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Antioxidant activity and protective effect of hydroxy derivatives of chalcones for sterlet (*Acipenser ruthenus*, Linnaeus, 1758) sperm

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ABSTRACT

The aim of this work is to study the effect of adding hydroxy derivatives of chalcones to the basic cryomedium on the ability of sterlet sperm to utilize superoxide and hydrogen peroxide, the intensity of lipid peroxidation of male fish germ cells, and their viability both before cryopreservation and after 3 days of freezing at liquid nitrogen temperature. The ability of phenolic derivatives of chalcones to increase the superoxide dismutase and catalase activities of sterlet sperm and to reduce the intensity of lipid peroxidation has been established. The antioxidant activity of the derivatives exceeds the effect of Trolox, which inhibits the functioning of the enzyme component of the antioxidant protection of fish sperm and promotes lipid peroxidation of fish sperm before cryopreservation. A beneficial effect of hydroxy derivatives of chalcones on the motility parameters of thawed sperm has been shown, indicating their ability to increase the cryoresistance of sperm in such a promising aquaculture species as sterlet.

1. Introduction

In the terms of biochemical composition and taste, sterlet is one of the most delicious species for fish products; therefore, this sturgeon species is promising for aquaculture. In addition, compared to other sturgeon species, sterlet is relatively small in size and is characterized by reaching marketable weight and sexual maturity earlier than the others. The development of effective cryotechnologies will make it possible to preserve the gene pool of this relict fish species, as well as to use thawed sperm for artificial insemination in commercial aquaculture.

In the current conditions of a dramatic decline in the abundance of wild sturgeon species populations in the Volga-Caspian basin, their artificial reproduction plays a decisive role in preserving the gene pool of these valuable fish species [27]. A current trend in aquaculture is the cryopreservation of fish reproductive cells [40]. Despite a long history of research into freezing sturgeon species sperm, existing cryopreservation technologies are still insufficiently effective for routine application in aquaculture due to the low fertility of thawed sperm [4].

One of the main factors damaging the viability of fish sperm during cryopreservation is the development of oxidative stress as a result of an increase in the concentration of reactive oxygen species (ROS) to toxic levels. Therefore, one of the strategies for increasing the cryoresistance of gametes during cryopreservation is the introduction of antioxidants (AO) into the basic cryomedia [31]. Specific features of the lipid profile of sturgeon species sperm [5], including a significant content of easily oxidized polyunsaturated fatty acids, determine the low cryoresistance of the reproductive cells of males of these relict fish species [16]. In recent years, the protective activity of polyfunctional phenolic compounds containing several different fragments with antioxidant activity has been studied during cryopreservation of sturgeon species sperm [14,21,23].

Hybrid structures based on chalcone, a precursor of flavonoids widespread in the plant world and possessing a wide range of biological activities [30], are of significant interest as potential cryoprotectors. Previously, in *in vitro* experiments, we established the antioxidant activity of hydroxy derivatives of chalcones [25,29], including antiradical activity against the initial ROS — superoxide anion radical ($O_2^{-\bullet}$).

The effectiveness of the antioxidant and cryoprotective action, as well as the toxicity of the compounds, is largely determined by their concentration [13]. Previously, we established the optimal

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concentration of phosphorus-containing phenol (0.1 mM), at which it not only reduces the intensity of lipid peroxidation in beluga sperm but also improves the quality of reproductive cells [22]. For this phenolic derivative, a 2-fold increase in the fertility of defrosted beluga sperm was shown at a concentration of 0.1 mM [14,23]. The use of other hybrid compounds at this concentration confirmed the effectiveness of their cryoprotective action [21,24].

The purpose of the current study is to research the effect of hydroxy derivatives of chalcones (Fig. 1) at a concentration 0.1 mM on $O_2^{-\bullet}$-utilizing activity (SOD-like) and H_2O_2-utilizing activity (CAT-like) of sterlet (*Acipenser ruthenus,* Linnaeus, 1758) sperm, the intensity of lipid peroxidation (LPO) in reproductive cells of male fish, and their viability before cryopreservation and after 3 days of freezing at liquid nitrogen temperature.

