incorporated into the STJ extender at concentrations ranging from 0.2% to 1.4%.

Semen was gradually diluted in STJ and STJ + PD extenders and then slowly cooled to $+4^\circ\text{C}$. A cooling equilibration period followed. The diluted semen was cryopreserved in pellet form, by dropping it into liquid nitrogen vapors. The resulting pellets were stored in liquid nitrogen at -196°C . Subsequently, pellets were thawed in water at 37°C for 30 seconds.

Semen evaluation was performed using the computerized CEROS system, analyzing: total motility (%), progressive motility (%), kinetic parameters (VAP, VSL, VCL; $\mu\text{m/s}$), morphological abnormalities (%), and acrosomal integrity (%). Results are expressed as mean \pm standard deviation (SD). Differences were assessed using the Student's t-test, with statistical significance set at $p \leq 0.05$ and $p \leq 0.01$.

RESULTS AND DISCUSSIONS

The widespread use of artificial insemination is largely attributed to the possibilities of semen dilution and preservation. Semen can be used for insemination in diluted form only after an appropriate equilibration period following dilution, as this process helps maintain sperm motility for a longer duration. The development of preservation methods, which allow semen to be used for insemination over variable time intervals, has been a decisive factor in the expansion of artificial insemination in livestock species. Semen preservation is achieved through dilution, gradual temperature reduction, and maintaining low temperatures throughout the storage period.

To identify the most suitable dilution medium, a series of variants have been proposed, with variable degrees of effectiveness. Greater progress can be achieved by incorporating biologically active substances into diluents, which have protective roles on spermatozoa during cryopreservation. Experimental data regarding the influence of the biologically active preparation PD on the morphological parameters of ram spermatozoa during their cryopreservation at -196°C are presented in Table 1.

Table 1. Morphological indices of ram spermatozoa (8 ejaculates)

Specification	Indices	Dilution media							
		Control STJ	Experimental STJ +PD(%)						
			0,2%	0,4%	0,6%	0,7%	0,8%	1,0%	1,4%
Reanimation	Motile, %	39,8 $\pm 1,8$	42,6 $\pm 2,0$	48,2 $\pm 2,7^*$	49,2 $\pm 1,7^{**}$	45,2 $\pm 2,1$	44,0 $\pm 2,1$	43,4 $\pm 1,2$	43,4 $\pm 1,6$
	Progressive, %	18,0 $\pm 1,7$	20,6 $\pm 2,0$	25,4 $\pm 2,5^*$	28,0 $\pm 2,4^{**}$	20,4 $\pm 1,5$	19,8 $\pm 0,7$	19,4 $\pm 1,2$	19,2 $\pm 1,2$

	Kinetic parameters, $\mu\text{m/s}$	VAP	92,1 $\pm 3,5$	92,2 $\pm 3,5$	101,2 $\pm 1,7^*$	105,5 $\pm 2,7^*$	104,0 $\pm 5,4$	104,6 $\pm 5,7$	103,4 $\pm 5,9$	96,8 $\pm 6,4$
		VSL	73,6 $\pm 3,1$	76,3 $\pm 3,7$	86,9 $\pm 3,1^*$	91,5 $\pm 4,5^*$	89,0 $\pm 4,2^*$	89,6 $\pm 3,9^*$	81,4 $\pm 4,5$	82,5 $\pm 2,7$
		VCL	145,4 $\pm 8,3$	160,4 $\pm 5,3$	165,0 $\pm 4,3$	170,7 $\pm 8,5$	161,9 $\pm 3,0$	154,5 $\pm 5,7$	141,1 $\pm 8,6$	137,6 $\pm 8,3$

* $P \leq 0,05$; ** $P \leq 0,01$.

Based on the data presented in Table 1, the introduction of the biologically active preparation PD into the STJ extender had a clearly beneficial effect on sperm motility after the thawing process. Compared to the control group, in which total motility reached $39.8 \pm 1.8\%$, the experimental variants containing PD showed significant increases in this parameter. The highest values were recorded at PD concentrations of 0.4% and 0.6%, where total motility reached $49.2 \pm 1.7\%$ ($p \leq 0.01$) and $48.2 \pm 2.7\%$ ($p \leq 0.05$), respectively. These results indicate that supplementation of the STJ extender with PD contributes to maintaining cell functionality following the thermal stress associated with cryopreservation.

Progressive motility followed the same trend, being significantly higher in the groups receiving 0.4% and 0.6% PD compared to the control. This increase suggests enhanced protection of the propulsion mechanisms of the sperm flagellum, which is essential for the fertilizing potential of spermatozoa.

Analysis of the kinetic parameters (VAP, VSL, and VCL) confirmed the results obtained for motility. In the PD-treated groups, the values of these parameters were consistently higher than in the control. For example, VAP reached $105.5 \pm 2.7 \mu\text{m/s}$ at the 0.6% PD concentration, compared with $92.1 \pm 3.5 \mu\text{m/s}$ in the control group. The VSL and VCL values showed a similar upward pattern, reaching their highest levels at PD concentrations of 0.4–0.7%.

