The effectiveness of using lipid- and water-soluble analogues of vitamin E as antioxidant protectors for cryopreservation of sperm of sterlet (*Acipenser ruthenus*) and Russian sturgeon (*Acipenser gueldenstaedtii*)

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Abstract. The aim of the research was to comparative study the effectiveness of synthetic antioxidants – lipid-soluble (butylated hydroxytoluene, BHT) and water–soluble (trolox) analogues of vitamin E for the cryopreservation of Russian sturgeon and sterlet sperm in the presence of the modified Stein's medium. The ability of phenolic antioxidants to reduce the level of lipid peroxidation of sturgeon sperm, beneficial effect on the motility of thawed sperm has been shown. The addition of lipid- and water-soluble antioxidants to a basic cryomedim increased the fertilization rate of Russian sturgeon and sterlet sperm cells about 10% compared to the control experiment. The insignificant protective effect of these antioxidants presumably is explained by the use of a cryogenic medium with additive of toxic DMSO, therefore, it is necessary to develop a new composition of the cryo-medium with a minimum concentration of DMSO.

1 Introduction

The anthropogenic load on reservoirs is increasing every year, and it is increasingly difficult for hydrobionts to resist the negative effects of the environment. The most vulnerable species are endangered, including sturgeon fish. Due to the impact of adverse factors (hydraulic engineering, poaching, pollution of reservoirs, etc.), the number of populations is decreasing, as well as their structure is changing. Thus, in the populations of Russian sturgeon and stellate sturgeon, juveniles predominate (70-90%), and the average weight has decreased to 5 and 4 kg, respectively [1]. Spawning is mainly for the first time spawning individuals. The share of Russian sturgeon females in the spawning part of the population decreased from 48.6% in 1981 to 9.2% in 2011, and the stellate sturgeon from 47 to 8% [2].

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Most of the individuals of sturgeon fish living in the rivers and seas of Russia are grown in artificial conditions [2]. However, artificial reproduction is not able to maintain the population of sturgeon fish at a sufficiently high level. One of the reasons is the use of a limited number of individuals from breeding herds in the spawning campaign. This leads to a decrease in population heterogeneity due to inbreeding, and may also cause species degeneration in the future.

In the current situation, it is advisable to use methods of cryopreservation of reproductive cells of fish for the formation of uterine herds at fish-breeding enterprises. The presence of a cryobank of fish sperm makes it possible to use a larger number of males in the spawning campaign, while reducing fish-breeding areas, as well as to exchange sex cells between farms. However, there is still no single method of cryopreservation of fish sperm, so the improvement of techniques and technologies aimed at improving the quality of defrosted germ cells continues. Sperm damage during cryopreservation causes reduced fertility of thawed sperm.

A significant part of the damage to cellular components during freezing/thawing of spermatozoa is explained by the increased rate of oxidation of important biomolecules due to excessive production of reactive oxygen species (ROS,) during cryopreservation [3]. Under these conditions, the antioxidant defense system present in reproductive cells, which is represented by enzymes and low molecular weight antioxidants, cannot cope with the removal of ROS and develops oxidative stress. This phenomenon is especially typical for fish sperm, which have a high content of polyunsaturated fatty acids - abundant substrates for free radical attack, while at the same time relatively weak endogenous antioxidant protection [4].

Classical cryoprotectants are generally unable to scavenge ROS, while a combination of cryoprotectants and antioxidants which are able to trap free radicals and suppress oxidation reactions can improve the efficiency of cryopreservation. Therefore, the introduction of compounds with antioxidant activity into freezing media, is an important factor affecting the fertility of defrosted fish sperm.

However, some antioxidants have certain properties that ultimately limit their use. So, characteristics such as high hydrophobicity and low stability limit application of a powerful antioxidant vitamin E- endogenous fat-soluble antioxidant contained in spermatozoa and seminal plasma of fish, including sturgeons [5]. Insolubility in water limits the effectiveness of a-tocopherol in removing ROS in the aquatic environment, especially inside cells.Compared with natural antioxidants, BHT - well-known antioxidant, synthetic analogue of vitamin E has the advantages of stable structure and high antioxidant action [6]. At low concentrations, BHT is widely used as an antioxidant additive (E321) in foods and food packaging. [7].Several studies have demonstrated that the addition of this lipophilic synthetic analogue of vitamin E to cryopreservation media protects human [8], various animal species spermatozoa [9], fish sperm from cryodamage [10-16]·trolox, a well-known water-soluble analogue of α-tocopherol, is also highly stable [17] and often used as a standard [18] or positive control in tests to determine the antioxidant activity of compounds. Trolox is known to be successfully used in the cryopreservation of human [19] and domestic animal sperm [20]. Studies of the protective activity of trolox during storage of fish at low temperatures are clearly not enough. In our previous work the protective activity of a water-soluble analog of vitamin E was studied in the preservation of beluga sperm [13].