2. Materials and methods

2.1. Reagents and solutions

The study of the cryoprotective effect of hydroxy derivatives of chalcones **1–3** (Fig. 1), which were synthesized by previously known methods [25], on sturgeon species sperm was conducted in comparison with a phenolic synthetic AO, a water-soluble analogue of vitamin E – Trolox.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and all other reagents were supplied by Sigma–Aldrich. The test compounds were dissolved in modified Stein medium (130 mM NaCl, 5 mM KCl, 20 mM $NaHCO_3$, 5.5 mM glucose, 12.5 % egg yolk, 12.5 % Me_2SO), which served as the basic cryoprotective medium, as described in our previous study [23]. The final concentration of compounds in the measurement media was 0.1 mM.

2.2. Sperm collection

The research object was sterlet (*Acipenser ruthenus,* Linnaeus, 1758) sperm obtained from sturgeon hatcheries in the Lower Volga during the spawning period (from late April to mid-May 2023). The males (10 fish) had an average weight of 2–3 kg (6–7 years). The sterlet males used in the study were given a single injection of Luteinizing Hormone – Releasing Hormone Ethylamide (LH-RHa, Surfagon, Mosagrogen LTD, Russia). All experimental animal protocols in the study were conducted in accordance with the recommendations of the BIOETHICS COMMISSION of the Federal State Budgetary Institution of Science 'Federal Research Centre the Southern Scientific Centre of the Russian Academy of Sciences' (SSC RAS) (Protocol No. 2 of July 05, 2024). According to the conclusion of the BIOETHICS COMMISSION, our experimental studies comply with the principles of bioethics and the rules for the use of animals for scientific purposes. The sperm was collected with a catheter, which was a polyethylene tube shaped like a truncated cone (tapered) on one side and made of high-quality primary polyethylene. The catheter length was 440 ± 5 mm, the outer diameter of the tube was 4.5 mm, the inner diameter of the tube was 3.5 mm, and the inner diameter of the opening of the narrowed side was 1 mm. The sperm samples were cooled to 8 ± 2 °C and delivered to the laboratory in a thermal container.

2.3. General procedure for sperm freezing and thawing

Sperm cryopreservation was carried out according to the method of Tsvetkova et al. (1996) [41]. Sperm diluted with modified Stein cryomedium in a ratio of 1:1 (v/v) was distributed into labelled 1.5 ml Eppendorf tubes and placed in a refrigerator (T = 4 °C) for 20 min for equilibration. Fifteen tubes with sperm were frozen from each male, taking into account five experimental groups and three replicates per group. The final concentration of spermatozoa in each tube was 0.26 ± 0.10 × 10^9/ml. After the equilibration stage, freezing was carried out in a PLANER cryofreezer (UK), from 5 °C to −70 °C at a rate of 20–25 °C/min (freezing time was approximately 3 min). Once the samples reached a temperature of −70 °C, they were placed in liquid nitrogen and stored in a Worthington Industries LS-6000 biological storage facility (USA) for 3 days. The freezing temperature was measured using an electronic thermometer. Sperm thawing was performed in a water bath for 30–40 s at a temperature of 38–40 °C.

2.4. Assessment of the ability of sterlet sperm to utilize $O_2^{-\bullet}$

The ability of sterlet sperm to utilize $O_2^{-\bullet}$ was determined in a model system of adrenaline (epinephrine) oxidation in an alkaline medium [37], by measuring the rate of formation of the intermediate product of adrenaline oxidation, adrenolutin [43] using spectrophotometry at a wavelength of 347 nm with a 96-well microplate spectrophotometer (*Multiskan Sky Thermo Fisher Scientifics*, USA). The spectrophotometer wells were loaded with bicarbonate buffer (0.2 ml) and adrenaline solution (0.01 ml, 0.1 %, 5.46 mM). The plate was placed in the spectrophotometer and the optical density was measured over 5 min, with periodic shaking of the plate. The control sample contained all components except the adrenaline solution. A sperm homogenate in 0.2 M Tris buffer at a ratio of 1:10 was prepared using a mechanical homogenizer. The homogenate was centrifuged (3000 rpm) in a Thermo Scientific SL16R centrifuge (Thermo Fisher Scientific, Germany) for 10 min to remove partially destroyed cells and nuclei. The resulting supernatant, containing spermatozoa, was used to measure the ability of sterlet sperm to utilize $O_2^{-\bullet}$.

