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Optimization of the process of cryopreservation of Beluga, *Huso huso* semen using various cryoprotectants

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ABSTRACT

Currently, the research on cryopreservation of the reproductive cells plays great importance in the conservation of genetic diversity of many economically and ecologically valuable fish species. In this study, optimization of beluga (*Huso huso*) semen cryopreservation was carried out by testing four different cryoprotectants, i.e., 5% ethylene glycol, 10% and 3% dimethyl sulfide oxide, and 5% glycerol. Moreover, the effects of cryotube size (0.2 and 1.9 mL) and type (Eppendorf tube and polypropylene straws) on the effectiveness of semen cryopreservation were tested. The quality of beluga cryoconserved semen was assessed using a five-point Persov scale, including spermatozoa activity time. In this study, the use of a 3% DMSO cryosolution resulted in the best sperm motility (79%) and activity time (305 s) that were significantly ($p < 0.05$) higher when compared to other cryoprotectants: 10% DMSO (48.1% and 254 s), 5% ethylene glycol (21.4% and 152 s), and 5% glycerin (21.8% and 139 s). It was shown that the use of smaller cryotubes (0.2 mL) significantly ($p < 0.05$) increases sperm motility (78%), when compared to 1.9 mL cryotubes (50.1%). No significant differences ($p > 0.05$) in semen motility were observed between polypropylene straws and Eppendorf vials.

Keywords: Cryopreservation, Cryoprotectant, Reproductive cells, Beluga, Sperm.
Article type: Research Article.

INTRODUCTION

One of the side effects of the development of civilization is the loss of biological diversity, which is caused by the extinction of many plant and animal species. The acceleration of the degradation of natural ecosystems that has been taking place since the end of the 20th century is considered as the main threat to life biodiversity on our planet (Bapsanova 2012). Currently, the most important task is to conserve the gene pool of threatened and endangered fish populations and species, especially those that are economically important for fishery catches in natural reservoirs or for aquaculture as promising production objects (Ananyev & Manohina 2007). To conserve and restore the size of fish populations, artificial reproduction biotechnologies have been developed for use with various fish species. Usually, the production of fish stocking material is based on breeders from broodstock kept on farms. This approach limits the possible number of individuals interbreeding with each other, which subsequently may lead to inbreeding (Belaya *et al.* 2018). Sperm cryopreservation is recognized as one of the most promising methods of conserving the genetic diversity of fish, and not only endangered species, but also those important for aquaculture production. The establishment of cryobanks makes it possible to preserve the hereditary potential and the genetic heterogeneity of fish species populations and strains, to create sperm insurance funds, to breed in the absence of mature males, and to use cryopreserved sperm for other scientific and breeding

