The volume of the sample as a factor of survival of sturgeon spermatozoa after cryopreservation

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Abstract. This research was carried out to examine the effect of various volumes (0.5, 0.75, 1.5 and 2 mL) of the frozen sample on cryopreservation of sturgeon sperm and also the possibility of using the method of vitrification of sperm under deep low-temperature cooling in the form of thin films on nets. The object of the study was the spermatozoa of the Russian sturgeon (*Acipenser gueldenstaedtii* Brandt, 1833) and the Siberian sturgeon of the Lena population (*Acipenser baerii* Brandt, 1869). There is a direct relationship between the volume of frozen material and the survival rate of defrosted sperm. With the increase in freeze sample preservation frozen-melted cells is falling, as is the range of cooling rate to freeze the sample, in which the majority of cells are frozen at a speed different from the optimal values. When cryopreservation of a sperm smear in the form of a thin film, the analysis of cell movement activity after defrosting showed the suitability of such sperm for use in the fish-breeding process. The highest life time of the sperm as it was observed during the freezing of the films on the plastic samples.

1 Introduction

Currently, in the context of global environmental problems, the problem of preserving biological diversity has acquired special significance and relevance [1-3]. To date, low-temperature preservation is one of the most affordable and acceptable methods of long-term storage of cells [4-7].

The accumulated data on low-temperature preservation of biological objects to date indicate that the process of long-term storage of biomaterial at low temperature does not significantly affect the preservation of cells after freezing-thawing [8-11].

From frozen sperm, viable fish juveniles are successfully obtained, which in quality is not inferior to juveniles obtained by traditional methods [12-14]. Solving the problem of cryopreservation of fish reproductive cells is the final link in the problem of preserving the genetic diversity of rare and endangered fish. The creation of collections and stocks of sperm and eggs of commercial fish whose populations are on the verge of extinction or in a stressed state will allow them to restore their numbers in compliance with the genetic

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heterogeneity of the gene pool. In many countries, the technique of cryopreservation of sperm is used for commercial purposes to increase the heterogeneity of the gene pool of cultivated species, both for commercial cultivation and for the restoration of natural populations.

During cryopreservation, cells are exposed to a complex of stress factors that cause structural and functional changes in various subcellular systems [15]. These processes can develop at the stage preceding freezing, in the zone of positive temperatures in the presence of cryoprotectors, as well as under the influence of cooling and/or thawing. The main causes that initiate cell damage when exposed to low temperatures are the formation of extracellular and intracellular ice crystals, which destroy cell structures and salt (osmotic) shock, which causes an increase in the concentration of salts in the cell [16, 17].

Optimization of methods for preparing cells and tissues for low-temperature preservation is carried out by applying combined techniques, refining existing approaches and technologies, as well as searching for non-standard techniques for transferring cells to a state of suspended animation. This approach opens up new opportunities and prospects for the conservation of biological material, both for the development of fundamental foundations and for practical application for reproduction.

To the best of our knowledge, there is limited information regarding role of volumes of the frozen sample on cryopreservation of sturgeon sperm. From this point of view, this research was carried out to examine the effect of various volumes (0.5, 0.75, 1.5 and 2 mL) and also the possibility of using the method of vitrification of sperm under deep low-temperature cooling in the form of thin films on nets.

2 Material and research methods

Russian sturgeon (*Acipenser gueldenstaedtii* Brandt, 1833) and Siberian sturgeon of the Lena population (*Acipenser baerii* Brandt, 1869) sperm, received from sturgeon hatchery in the spawn period, was used in the study. For stimulation of sturgeon maturation, the Luteinizing Hormone – Releasing Hormone Ethylamide (LH-RHa, Surfagon) was applied in 0.5-1 mg/kg concentration at water temperature 14°C by injection. Males were injected once. The sperm was collected into glass containers by catheter. The seminal fluid collected for the experiments were cooled (4 ± 2 °C) and delivered to the laboratory in the thermal container.

The motility of sperm was estimated using binocular microscope Micmed-5 after addition of river water as an activating solution to the post-thaw sperm at a ratio of 1:250, and the fresh sperm was activated at a ratio of 1:1000. Duration time (total period of sperm movement, min.) was defined as the time from the activation to the stop of movement using stopwatch.

The sperm diluted with the cryoprotective medium was distributed in labeled Eppendorf tubes. The ratio of sperm and cryomedium was 1:1. In the study of the effect of the volume of the freezing container on the survival of frozen-thawed cells, different volumes of Eppendorf tubes were used – 0.5 ml, 0.75 ml, 1.5 ml and 2 ml. When freezing sperm in the form of films, we took nets made of different materials – metal (aluminum) and plastic (polymer glass fiber coated with polyvinyl chloride). The nets were immersed in a mixture of sperm and cryoprotective solution and placed in liquid nitrogen.

