ТОВАРНАЯ АКВАКУЛЬТУРА И ИСКУССТВЕННОЕ ВОСПРОИЗВОДСТВО РЫБ

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ALTERNATIVE METHODS OF PREPARATION OF FISH SPERM TO FREEZE AT ULTRA-HIGH VALUES OF COOLING RATE

Abstract. The sexual products of the Russian sturgeon (*Acipenser güldenstädtii* Brandt, 1833), the Siberian sturgeon of Lena population (*Acipenser baerii* Brandt, 1869) and white salmons (*Stenodus leucichthys* Güldenstädt, 1772) were used as an object of this research. The aim of the represented research was to study the possibility of freezing of fish men reproductive cells in the form of "thin films" on meshes at ultra-high cooling rates, and also by preliminary drying of a sperm smear in the thermostat. When freezing sturgeon semen in the form of a "thin film", the analysis of motility of post-thawed spermatozoa showed the efficiency of application of this method of preparation of sperm to cryopreservation. The lifespan in all test samples was more than 14 minutes, indicating the suitability of frozen-thawed reproductive cells for aquaculture purposes. The highest spermatozoa lifespan of both Russian and Siberian sturgeons is detected when freezing on plastic samples. In preparation for cryopreservation by preliminary drying of a sperm smear in the thermostat, there was a fluctuation motion of 20 % of sperm. This method of preparation of cells for cryopreservation requires modification and improvement, but the fact that there has been received a certain number of viable spermatozoa, suggests the possibility of application of the given method.

Key words: fish spermatozoa, films, drying, cryopreservation, ultra-high cooling rate.

Introduction

Accumulated data on cryopreservation of biological objects indicate that the process of long storage of biomaterials at low temperature does not significantly impact on the preservation of cells after freezing – thawing [1–6].

Consequently, the problem of long preservation of biological objects in a viable state could be considered to be resolved if there would be the ability to cool them quickly to the temperature of liquid nitrogen (–196 °C), and then to warm as fast to the initial temperature [1]. Based on such considerations, in the 50–60-ies an American cryobiologist B. J. Luyet and his team studied the possibility of providing structural and functional integrity of cells with the help of ultra-fast cooling carried out, for example, by the dispersion of fine drops of cell suspension directly in liquid nitrogen spraying on a cold metal plate, etc. Theoretical evaluation shows that for stabilization of the original structure of aqueous solution (i. e. vitrification) its cooling rate should be expressed as a value not less than 10^4 °C/s. In some cases they managed to achieve partial vitrification in viscous solutions and cell suspensions in the process of ultra-fast cooling. However, in the process of warming, especially when the temperature was close to the melting temperature, metastable vitreous phase, as a rule, passed in thermodynamically stable, crystal phase, so the structure of the solution did not remain unchanged during the whole cycle of cooling-thawing. It is not excluded, however, that the method of ultra-fast cooling may be acceptable for cryopreservation of individual cells, i. e. in those cases when the sample size is extremely small, for decreasing the volume of a sample, the probability of fluctuational emergence of crystals in it also decreases, and the tendency to hypothermia increases [1].

Obviously, the smaller the volume of frozen samples, the less mechanical ski damages the cells undergo in the procedure of freezing – thawing [7, 8].

By increasing the cooling rate the area and the perimeter of micro-particles of ice reduce. With ultra-fast cooling (3000–4000 °C/min) the vitrification of medium occurs in a thin layer (0.1 mm) [9].

Currently the improvement of the methods of cryopreservation is done by optimizing the existing methods, applying of combined methods, as well as searching for new approaches to convert cells to the state of rest. These research works give new perspectives to the preservation of biomaterials as for the development of fundamental bases, and for practical purposes for reproduction [10].

Usually stepwise cooling is used in the process of cryopreservation of fish semen. In this case the cooling rates vary at different stages of freezing in certain strictly limited intervals of temperature values [11]. However, F. I. Shintimirova with her coauthors showed that for some fish species ultra-fast freezing is optimal [12]. Recently, the researchers have paid attention to the development of the technology of vitrification of sperm even without the use of cryoprotectants [13, 14]. Application of the method of vitrification allows to eliminate cell injury when freezing by ice crystals. In addition, this allows to exclude from the process of freezing the use of toxic cryoprotectants, such as dimethyl sulfoxide (DMSO), or significantly reduce their concentration [13].

In connection with foregoing, the aim of this research was to study the possibility of freezing of fish male reproductive cells in the form of thin films on meshes at ultra-high values of cooling rate, and by preliminary sperm smear drying in the thermostat.