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purposes (Dokina *et al*. 2006). The use of cryopreserved spermatozoa for artificial reproduction in aquaculture enterprises allows for the production of genetically heterogeneous offspring. Moreover, it enables for the reduction of costs related to maintaining of males, during the production of monosex, all-female fish stocks (Chipinov & Bogatyreva 2011). Cryopreserved reproductive cells can be used at any time, without the risk of the untimely maturation of breeders or the receipt of inadequate-quality reproductive products from breeders. Sturgeons are considered to be one of the most commercially important fish groups in the global aquaculture; they are cultured for caviar, meat and insinglass (Baryshev & Krivoshein 2007). They are also one of the most endangered fishes in the world (Khodorevskaya *et al*. 2012). In Caspian-bordering countries, including the Republic of Kazakhstan, sturgeons are a symbol of national wealth (Tumenov & Bakiyev 2015). Unfortunately, the intensive fisheries exploitation of sturgeons in the Caspian basin has resulted in a sharp decline in their population sizes within the recent decades. As a result, presently, fishery catches of commercially important species such as the Russian sturgeon (*Acipenser güeldenstäedtii*), stellate sturgeon (*A. stellatus*), beluga (*Huso huso*) and bastard sturgeon (*A. nudiventris*) are completely prohibited in the area. Among them, the beluga is considered to be one of the most endangered species in the Caspian basin (Vasilyeva *et al*. 2012). Therefore, the development and optimization of beluga semen cryobanking is crucial for the effective conservation of the species gene pool in Caspian reservoirs and for artificial reproduction in aquaculture conditions (Ponomareva *et al*. 2016; Ivanov *et al*. 2022). In contemporary worldwide research practice, study on the conservation and use of frozen fish sperm is being carried out quite widely (Ponomareva *et al*. 2009; Matishov *et al*. 2012; Huang *et al*. 2014; Niu *et al*. 2022; Pu-Yuan *et al*. 2022). Over the past few decades, our scientific knowledge on the specifics of fish spermatozoa cryopreservation procedures has significantly increased. Currently, many methods of sperm cryopreservation suitable for use in sturgeons are known (Sadeghi *et al*. 2013; Sadeghi *et al*. 2013; Sadeghi *et al*. 2013; Dzyuba *et al*. 2014; Aramli *et al*. 2015; Judycka *et al*. 2015; Kibalova *et al*. 2017; Nascimento *et al*. 2021). Nevertheless, for the successful implementation of these methods in the practice of aquaculture production and species conservation, the standardization of fish sperm cryopreservation protocols is necessary. The concentration of spermatozoa in samples during cryopreservation is one of the most important parameters. Nascimento *et al*. (2021) conducted a number of studies regarding artificially increase in the concentration of spermatozoa, which positively affected the cryopreservation of the sperm of sturgeons. Using antioxidants in the cryopreservation of sturgeon sperm enhanced the motility of spermatozoa (Osipova *et al*. 2016; Savushkina 2016). Similar effect was observed when using glucose and sucrose as cryoprotective additives in the cryopreservation of beluga sperm (Golshahi *et al*. 2016). According to the experiments carried out by many authors, the optimization of cryopreservative technology of beluga semen can be best achieved when penetrating cryoprotectants such as dimethyl sulfoxide (DMSO) and methanol (MeOH) are used (Sadeghi *et al*. 2013; Sadeghi *et al*. 2013; Isaev *et al*. 2016). Along with the study of protection methods, it is necessary to study ways to increase the resistance of cells to the cryopreservation procedure, the effect of which would complement the protective effect of cryoprotectors. One of such research can be the determination of the frozen semen volume on its quality (Krasilnikova 2015). As the cryopreservation of fish reproductive cells is an important research direction in the conservation of genetic biodiversity, as well as in the development of the aquaculture production, the main purpose of our study was to optimize the process of beluga semen cryobanking.

MATERIALS AND METHODS

The research described in this study was carried out in 2021 by the scientific research centre "Fisheries" at S. Seifullin Kazakh Agro Technical University. The reproductive cells used in this study were acquired from nine beluga males kept in the Ural-Atyrau Sturgeon Fish Hatchery. Milt was obtained by decantation during the spawning period. The collected semen was immediately subjected to quality assessment according to a 5-point scale developed by G.M. Perov (Matrossova 2020), which included milt colour, consistency, motor activity time and motility. For assessment of the sperm motor activity time and motility each semen sample was activated by water and then examined under a microscope equipped with a camera (CEROS) and personal computer (CASA, IMV-technologies, France). Sperm motility assessment was relied on the determination of the percentage of spermatozoa with rectilinear movement. Only the sperm samples that scored at least 4 points in quality assessment and with motility at least 90% were used for further cryopreservation studies. The validated semen samples from each three different males were mixed, resulting in three separate replication groups, which subsequently were used for further cryopreservation experiments. The low-temperature conservation of the reproductive cells of male