Cryopreserved sperm was taken from cryobank of Southern Scientific Center of Russian Academy of Sciences (SSC RAS). Sperm samples were stored in cryostorage Cryo Diffusion (France) in liquid nitrogen during 5 days. Thawing of sperm was carried out in a water bath during 30 - 40 s at a temperature of 38-40°C. Motility in experiment were analyzed using an unpaired Student's *t*-test. Statistical significance was set at $p < 0.05$.

3 Results and discussion

The ability to effectively stop the processes of natural decay and degradation of cells with their subsequent long-term storage in a biostabilized state is a necessary logistical element of almost any cell technology. Cells do not survive in ice crystals, they do not live long and in a liquid state (if not specially cultivated). The active development of methods for cryopreservation of fish sperm in recent years has shown that various approaches to the implementation of the same technological procedures for deep freezing are not always reproducible and do not give identical results. This also applies to such an important issue as sample volumes. The results of the experiment to identify the relationship between the volume of the sample for freezing and the survival of spermatozoa are shown in figures 1 and 2.

Fig. 1. The motility of sperm depends at the volume of sample, %

Fig. 2. Total period of sperm movement depends at the volume of sample, min.

The highest percentage of motility and total period of sperm movement was in samples frozen in 0.5 ml Eppendorf tubes. When cryopreservation of seminal fluid in larger test tubes (0.75 ml, 1.5 ml and 2 ml), the results are slightly worse.

This is probably due to the fact that a small volume object has a smaller temperature gradient, i.e. the temperature drop in the sample during freezing occurs more evenly, compared to containers of large volumes. In this regard, cells located at different points of the frozen sample are cooled at significantly different speeds. Thus, the inhomogeneity of the temperature field and the variation of cooling rates in the sample increases with increasing sample volume.

A similar pattern occurs when thawing samples. Defrosting of samples of smaller volume occurs faster, i.e. the speed variation during thawing is minimal. In large test tubes, defrosting occurs unevenly: the contents of the sample, located closer to the periphery, already have a liquid fraction, while the "core" of the sample has not yet thawed.

Thus, with the increase in freeze sample preservation frozen-thawed cells is falling, as is the range of cooling rate to freeze the object in which the majority of cells are frozen at a speed different from the optimal values.

In the process of cryopreservation, with a slow decrease in temperature that causes hypothermia, there is a strong dehydration of cells and an increase in the concentration of intracellular substances, leading to denaturation of proteins and damage to the membranes. Cells are destroyed due to contact with the hyperconcentrated environment of electrolytes, in which there can be sharp changes in the pH and ionic strength of the medium. Cell membranes can also be damaged because the cell reaches a minimum volume. Such damages can be aggravated by recrystallization processes during slow thawing and cooling of objects with low speeds up to minus 150 °C. In the case of step-by-step freezing, each type of cell and tissue requires its own freezing algorithms (programs), which are often quite complex and require expensive software freezers. The phenomenon of "vitrification" is an alternative approach to freezing biological material. According to the theoretical provisions, at very high concentrations of cryoprotectors in the medium and a rapid

decrease in temperature (i.e. almost instantaneous immersion of the carrier in liquid nitrogen), the entire sample passes into a glassy state, bypassing the crystallization phase. This allows us to open new areas of research in the field of cryopreservation of fish reproductive cells and ensure the improvement of previously known technologies.[18-19]

When using the method of vitrification of sperm under deep low-temperature cooling in the form of thin films on nets, the results were obtained (Figure 3, 4).

Fig. 3. The motility of sperm depends at the matileal of nets, %

Fig. 4. Total period of sperm movement depends at the matileal of nets, min.

When applying the seminal fluid to the net frame, a thin film was formed. The motility time of warmed spermatozoa has shown the effectiveness of this method of preparing cells for low-temperature preservation. The activity time in all experimental samples was more than 10 minutes, which indicates the suitability of the sperm for fish-breeding purposes, since the standard fertilization time for sturgeon during artificial reproduction is 3 minutes. When using polymer fiberglass nets, the percentage of motility and motility time of spermatozoa was the highest. When using aluminum nets, the duration of motility in Russian sturgeon spermatozoa decreased by 1.5 times, and in Siberian sturgeon spermatozoa in the Lena population by 1.3 times. However, no statistically significant differences were found between the use of 2 types of grids in any species. The use of

aluminum samples was less convenient, the mixture of sperm and cryoprotective medium was whipped into a foam with rigid mesh fibers, which significantly complicated the work.

Thus, the experiments established a direct relationship between the volume of frozen material and the survival rate of defrosted sperm. In the process of freezing a cell suspension in a container, the field of cooling rates is inhomogeneous, so that different cells are cooled at different speeds, differently damaged depending on their position in the container in which the cell suspension was frozen. The results obtained allow us to conclude that the preferred use of a smaller volume of the container for low-temperature preservation of fish sperm. It is obvious that this will help to increase the efficiency of the developed methods of low-temperature preservation of genetic material.[20]

Work in the field causes great difficulties with the use of programmable biofreezer, the Results of experiments have shown the possibility and prospects of using alternative methods of cryopreservation of sperm in the form of thin films on nets.

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