The aim of the study was to comparative study of the effectiveness of synthetic antioxidants - lipid-soluble (butylated hydroxytoluene) and a water–soluble (trolox) analogues of vitamin E – as cryoprotectors in the process of low-temperature preservation of Russian sturgeon and sterlet sperm.

2 Material and research methods

BHT (2,6-di-*tert*-butyl-4-methylphenol, 99%), trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%), all other reagents were purchased from Sigma–Aldrich. Phenolic compounds were dissolved in cryomedium (130 mM NaCl, 5 mM KCl, 20 mM $NaHCO_3$, 5.5 mM glucose, 12.5% egg yolk, 12.5% DMSO) [21], to make a stock solution with the sperm at a concentration of 0.1 mM.

The Russian sturgeon (*Acipenser gueldenstaedtii* Brandt, 1833) and sterlet (*Acipenser ruthenus* Linnaeus, 1758) 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 percentage of motile sperm cells was estimated using binocular microscope Micmed-5 with video-eyepiece HB-200 (LOMO, Russia) with x 800 total magnification 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. For cryopreservation we used the sturgeon sperm with 4 and 5 points activity determined according to the Persov scale [22], the concentration of spermatozoa in the sperm (Goryaev camera) was $2.01 \cdot 10^9$ cells/ml (Russian sturgeon) и $2.81 \cdot 10^9$ cells/ml (sterlate sturgeon). Motility in experiment were analyzed using an unpaired Student's *t*-test. Statistical significance was set at $p<0.05$.

The sperm diluted with the cryoprotective medium was distributed in labeled Eppendorf tubes. The dilution ratio of sperm and cryomedium was 1:1. Before cryopreservation for mix sperm and cryomedia was acoustico-mechanical influences 500 Hz 1 minute. Equlibration time was 20 minutes. The samples were cryopreserved by programmable freezer with cooling rate 2°C/min. to -20°C, 20°C/min. to -70°C.

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.

The level of lipid peroxidation (LPO) of sperm was determined by the accumulation of secondary carbonyl products, which reacted with thiobarbituric acid (TBARS), using the traditional method [13]. The calculation was carried out according to the formula:

$$X = (E * 3 * 3.2) / (0.156 * 2), \quad (1)$$

where X is the content of TBARS in native and defrosted sperm, nmol; E is the extinction of samples; 3.2 is the total volume of the studied samples, ml; 2 is the volume of the supraventricular fluid taken to determine TBARS, ml; 3 — sample volume, ml; 0.156 — extinction of 1 nmol TBARS in 1 ml at 532 nm. The content of TBARS was expressed as nano-moles per 109 cells.

The effectiveness of the antioxidant action (EAA) of compounds was calculated by the formula:

$$EAA = [(C0 - C1) / C0] * 100\% \quad (2)$$

where C_0 is the concentration of TBARS in semen (control), C_1 is the concentration of TBARS in semen containing the compound under study. A positive value of the EAA indicator indicates the manifestation of an antioxidant effect by the substance; a negative value indicates the manifestation of a pro-oxidant effect.

Fertilization test with thawed sperm of Russian sturgeon and sterlet in laboratory conditions was carried out on the tanks of Kagalnik fish hathery (Rostov region, Russia) in Petri dishes. This method allows for fertilization and incubation of embryos without destickness eggs, which is very important when experimenting on small amounts of material. From 150 to 180 eggs of Russian sturgeon or sterlet were placed in Petri dishes. Then petri dishes were placed in pools with running water and after 18-24 hours the percentage of fertilization of experimental batches was determined.

3 Results

The work established the effect of BHT and trolox on the LPO level in the semen of Russian sturgeon and sterlet before and after freezing.The data on the LPO level obtained in the work show that in the control experiment, both in native and defrosted sturgeon sperm, the level of accumulation of toxic LPO secondary products practically does not differ for Russian sturgeon and sterlet.