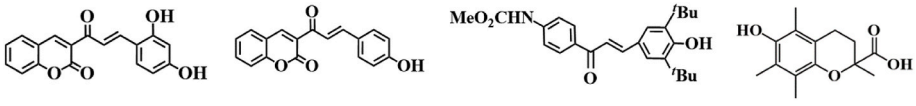
All procedures were performed at a temperature of 0–4 °C. The rate of adrenaline oxidation in the absence and presence of a biological product was assessed over a 5 min period. A decrease in the rate of the process in the presence of a biological product indicates the ability of fish sperm to utilize $O_2^{-\bullet}$. The activity was calculated relative to the control (%), with the oxidation of adrenaline in the alkaline bicarbonate buffer without the addition of a biological product was taken as 100 %. The experiments were repeated three times. Inhibitory activity (*I %)* was calculated using formula:

$$I\,(\%) = [(1 - A_i\ /\ A_0\ ) \times 100\%]$$

where A_i – optical density in the sample with the addition of tested compounds, and A_0 – optical density in the control (without added compounds).

2.5. Assessment of the ability of sterlet sperm to utilize H_2O_2

A mixture was prepared containing 1 ml of sperm (native or with the addition of a solution of the tested compounds in Me_2SO at the final



1 **2** **3** Trolox

Fig. 1. Structures of hydroxy derivatives of chalcones **1**–**3** and Trolox.

concentration of 0.1 mM) and 7 ml of phosphate buffer (pH 7.4). The mixture was centrifuged at 3500 rpm for 10 min and then the supernatant was collected. The reaction mixture contained 100 μl of 30 mM H_2O_2 solution in phosphate buffer and 100 μl of the supernatant in phosphate buffer. The control sample contained 100 μl of phosphate buffer instead of the supernatant. The H_2O_2 concentration was determined by spectrophotometry (λ = 240 nm) [19] for 10 min. For each compound, three parallel independent measurements were conducted. Based on spectrophotometric data, kinetic curves of H_2O_2 consumption were obtained and linearized using the coordinates of the first-order equation. Based on the slope of the obtained straight lines, the rate constants for the first-order decomposition reaction of H_2O_2 were calculated. A decrease in the reaction rate in the presence of the compound indicates its inhibitory activity.

2.6. Determination of the level of lipid peroxidation of sterlet spermatozoa and the effectiveness of the antioxidant effect of the studied compounds

The intensity of LPO in sterlet sperm was assessed by the level of accumulation of carbonyl oxidation products reacting with thiobarbituric acid – ThioBarbituric Acid Reactive Species (TBARS), using a well-known method [28]. We have previously published a method for determining the level of TBARS in fish sperm with cryomedium, both before and after cryopreservation [14]. The content of TBARS in fish sperm lipids was expressed in nanomoles per 10^9 cells. Cooled homogenized fish sperm (25 μl, native or with additives of the tested compounds at a final concentration of 0.1 mM) was added to 2 ml of a 1.2 % KCl solution, cooled to 0–4 °C. It was previously shown that Me_2SO had no effect on LPO levels relative to the control without additives. The resulting mixture, together with solutions of ascorbic acid (0.1 ml, 2.6 mM), Mohr's salt (0.1 ml, 0.04 mM), and trichloroacetic acid (1 ml, 40 %) was poured into polypropylene tubes and placed in a thermostated water bath (37 °C) for 10 min. The mixture was then centrifuged (3000 rpm) for 10 min. The supernatant (2 ml) was collected into clean tubes, and 1 ml of TBA solution (0.8 % l) was added. At the next stage, the tubes were placed in a boiling water bath for 10 min. After cooling to room temperature, chloroform (1.0 ml) was added to the samples until a clear solution was obtained, and the samples were centrifuged (3000 rpm) for 15 min. The clear supernatant was collected, and the optical density of the sample was measured using a *SF-103* spectrophotometer (λ = 532 nm). The TBARS level was calculated using the formula:

$$X = (A \cdot 3.0 \cdot 3.2) \,/\, (0.156 \cdot 2.0)$$

where X is the content of TBARS in sperm (nmol/10^9 cells); A – optical density of samples; 3.0 – sample volume (ml); 3.2 – total volume of tested samples (ml); 0.156 – extinction of 1 nmol of TBARS in 1 ml at 532 nm; 2.0 — volume of supernatant taken for determination of TBARS (ml).