The first stage of the experimental work was devoted to studying the possibility of freezing of sturgeon fish semen in the form of a "thin film". The object of the research was sexual products of Russian sturgeon (*Acipenser güldenstädtii* Brandt, 1833) and Siberian sturgeon of Lena population (*Acipenser baerii* Brandt, 1869), received from the Donskoy sturgeon plant (Rostov region) during the period of spawn.

The experiment was planned as a one-factor and unit experiment. Sperm from different males was determined as a unit, and the material of meshes – metal (aluminum) and plastic (polymeric fiber (fiberglass) coated with polyvinyl chloride) was determined as a factor (Fig. 1).

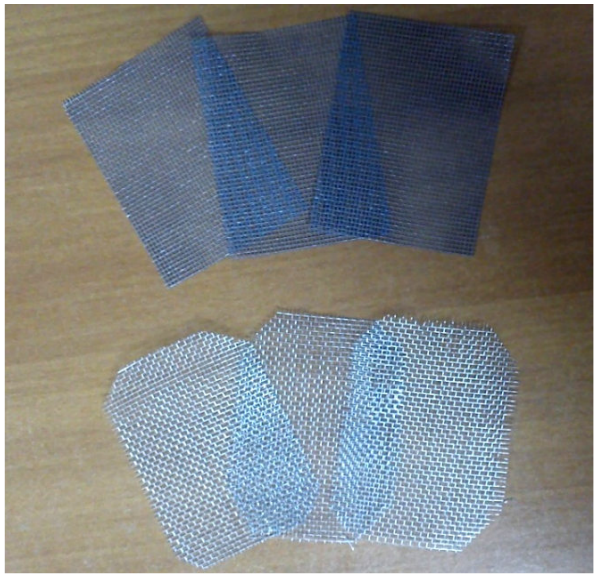

Fig. 1. Plastic meshes: a polymer fiber (fiberglass)
coated with PVC (top) and a metal mesh (aluminum) (bottom)

The semen was compounded with the protectant in the ratio of 1 : 1 with the use of electrical stimulation of membranes by means of a low-frequency rectangular exciter with amplitude of 150 mV and frequency of 20 Hz for 1 minute [2, 15–23]. Next the meshes were dipped into a compound of sperm and cryoprotective solution, and then froze at ultra-high cooling rates. Experiments were performed three times. Thawing was performed at the temperature of 20 °C. The output value was spermatozoa lifespan. The significance of the differences between factors and units was detected according to *F*-test [24].

The results of experimental series are presented in Fig. 2.

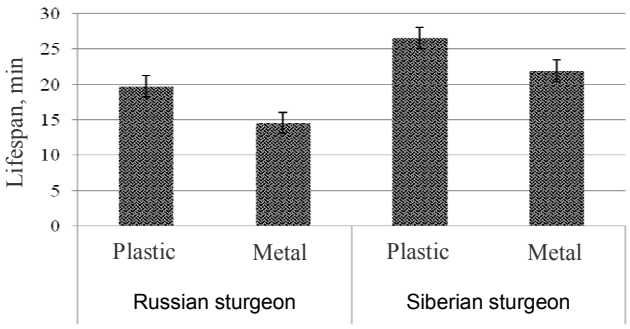

Fig. 2. The lifespan of spermatozoa freezing on meshes of different materials

The analysis of sperm motility after thawing showed the effectiveness of this method of preparation of semen for cryopreservation. The lifespan in all test samples was more than 14 minutes, indicating the suitability of frozen-thawed reproductive cells for aquaculture purposes. The greatest spermatozoa lifespan both of Russian and Siberian sturgeons was detected when freezing on plastic meshes.

Freezing on metal meshes was less comfortable in application due to the fact that rigid fibers of mesh frothed up the compound of sperm and cryoprotective solution, which complicated the work.

The second experimental series of experiments was aimed at studying the opportunities of freezing fish sexual products without cryoprotectans by drying a sperm smear in the thermostat. The material for experimental studies was sexual products of white salmon (*Stenodus leucichthys* Güldenstädt, 1772) collected in the period of spawning campaign at the Alexandrovsky sturgeon fish hatchery (Astrakhan region).

A drop of semen was placed on a plexiglass and we took a smear, which was dried in the thermostat at different temperatures – 20, 27 and 35 °C, determining the time of drying. The tests were performed three times. After drying the spermatozoa were activated by water. The results of the experimental series on the determination of optimal parameters of sperm smear drying presented in Fig. 3.