The largest level of TBARS accumulates in defrosted sturgeon sperm without the use of antioxidants. The EAA values of the antioxidants used in the process of lipid peroxidation of native and defrosted sperm of both sturgeon species indicate that lipid-soluble antioxidant BHT is more active in reducing the accumulation of TBARS compared to water–soluble antioxidant trolox (Fig. 1).

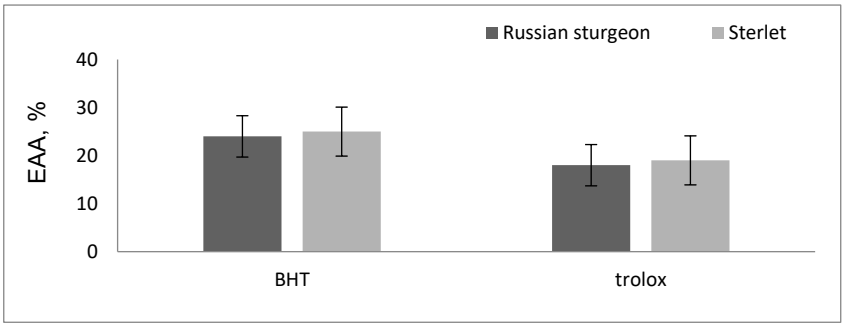

Fig. 1. The effectiveness of the antioxidant action (%) of phenolic compounds in the process of sturgeon sperm lipid peroxidation after cryopreservation. TBARS level in fresh: Russian sturgeon sperm in control - 0.70 (nmol/109 sperm); sterlet sperm in control - 0.65 (nmol/109 sperm). The concentration of the studied compounds is 0.1 mM. The average values for a series of experiments are given; differences from the control experimental group ($p < 0.05$). The values are expressed as mean ± SD.

The effect of BHT and trolox on the motility duration time of fresh sperm. as well as sperm after the stage of equilibration, which is necessary for the penetration of cryoprotectors into the cell [23], and defrosted sturgeon sperm was studied. The motility of fresh sterlet sperm (98%) was slightly higher than that of Russian sturgeon sperm (90%) (Fig. 2).

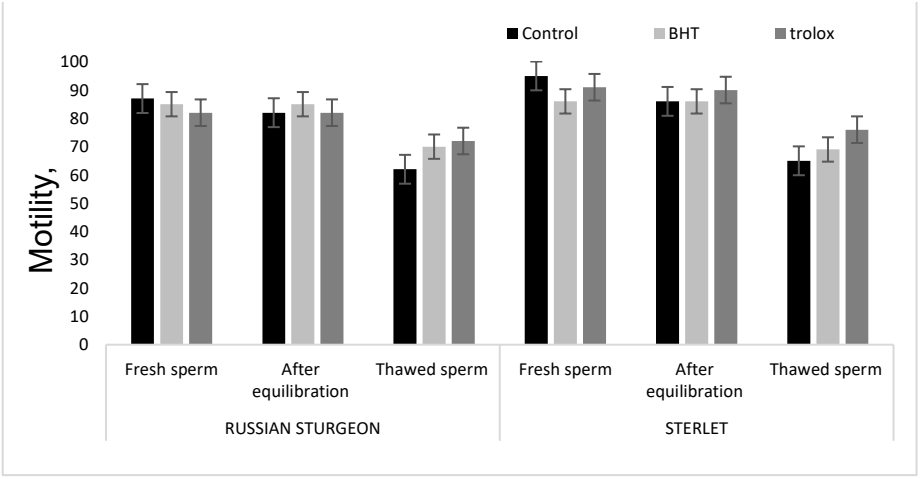

Fig. 2. Effect of the BHT and trolox on the sturgeon sperm motility during low-temperature preservation. The average values for a series of experiments are given; differences from the control experimental group ($p < 0.05$). The values are expressed as mean ± SD.

BHT did not affect the mobility of fresh sperm of Russian sturgeon and sterlet. The addition of trolox also did not affect the motility of fresh Russian sturgeon sperm, but it led to a decrease in the motility of fresh sterlet sperm compared to the control experiments.