The effectiveness of antioxidant action (EAA, %) of the tested compounds was calculated using the formula:

$$EAA = [(C_0 - C_1) \,/\, C_0\,] \times 100\%$$

where C_0 is the concentration of TBARS in control sperm; C_1 is the concentration of TBARS in sperm containing the tested compound. A positive EAA value indicates that the substance exhibits an antioxidant effect, while a negative value indicates the manifestation of a prooxidant effect. The experiments were repeated three times.

2.7. Motility and life time of sterlet spermatozoa after activation

The proportion of motile sterlet spermatozoa was assessed using a Mikmed-5 binocular microscope with an NV-200 video eyepiece (LOMO, Russia) after adding river water to the sperm as an activating solution at a ratio of 1:1000. Fish sperm with activity levels of 4 and 5, as determined according to the Persov scale [1], were used for the study.

The spermatozoa concentration in sterlet sperm was 0.52 ± 0.10 × 10^9/ml. Tested compounds were added to sperm (final concentration of 0.1 mM) and incubated for 20 min at 4 °C; the control group consisted of sperm without additives. The duration (the total period of spermatozoa movement, in seconds) was determined as the time from activation until the movement of the last spermatozoa stopped as observed in full view under the microscope, using a stopwatch. The same operator measured spermatozoa motility and lifespan after activation three times.

2.8. Statistical analysis

Statistical analysis, including verification of the conformity of the analyzed data to the normal distribution law, was performed using Statistica software for Windows, version 9.0 (StatSoft, Inc.). It was established that for all parameters the studied data have a normal distribution using the Kolmogorov-Smirnov, Lilliefors, and Shapiro-Wilk's tests. The obtained data were expressed as the mean ± SD. The parameters of antioxidant activity of the studied compounds, the concentration of TBARS, motility, and time of movement of fish sperm were analyzed using Student's t-test. Statistical significance was set at a level of $p < 0.05$.

3. Results

3.1. The effect of hydroxy derivatives of chalcones and trolox on the ability of sterlet sperm to utilize $O_2^{-\bullet}$ and H_2O_2 before and after cryopreservation

According to the results obtained (Table 1), the addition of hydroxy derivatives of chalcones **1–3** to a cryoprotective medium leads to an increase in $O_2^{-\bullet}$-, H_2O_2-utilizing activities of sterlet (*Acipenser ruthenus,* Linnaeus, 1758) sperm, both before (after equilibration) and after 3 days of freezing at liquid nitrogen temperature.

Compound **1** demonstrates the greatest stimulation of $O_2^{-\bullet}$–utilizing activity of fresh and thawed sterlet sperm, while compound **3** demonstrates the greatest stimulation of H_2O_2–utilizing activity. The addition of Trolox leads to a decrease in SOD-like activity of sperm, both before and after cryopreservation, by 2 and 2.4 times, respectively. In the presence of this reference compound, the CAT-like activity of thawed sperm also decreases (by 1.6 times), while the H_2O_2–utilizing activity of fresh sperm before cryopreservation increases by 1.7 times.

A decrease in SOD–, CAT–like activities of thawed sterlet sperm, compared to sperm before cryopreservation was determined, with decreases of 1.3 and 1.4 times, respectively.

Table 1
SOD–, CAT–like activities of sterlet sperm after equilibration (I) and after cryopreservation (II) in the presence of studied hydroxy derivatives of chalcones **1–3** and Trolox.

Compound	SOD-like sperm activity, % inhibition		CAT–like sperm activity, rate constant of H_2O_2 decomposition (k •10^5, s^{-1})	
	I	II	I	II
control	51.36 ± 1.19	38.46 ± 0.67[c]	11.16	7.97[c]
1	73.18 ± 1.67[a]	62.04 ± 0.99[b,c]	20.68[a]	9.01[c]
2	55.29 ± 1.28[a]	42.16 ± 0.61[b,c]	19.00[a]	9.12[c]
3	57.31 ± 1.44[a]	41.37 ± 0.43[b,c]	26.86[a]	14.48[b,c]
Trolox	24.60 ± 0.84[a]	16.08 ± 0.42[b,c]	18.47[a]	5.08[b,c]

The control values are for Stein's medium without additive compounds **1–3** and Trolox. The average values for a series of experiments are given; [a] differences from the control and experimental groups after equilibration ($p < 0.05$); [b] differences from the control and experimental groups after cryopreservation ($p < 0.05$); [c] differences in the experimental groups after equilibration and after cryopreservation ($p < 0.05$). The values are expressed as mean ± SD.