These significant increases in movement velocities indicate improved preservation of the structural and functional integrity of the flagellum, suggesting a protective effect of PD on sperm membranes and energetic mechanisms during freezing.

The experimental data are presented in Table 2.

Table 2. Morpho-functional abnormalities of ram spermatozoa (8 ejaculates), (%)

Specification		Indices							
		Martor STJ	Experimental STJ +PD(%)						
			0,2	0,4	0,6	0,7	0,8	1,0	1,4
Reanimare	Macrocephaly	0,8 $\pm 0,4$	0,6 $\pm 0,2$	0,4 $\pm 0,2$	0,6 $\pm 0,4$	0,6 $\pm 0,2$	0,6 $\pm 0,2$	0,4 $\pm 0,2$	0,6 $\pm 0,4$
	Microcephaly	0,8 $\pm 0,4$	0,6 $\pm 0,4$	0,4 $\pm 0,2$	0,4 $\pm 0,2$	0,4 $\pm 0,2$	0,4 $\pm 0,2$	0,4 $\pm 0,2$	0,4 $\pm 0,2$
	Broken neck	8,4 $\pm 2,0$	6,2 $\pm 1,8$	6,4 $\pm 1,3$	8,0 $\pm 2,1$	7,8 $\pm 1,8$	6,6 $\pm 1,8$	7,0 $\pm 1,9$	7,4 $\pm 1,4$
	Coiled tail	8,2 $\pm 1,5$	6,8 $\pm 1,7$	6,6 $\pm 1,9$	6,2 $\pm 1,5$	6,6 $\pm 1,9$	7,6 $\pm 1,7$	7,2 $\pm 1,9$	7,4 $\pm 2,1$
	Headless	8,0 $\pm 0,5$	7,0 $\pm 1,8$	7,4 $\pm 1,9$	7,6 $\pm 1,6$	7,4 $\pm 1,3$	7,8 $\pm 0,8$	5,4 $\pm 1,0$	7,6 $\pm 1,3$
	Tailless	9,6 $\pm 0,5$	8,2 $\pm 2,2$	7,8 $\pm 1,6$	8,4 $\pm 1,4$	7,8 $\pm 2,1$	8,4 $\pm 0,9$	7,8 $\pm 2,0$	6,8 $\pm 1,1$
	Total	35,8	29,4	29	31,2	30,6	31,4	28,2	30,2
Total, %	17,9	14,7	14,5	15,6	15,3	15,7	14,1	15,1	

Analysis of Table 2 shows that the total percentage of morphological abnormalities increased after the cryopreservation process in all variants, which is characteristic of the freeze–thaw technology used for ram semen. However, the experimental variants treated with PD exhibited lower values of abnormalities compared with the control group, indicating a beneficial effect of the biologically active preparation on the structural stability of sperm cells.

In the control group, the total percentage of abnormalities was 17.9%, whereas in the experimental groups the values ranged between 14.1% and 15.6%, with the lowest percentage recorded in the 1.0% PD variant (14.1%). These differences suggest that PD may contribute to maintaining the morphological integrity of spermatozoa, reducing the occurrence of defects frequently induced during processing, such as broken neck, coiled tail, headless, or tailless spermatozoa.

The presence of bioactive compounds in PD, with antioxidant and membrane-stabilizing potential, may explain the reduction in morpho-functional alterations observed in this study.

In another series of experiments, the effect of the complex biologically active preparation PD was evaluated on the percentage of spermatozoa with damaged acrosomes in cryopreserved ram semen. The experimental data are presented in Figure 1.