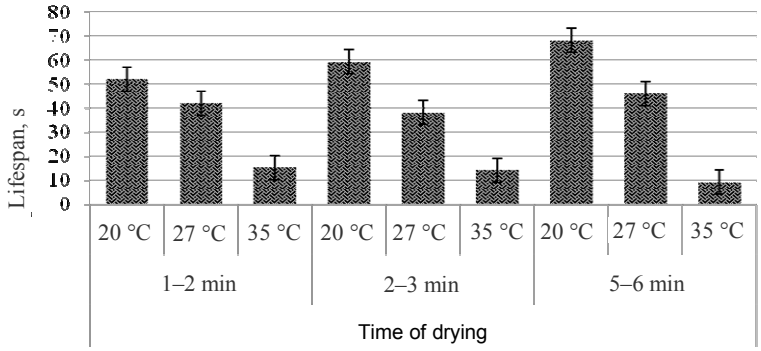

Fig. 3. The lifespan of spermatozoa depending on the temperature and the time of drying

It was necessary to achieve the degree of dehydration of cells, which, on the one hand, would be sufficient to exclude the possibility of intracellular ice formation, and on the other, would not reach a critical level, which could lead to the inevitable damage to cells. The analysis of the results elicited the following regularity: the less the temperature of sample drying, the higher the sperm activity after its activation by water, regardless of the time spent in the thermostat. The highest sperm cells lifespan was set at drying time of 5–6 minutes at temperature of 20 °C.

After the determination of the optimal drying mode, the freezing of plexiglass meshes with semen smears on them, which had been previously dried at temperature of 20 °C for 5 minutes, was performed. Freezing was performed with ultra-high values of cooling without cryoprotectants, because it was assumed that this method would allow to avoid toxic effects of penetrating protectants on the fish semen and improve the survival of post-thawed cells.

After the storage of the samples in liquid nitrogen the meshes were thawed at a room temperature for 30 seconds and the sperm was activated by water. Output value was the lifespan of spermatozoa.

After thawing and activation of specimen, with an average lifespan of 41 seconds, there was a fluctuation motion of 20 % of sperm, confirming once again the fact that in selective influence of low

temperatures the most powerful sperm survives. Apparently, preliminary dehydration of the cells caused structural damages to the main amount of sperm cells due to dehydration. As cryopreservation was performed after thermotreatment and without the use of cryoprotectants, most of the cells were not able to experience double thermal shock, and as a result male reproductive cells proved to be inviable. This method of the preparation of cells for cryopreservation requires modification and improvement, but the fact that there has been received a certain number of viable sperm yet, suggests the possibility of application of the given method.

Conclusion

The results of the described above experimental unit show the prospects of application of alternative methods of preparation of fish sperm to the impact of low temperatures. Cryopreservation of fish sperm at ultra-high values of cooling can reduce the number of cryoprotectants that, in turn, will reduce or avoid the toxic effect of the latter on the object.

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АЛЬТЕРНАТИВНЫЕ СПОСОБЫ ПОДГОТОВКИ СПЕРМАТОЗОИДОВ РЫБ К ЗАМОРАЖИВАНИЮ ПРИ СВЕРХВЫСОКИХ ЗНАЧЕНИЯХ СКОРОСТИ ОХЛАЖДЕНИЯ

Объект исследований – половые продукты русского осетра (*Acipenser güldenstädtii* Brandt, 1833), сибирского осетра ленской популяции (*Acipenser baerii* Brandt, 1869) и белорыбицы (*Stenodus leucichthys* Güldenstädt, 1772). Цель исследований – изучение возможности замораживания мужских репродуктивных клеток рыб при сверхвысоких значениях скорости охлаждения в виде «тонких пленок» на сетках, а также путем предварительного высушивания мазка спермы в термостате. При замораживании семенной жидкости осетровых рыб в виде «тонкой пленки» анализ двигательной активности сперматозоидов после дефростации показал эффективность применения данного способа подготовки семенной жидкости к низкотемпературному консервированию. Время жизни во всех опытных образцах составило больше 14 минут, что свидетельствует о пригодности замороженно-оттаянных репродуктивных клеток для рыбоводных целей. Наибольшее время жизни сперматозоидов как русского, так и сибирского осетров отмечено при замораживании на пластиковых образцах. При подготовке к криоконсервации путем предварительного высушивания мазка спермы в термостате наблюдали колебательные движения 20 % спермиев. Этот способ подготовки клеток к криоконсервации требует доработки и совершенствования, но тот факт, что все-таки было получено определенное количество живых сперматозоидов, свидетельствует о возможности применения данной методики.

Ключевые слова: сперматозоиды рыб, пленки, высушивание, криоконсервация, сверхвысокие скорости охлаждения.

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