3.2. The effect of hydroxy derivatives of chalcones and trolox on the level of TBARS accumulation in sterlet sperm before and after cryopreservation

As shown by the results obtained (Table 2), the addition of the studied hydroxy derivatives of chalcones to the basic cryo-medium reduced the level of TBARS in sterlet sperm both before and after cryopreservation. However, this change was not statistically significant ($p > 0.05$).

In contrast, the addition of the reference antioxidant (Trolox) increased the intensity of LPO in sterlet sperm before cryopreservation compared to the control experiment.

After cryopreservation, this compound showed AO, but the EAA of Trolox for thawed sterlet sperm was significantly lower than the EAA for hydroxy derivatives of chalcone (Fig. 2).

The highest EAA is demonstrated by compound **3**, in the presence of which the level of TBARS in thawed sterlet sperm is lower than the LPO level in control sperm before cryopreservation (Table 2). It is important to note that the EAA of new phenolic derivatives increases after thawing of germ cells; the largest increase (by 4.3 times) was observed for compound **2**.

3.3. The effect of hydroxy derivatives of chalcones and trolox, on sperm motility parameters of sterlet before and after cryopreservation

We studied the effect of additives of hydroxy derivatives of chalcones, as well as Trolox, on the activity indices (the proportion of spermatozoa with progressive movement and their movement time) of native sterlet sperm, sperm with cryomedium before and after the equilibration stage (20 min, T = 4 °C), as well as after thawing sperm frozen at the temperature of liquid nitrogen. The motility of native sperm (without additives of antioxidants and cryomedium) of sterlet is 100 %, with a movement time is 1185 s. The addition of Stein's cryomedium without antioxidants to native sperm leads to an increase in sperm motility time before equilibration to 1522 s. After equilibration, a slight decrease in the lifespan by 69 s is noted (Fig. 3).

After the introduction of hydroxy derivatives of chalcones **1** and **3**, the proportion of spermatozoa with progressive movement both before and after sperm equilibration did not change statistically significantly ($p > 0.05$). The addition of compound **2** and Trolox led to a decrease in motility before equilibration by 4 % and 10 %, and after equilibration, by 8 % and 15 %, respectively (Fig. 4).

The work revealed a significant decrease in the parameters of fish sperm motility and lifespan after cryopreservation, relative to the values after the equilibration stage ($p < 0.05$) (Table 3). The percentage of motile sterlet spermatozoa in the control decreased from 100 % to 2.4 % after cryopreservation. In the presence of all the tested compounds after cryopreservation, sperm motility also decreased compared to the variant

Table 2
The level of accumulation of ThioBarbituric Acid Reactive Species (TBARS) in sterlet sperm in the presence of tested compounds after equilibration (I) and after cryopreservation (II).

Compound	TBARS, nmol/10^9 cells	
	I	II
control	1.66 ± 0.14	2.03 ± 0.1 0[c]
1	1.55 ± 0.15	1.73 ± 0.1 0[b]
2	1.63 ± 0.13	1.88 ± 0.20
3	1.32 ± 0.15[a]	1.51 ± 0.1 4[b]
Trolox	1.80 ± 0.18	1.95 ± 0.1 0

The control values are for Stein's medium without additive compounds **1–3** and Trolox. The average values for a series of experiments are given; [a]differences from the control and experimental groups after equilibration ($p < 0.05$); [b]differences from the control and experimental groups after cryopreservation ($p < 0.05$); [c]differences in the experimental groups after equilibration and after cryopreservation ($p < 0.05$). The values are expressed as mean ± SD.