belugas was carried out in accordance with the methodology developed by Belaya *et al*. (2018). For this purpose, the beluga semen was first diluted with a cryoprotectant at the ratio of 1:1, which was added slowly while being continuously stirred. Four different cryosolutions were tested, i.e., (i) 10% dimethyl sulfoxide (DMSO) + sucrose and mannitol; (ii) 3% DMSO + sucrose and mannitol; (iii) 5% ethylene glycol + sucrose and mannitol and (iv) 5% glycerin + sucrose and mannitol. The cryosolutions were prepared in a cool room at 16-18 ℃. The resulting milt mixtures were dispensed into different types of cryovials, i.e., Eppendorf tubes with volumes of 0. 2 mL and 1.9 mL as well as polypropylene straws with volume of 0.2 mL to optimize the use of cryovials with different volumes and types. Cryovials filled with seminal fluid were frozen in liquid nitrogen vapours. Cryopreservation was carried out in three stages, i.e., initial freezing (2-5 ℃/min), main freezing (10-20 ℃/min) and final deposition of cryovials in a Dewar vessel filled with liquid nitrogen. The quality of the defrosted beluga sperm was examined in the laboratory of JSC, "Republican Centre for livestock breeding "Asyl Tulik", 3 months after being frozen and stored at -196 ℃. Sperm was thawed by removing the test tubes with frozen sperm from liquid nitrogen and placing them in a water bath at a temperature of 38-40 °C. For each thawed sample variant of cryoprotector as well as cryovial volume and type, the sperm motility and motor activity time were determined. Statistical processing was carried out according to the guidance of GF Lakin (Lakin 1990) using the Microsoft Excel software (Korossov & Gorbach 2007). Student's t-test was employed to determine the significance of recorded differences in values of sperm motility and motor activity time between examined experimental variants.

RESULTS

The recorded assessment results on the quality of beluga sperm before and after freezing showed that the use of various cryoprotectants affects the motility of beluga sperm (Table 1). The most effective cryoprotectant was DMSO 3%. The motility of beluga spermatozoa in the three replicate groups was in the range of 77.6-81.1%, with an average value of 79.0%. Increased concentration of DMSO to 10% resulted in significantly ($p < 0.05$) lower sperm motility ranging from 46.1 to 50.1%, with average of 48.1%.

Table 1. Efficiency of beluga sperm cryopreservation using different cryoprotective media.

Replicate group	Cryoprotective medium	Sperm motility (%)	
		Fresh sperm	After defrosting
1 (sperm mixture from 3 males)	DMSO 10%	96.1 ± 0.3	46.1 ± 0.2
	DMSO 3%		78.3 ± 0.3
	Ethylene glycol 5%		24.3 ± 0.2
	Glycerol 5%		22.4 ± 0.1
2 (sperm mixture from 3 males)	DMSO 10%	95.7 ± 0.1	50.1 ± 0.3
	DMSO 3%		81.1 ± 0.2
	Ethylene glycol 5%		21.3 ± 0.1
	Glycerol 5%		22.5 ± 0.2
3 (sperm mixture from 3 males)	DMSO 10%	95.2 ± 0.2	48.1 ± 0.3
	DMSO 3%		77.6 ± 0.4
	Ethylene glycol 5%		18.6 ± 0.2
	Glycerol 5%		20.5 ± 0.3
The average value for the three groups	DMSO 10%	95.7 ± 0.3	48.1 ± 1.3
	DMSO 3%		79.0 ± 1.4
	Ethylene glycol 5%		21.4 ± 1.9
	Glycerol 5%		21.8 ± 0.87