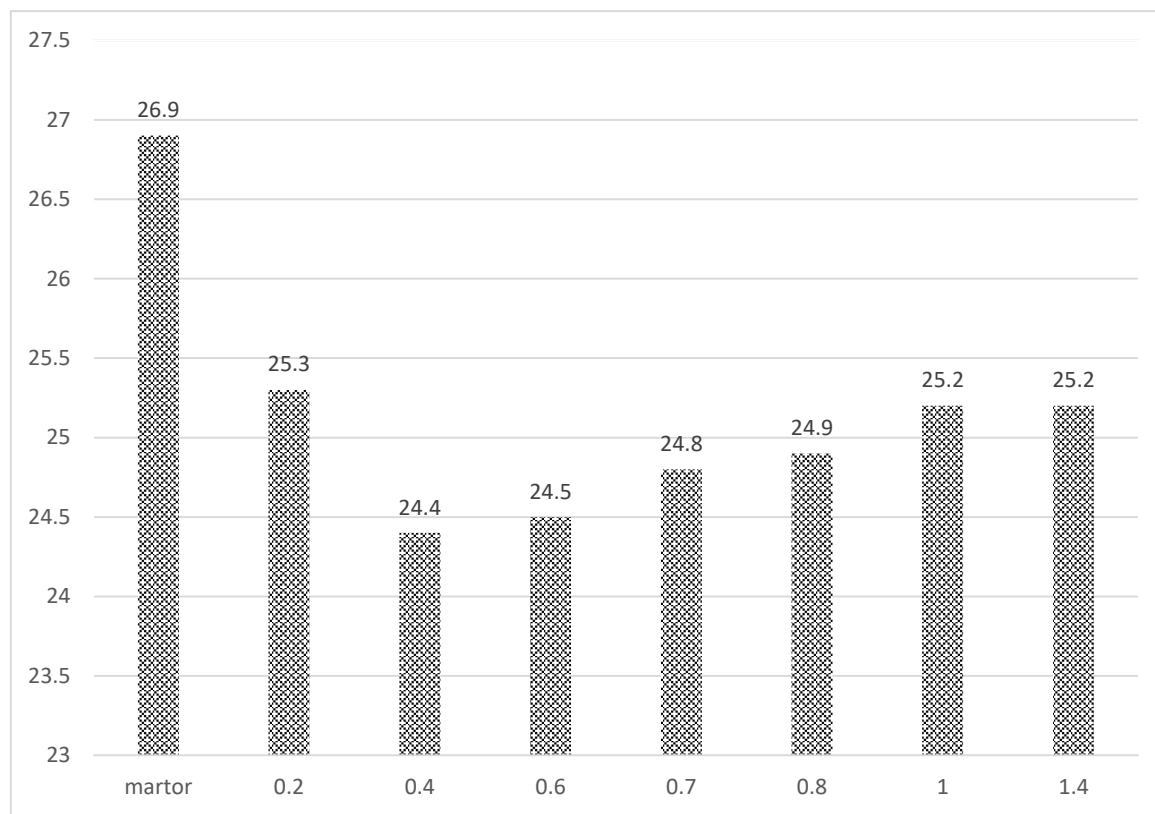


Figure 1. Spermatozoa with damaged acrosome in cryopreserved ram semen (8 ejaculates), %.
(* $P \leq 0,05$)

The acrosome is a critical structure for the fertilizing capacity of spermatozoa, and its deterioration is a sensitive indicator of cryogenic stress. Analysis of the results regarding acrosomal integrity revealed more pronounced damage in the control group after the freeze–thaw process, whereas the variants treated with PD exhibited lower percentages of spermatozoa with damaged acrosomes.

The best results were obtained at PD concentrations of 0.4–0.7%, where the proportion of sperm cells with acrosomal damage was significantly lower than in the control. This indicates a clear protective effect on the acrosomal membrane, likely due to the bioactive compounds in PD that reduce oxidative stress and stabilize the membrane structures of spermatozoa.

This improvement in acrosomal integrity is consistent with the results obtained for motility and kinetic parameters, confirming the beneficial effect of PD on the viability and functional performance of sperm cells following cryopreservation.

The results confirm the protective role of the biologically active preparation PD on ram spermatozoa during the cryopreservation process. The natural compounds contained in PD likely contribute to reducing oxidative stress and stabilizing the sperm membrane, which explains the superior values of motility and kinetic parameters observed.

The concentrations of 0.4–0.6% exhibited the most pronounced effect, which is consistent with other studies reporting that natural extracts rich in polysaccharides and amino acids can ameliorate cold shock and mechanical damage to the sperm flagellum (Leahy & Gadella, 2015; Yimer et al., 2022).

The reduction in morphological abnormalities and the protection of the acrosome further confirm the efficiency of PD as a biologically active additive. These results are in agreement with international literature (Paulenz et al., 2018), which highlights the importance of optimizing semen dilution media to achieve superior post-thaw viability.

CONCLUSIONS

The results obtained in this study demonstrate that the introduction of the biologically active preparation PD into the STJ base extender has a significantly favorable impact on the quality of cryopreserved ram semen. The best performance was recorded at PD concentrations of 0.4–0.7%, where clear improvements were observed in total and progressive motility, kinetic parameters (VAP, VSL, VCL), and acrosomal protection compared to the control extender. These findings indicate that PD can effectively contribute to maintaining the functionality and integrity of spermatozoa exposed to the freeze–thaw process, reducing cryogenic stress–induced damage and optimizing their fertilizing potential. Throughout the entire technological process, from dilution to thawing, the biologically active preparation PD did not exert any negative effects on semen quality. On the contrary, its use as an additive in the dilution media contributed to reducing the frequency of morphological abnormalities and to preserving membrane and acrosomal integrity. Therefore, PD proves to be a safe and effective biologically active additive, and its integration into cryopreservation media represents a promising direction for improving the efficiency of artificial insemination in sheep. These results support the need for further applied research, including the evaluation of PD's impact on fertility rates under field conditions

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Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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