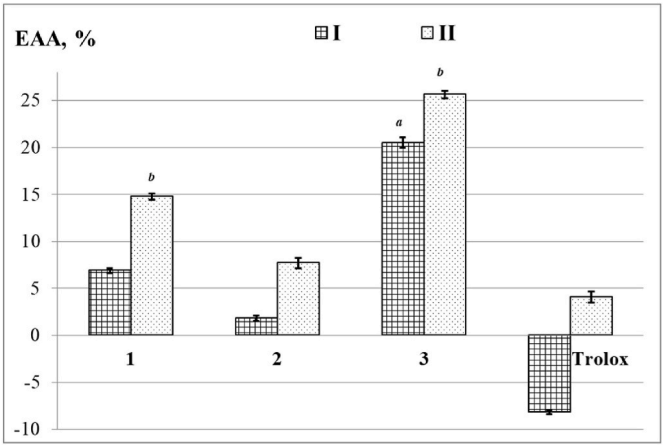


Fig. 2. Efficiency of antioxidant action (EAA, %) of the compounds **1–3** and Trolox after equilibration (I) and after cryopreservation (II) of sterlet sperm. *The control values are for Stein's medium without additive compounds **1–3** and Trolox. The average values for a series of experiments are given;* [a] *differences from the control and experimental groups after equilibration ($p < 0.05$);* [b] *differences from the control and experimental groups after cryopreservation ($p < 0.05$). The values are expressed as mean ± SD.*

before freezing, and in the case of compound **3,** it also decreased compared to the control variant. However, with the addition of compounds **1**, **2** and Trolox, sperm motility increased compared to the control.

In the present work, a decrease in sperm movement time after cryopreservation was established, both in the control and in the presence of all the studied compounds. The greatest decrease in this motility parameter is observed when compound **3** is added to the basic cryomedium, where the sperm movement is shorter by 2 min compared to the control variant. In the presence of all other studied compounds, the lifespan of thawed spermatozoa exceeds the sperm motility value observed in the control sterlet, indicating their cryoprotective activity.

4. Discussion

To improve the quality of fish sperm during cryopreservation, both low-molecular-weight AO and high-molecular-weight antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), are used [11,33]. SOD catalyzes the reaction of disproportionation of $O_2^{\bullet}$ to molecular oxygen and H_2O_2 [35]. CAT, by accelerating the decomposition of H_2O_2 to H_2O and O_2, plays a critical role in protecting fish sperm membranes from ROS [18].

In this work, an increase in the ability of sterlet sperm to utilize $O_2^{\bullet}$ and H_2O_2 before (after the equilibration stage) and after cryopreservation in the presence of compounds **1–3** and Trolox was established. It is known that $O_2^{\bullet}$ and H_2O_2 act as signaling agents at low concentrations [45] and are involved in the regulation of fish sperm motility [20], but are toxic at high concentrations [38]. Thus, according to Shalyutina-Koleshova et al. (2015) [34], the effect on frozen-thawed sterlet sperm of the enzymatic system generating $O_2^{\bullet}$ and H_2O_2 (xanthine-xanthine oxidase, pH 7.4) [10] was accompanied by a decrease in sperm motility, an increase in DNA fragmentation and activity of antioxidant enzymes, as well as intensification of LPO and protein oxidation in reproductive cells.

Previously, several studies characterized the enzymatic link of the antioxidant protection system in sterlet sperm [3,17,32]. According to published data and references, the sperm of this sturgeon species, in addition to SOD and CAT, also contain antioxidant enzymes such as glutathione peroxidase and glutathione reductase, which, in addition to CAT, are involved in the utilization of H_2O_2. Moreover, the ability of fish