The application of cryoprotectants based on ethylene glycol and glycerine were significantly less effective ($p < 0.05$) than 3% DMSO, for which the recorded an average sperm motility were 21.4% and 21.8%, respectively. We also studied the effect of cryotube volume on sperm motility (Table 2). When freezing the beluga's seminal fluid in cryovials with different volumes, the best results on sperm motility were obtained using polypropylene straws and Eppendorf cryovial with volumes of 0.2 mL (78.8 and 75.9%, respectively), which were significantly higher ($p < 0.05$) when compared to that achieved using a 1.9 mL (50.1%) Eppendorf cryovial. The differences in sperm motility between 0.2 mL straws and Eppendorf cryotubes were not significant ($p > 0.05$). The sperm activity times before and after cryopreservation by different cryoprotestors are shown in Fig. 1. The recorded spermatozoa activity time in fresh beluga sperm (356 s) was significantly ($p < 0.05$) higher than cryopreserved sperm (139-305 s). Among cryopreserved sperm, the best results on spermatozoa activity time were obtained when using a DMSO-based cryoprotectant with a concentration of 3% (305 s), being significantly higher than

activity time of sperm cryopreserved by other cryoprotectants ($p < 0.05$). When using other cryoprotectants, the sperm activity times were as follows: DMSO 10%: 254 s; ethylene glycol 5%: 152 s; glycerin 5%: 139 s. When comparing the use of DMSO cryoprotectants with different concentrations, DMSO 3% showed significantly ($p < 0.05$) higher result than DMSO 10%. The cryoprotectant based on DMSO with a concentration of 3% was shown to have the most beneficial effect on the lifetime of beluga sperm, which was confirmed statistically ($p < 0.05$).

Table 2. The effect of cryotube volume on sperm survival (%).

Replicate group	Type of cryotubes	Volume of cryotubes (mL)	Sperm motility (%)
1 (sperm mixture from 3 males)	Eppendorf cryotubes	0.2	76.1 ± 0.3
	Eppendorf cryotubes	1.9	52.3 ± 0.2
	polypropylene straws	0.2	80.5 ± 0.2
2 (sperm mixture from 3 males)	Eppendorf cryotubes	0.2	72.3 ± 0.2
	Eppendorf cryotubes	1.9	48.4 ± 0.1
	polypropylene straws	0.2	78.6 ± 0.2
3 (sperm mixture from 3 males)	Eppendorf cryotubes	0.2	79.3 ± 0.2
	Eppendorf cryotubes	1.9	49.7 ± 0.2
	polypropylene straws	0.2	77.8 ± 0.3
The average value for the groups	Eppendorf cryotubes	0.2	75.9 ± 1.4
	Eppendorf cryotubes	1.9	50.1 ± 1.3
	polypropylene straws	0.2	78.9 ± 1.1

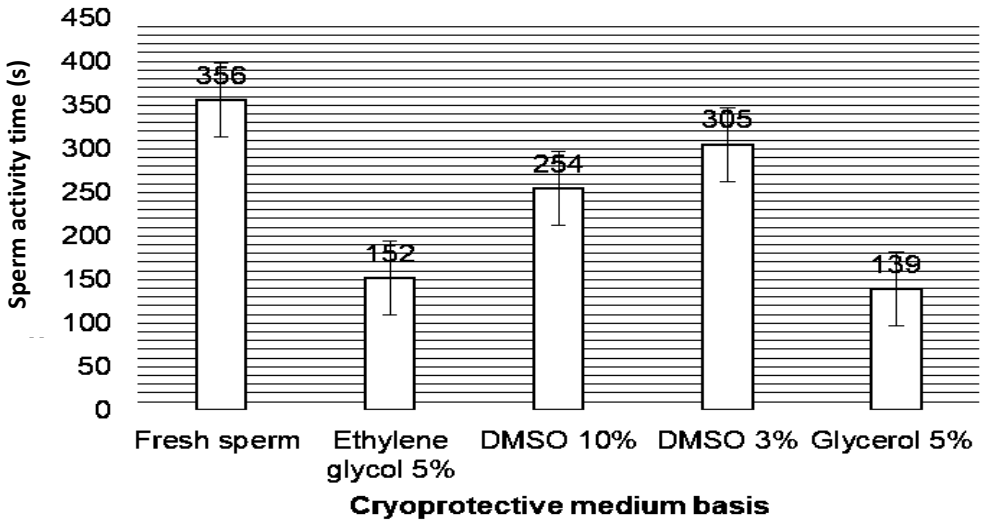

Fig. 1. Sperm activity times based on different cryoprotective media.