The work found that a 30-minute equilibration did not have any significant effect on sperm motility as in control, and in the presence of antioxidants, there was only a slight decrease in this indicator in native sterlet sperm. The cryopreservation procedure resulted in a decrease in the percentage of motile spermatozoa in both species. The sperm motility of the Russian sturgeon in the control decreased by 25%, with the addition of BHT and trolox, by 18% and 15%, respectively. The motility of thawed sterlet sperm in the control decreased by 27%, in the presence of BHT and trolox, by 23 and 18%, respectively. Thus, both phenolic antioxidants had a protective effect on the motility of defrosted sperm of the studied sturgeon species.

This study shows that the application of BHT and trolox as antioxidant - cryoprotectors had the positive effect on the fertilizing capacity of sterlet and Russian sturgeon sperm (Fig. 3).

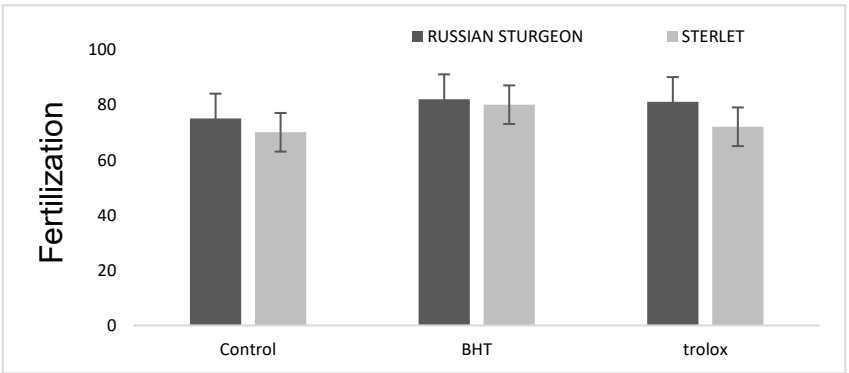

Fig. 3. Effect of the BHT and trolox on the fertilization rate after 5 days of freezing in liquid nitrogen. The average values for a series of experiments are given; differences from the cont rol experimental group ($p < 0.05$). The values are expressed as mean ± SD.

The addition of lipid-soluble antioxidant to a basic cryomedim increased the fertilization rate of Russian sturgeon and sterlet sperm cells by 10 %, compared to the control experiment. The addition of a water-soluble antioxidant to the basic cryo-medium increased the rate of fertilization of Russian sturgeon and sterlet sperm by 8% and 4%, respectivelly, compared to the control experiment. Thus, the greatest increase in sperm fertility with the addition of phenolic antioxidants to the cryo-environment was found for germ cells of Russian sturgeon with the addition of BHT.

4 Discussion

It is known that the antioxidant capacity of spermatozoa during cryopreservation is very limited due to the loss of most of the cytoplasmic components containing antioxidants during spermiogenesis. In addition, during cryopreservation of fish spermatozoa semen dilution also reduces the concentration of antioxidants available to spermatozoa, wherein, superphysiological levels of ROS are generated, which predetermines a high probability of the development of oxidative stress in reproductive cells. Therefore, an important strategy for protecting sperm from cryodamage is the introduction of antioxidants into basic cryoprotective media. Therefore, much interest has been focused on finding antioxidants to prevent the radical-induced oxidative damage to fish sperm during cryopreservation.

Results from the present study demonstrated that the addition of BHT and trolox leads to a decrease in the level of LPO both in Russian sturgeon and sterlet sperm. In the present study, we did not find significant differences in the level of lipid peroxidation in the spermatozoa of Russian sturgeon and sterlet spermatozoa, which, presumably, may be due to the fact that these sturgeon species have a similar fatty acid profile of sperm biomembranes. According to the literature data, sperm lipids of both sterlet and Russian sturgeon are characterized by the predominance of the sum of omega-6 acids over the sum of omega-3 acids. [24, 25].