DISCUSSION

In the studies conducted on the cryopreservation of beluga sperm using cryoprotectants based on DMSO, ethylene glycol and glycerine, various results were obtained. In our study, the best result for sperm motility was obtained using 3% DMSO, with the average of 79.0%. Compared to a cryoprotectant with 10% DMSO, the use of 3% DMSO showed 64.2% higher results ($p < 0.05$). In the research of Ponomareva *et al*. (2009) regarding the cryopreservation of the sturgeon's sperm, a DMSO concentration of 10% was preferred in comparison with Stein medium. Similarly, good results regarding the motility of beluga and sterlet sperm were obtained by Sadeghi *et al*. (2013) when using 10% DMSO as a cryoprotectant. However, Ponomareva *et al*. (2016) reported that application of 3% DMSO also results in good motility of beluga semen. In the present study, a poor result regarding the motility of beluga spermatozoa was obtained when using 5% ethylene glycol as a cryoprotectant, with an average value of 21.4%. Boryshpolets *et al*. (2011) provided data on the use of DMSO, dimethylacetamide, ethylene glycol and methanol as cryoprotectants at concentrations of 5% and 10%, in which the best results were achieved using 10% methanol, and the unsuitability of ethylene glycol as a cryoprotectant was shown. Similar results were reported for sterlet (*A. ruthenus*) by Shaliutina-Kolešová *et al*. (2015). When studying the effect of the volume of frozen material on the motility of male beluga reproductive cells by the current authors, the best results were obtained with the use of polypropylene straws with volumes of 0.2 mL, (78.9%). This was 1.6 times higher compared to the rate achieved with 1.9 mL Eppendorf cryovials, (50.1%). Similar

experiment was carried out by Krasilnikova *et al.* (2015) regarding the effect of test tubes with different volumes on the activity time of Siberian sturgeon sperm. The longest sperm activity time was recorded in a sample with a volume of 0.5 mL (29.7 min), which was 1.5 times higher when compared to that in a 0.75 mL test tube (19.7 min) and 2.56 times higher than that in a 1.5 mL container (11.6 min).

CONCLUSION

In our study, the best results regarding the cryopreservation of beluga sperm were demonstrated for DMSO with a concentration of 3%, for which an average value of sperm motility was 79%. The application of 0.2ml Eppendorf vials and polypropylene straws yielded the best results on motility of cryopreserved beluga semen (78.9%). The activity time of beluga spermatozoa among all the experimental groups was best when using 3% DMSO as a cryoprotectant, with an average activity time of 305s. This stock solution was shown to have a positive effect on the habitat of spermatozoa, both by penetrating inside the cell before freezing and preventing destruction during deep freezing and after thawing, acting as a nutrient medium for cells after a temperature shock.

AUTHOR CONTRIBUTIONS

Conceptualization, Zh.B.K. and A.D.M.; methodology, A.S.A., K.N.S., Zh.B.K. and A.D.M; software, G.K.B. and S.E.M.; validation, A.S.A., K.N.S. and G.A.A.; formal analysis, A.S.A., Zh.B.K., G.K.B. and G.A.A.; investigation, Zh.B.K., A.D.M. and S.E.M.; resources, A.S.A. and Zh.B.K.; data curation, Zh.B.K.; writing—original draft preparation, A.S.A., G.K.B., Zh.B.K. and A.D.M.; writing—review and editing, A.S.A., K.N.S. and G.A.A.; visualization, Zh.B.K. and A.S.A.; supervision, A.S.A. and K.N.S.; project administration, A.S.A. and Zh.B.K.; funding acquisition, A.S.A. and Zh.B.K. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest. This manuscript has not been published or presented elsewhere in part or in its entirety and is not under consideration by another journal. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these.

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