Shown, that the effectiveness of the antioxidant action of lipid-soluble antioxidant is higher, than water-soluble antioxidant (Fig. 1). The latter can be explained by that BHT, being a fat-soluble compound, acts directly in biomembranes containing a large amount of polyunsaturated carboxylic acids, which are primarily subjected to peroxide oxidation, while water-soluble antioxidants, including trolox, reduce peroxidation outside the cell, but have little effect in the membranes or inside the cell. It is known that BHT can also reduce the harmful effects of lipid peroxyl radicals by converting them into hydroperoxides, which do not have a toxic effect on sperm [26]. Comparative experimental and theoretical study by Boulebd, [27] of the radical scavenging behavior of BHT and trolox showed that the activity of BHT exceeds the activity of trolox

In addition, the protective effect of BHT may be due to its ability to protect spermatozoa from cold shock, since it can be incorporated into the membranes of spermatozoa and provide them with flexibility and fluidity.It should also be taken into account that BHT has antiviral and antibacterial activity [28, 29], which is important, since such a component of the basic cryomedia as egg yolk, which is of animal origin, can be a source of various microorganisms that can disrupt the fertile potential of spermatozoa [30]. It is also known that BHT is a co-antioxidant, since it is able to regenerate of a-tocopherol [31] endogenous fat-soluble antioxidant contained in spermatozoa and seminal plasma of fish, including sturgeons [32]. By reducing the level of LPO, the antioxidants used prevent the toxic effect of the products of this process. Thus, TBARS are able to form various adducts and insoluble conglomerates with cellular components, including deoxyadenosine and deoxyguanosine in the DNA molecule, thereby carrying a mutagenic potential. Lipoperoxidation processes occurring in the biomembranes of sperm cells promotes the loss of motility and fertilizing ability of defrosted fish sperm [33]. It is established that the use of BHT during cryopreservation of

Russian sturgeon and sterlet sturgeon spermatozoa in the presence of modified Stein's medium led not only to a decrease in the level of the sperm lipid peroxidation-derived carbonyl by-products, but also an increase in motility and in the percentage of fertilization of eggs**.** The effect of the water-soluble analogue of vitamin E on the Russian sturgeon sperm during low-temperature preservation was similar to the effect of BHT. However, the introduction of trolox into the basic cryo-medium during cryopreservation of sterlet sperm also led to a decrease in the intensity of lipid peroxidation and an increase in the motility of defrosted sperm, but did not improve the fertility of the reproductive cells of this sturgeon species compared to the control experiment. Our previous studies have shown a beneficial effect of BHT on beluga sperm motility and fertility, as well as an increase in the level of TBARS in defrosted beluga sperm and 10% decrease of the fertilization rate compared to the control experiment when using trolox [13].

The results on the effect of trolox on the fertility of thawed sterlet sperm do not agree with the literature data indicating that spermatozoa motility is the main indicator of their fertility [34]. According to Zhu et al. [20] the introduction of trolox (200 μM) into the cryogenic medium (Tris-citrate-glucose extender supplemented with egg yolk and DMSO 4% (v/v)) during cryopreservation of rabbit sperm reduces the levels of ROS and LPO in the sperm, significantly improved post-thaw motility, could protect the defrosted sperm acrosome, membrane integrity from cryodamage during the freezing-thawing process. The low cryoprotective activity of IRE and trolox, established in this study, may be due to the presence of a rather high concentration of DMSO (12.5%) in Stein's basic medium. In some studies of the process of cryopreservation of sturgeon sperm using DMSO, it was found that thawed spermatozoa, having high mobility, have low fertility [35, 36]. Later, a harmful specific effect of DMSO on the sterlet sperm acrosome was established [37]. The acrosome is a specific sperm structure responsible for penetrating the egg,. It is liely that DMSO triggers this structure prematurely, long before any contact with the egg. Recently developed and tested the first low toxicity cryoprotectant containing only 2% DMSO [38]. Thus, it is important to search for the optimal concentration of DMSO in the composition of the cryo-medium with the addition of the studied phenolic compounds, which allows minimizing the negative effect of this solvent on sturgeon sperm.

5 Conclusions

The problem of improving the quality of defrosted germ cells of valuable fish species, including sturgeons, remains very relevant today. In the course of the studies, positive results were obtained using both a fat-soluble vitamin E analog (BHT) and a water-soluble one (trolox) for sperm Russian sturgeon and sterlet. The antioxidant activity of BHT and trolox was established to reduce the level of lipid peroxidation in the sperm of Russian sturgeon and sterlet after freezing/thawing. An increase in sperm motility was shown when lipid- and water-soluble antioxidants were added to the cryomedium, and the rate of fertilization of Russian sturgeon and sterlet sperm. However, the protective effect of these antioxidants was weakly expressed, which, in our opinion, is explained by the use of a cryogenic medium with a high concentration of DMSO. In the future, it is necessary to carry out research on the modification of the basic cryomedium with a minimum concentration of toxic DMSO.

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