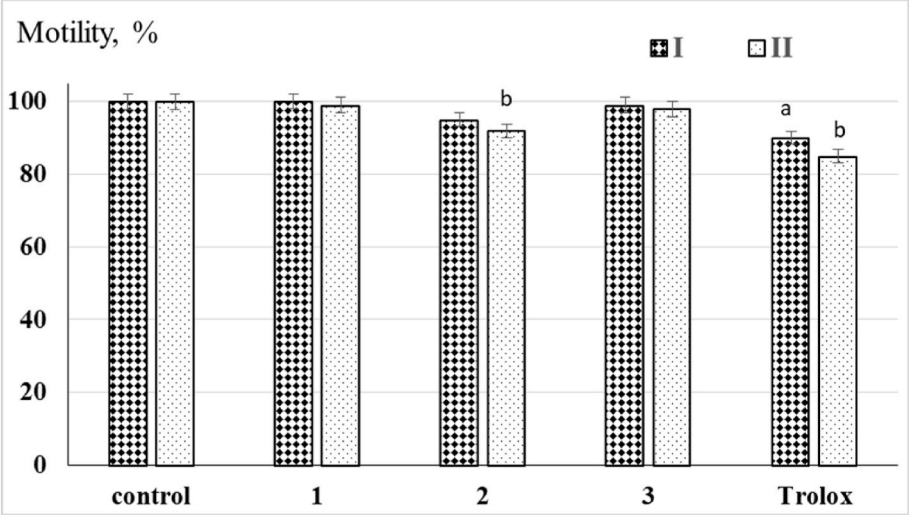


Fig. 3. The effect of the studied compounds on the motility of sterlet sperm before (I) and after equilibration stage (II). *The control values are for Stein's medium without additive compounds **1**–**3** and Trolox. The average values for a series of experiments are given;* [a] *differences from the control and experimental groups before equilibration ($p < 0.05$);* [b] *differences from the control and experimental groups after equilibration ($p < 0.05$); differences in the experimental groups before and after equilibration ($p > 0.05$). The values are expressed as mean ± SD.*

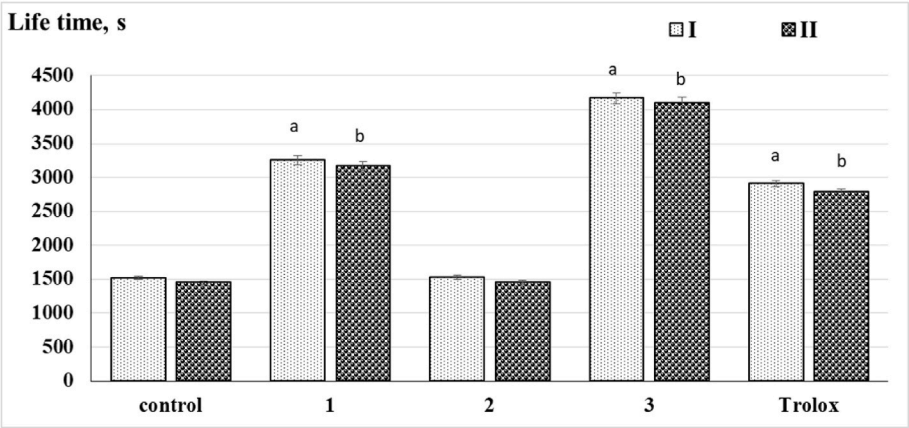


Fig. 4. The effect of the studied compounds on the life time of sterlet sperm before (I) and after equilibration stage (II). *The control values are for Stein's medium without additive compounds **1**–**3** and Trolox. The average values for a series of experiments are given;* [a] *differences from the control and experimental groups before equilibration ($p < 0.05$);* [b] *differences from the control and experimental groups after equilibration ($p < 0.05$); differences in the experimental groups before and after equilibration ($p > 0.05$). The values are expressed as mean ± SD.*

Table 3
The effect of the studied compounds on the parameters of sterlet sperm motility after cryopreservation.

Compound	Motility, %	Life time, s
control	2.4 ± 0.1^{b}	154 ± 3^{b}
1	$4.1 \pm 0.1^{a.b}$	$184 \pm 3^{a.b}$
2	$6.5 \pm 0.2^{a.b}$	$295 \pm 6^{a.b}$
3	$1.2 \pm 0.1^{a.b}$	$25 \pm 1^{a.b}$
Trolox	$3.1 \pm 0.1^{a.b}$	$196 \pm 2^{a.b}$

Effect of compounds on the proportion of translational motility of sterlet sperm after cryopreservation after 3 days of freezing in liquid nitrogen. The control values are for Stein's medium without additive compounds **1–3** and Trolox. The average values for a series of experiments are given; [a]differences from the control and experimental groups after cryopreservation ($p < 0.05$); [b]differences in the experimental groups after equilibration and after cryopreservation ($p < 0.05$). The values are expressed as mean ± SD.

sperm to utilize ROS may also be associated with the activity of low molecular weight antioxidants (uric acid, ascorbic acid, α-tocopherol, glutathione, and cysteine) [39]. Therefore, $O_2^{-\bullet}$-, H_2O_2-utilizing activities of fish sperm, designated in this work as SOD-like and CAT-like activities, respectively, maintain the concentration of these ROS at the optimal level, which prevents the development of oxidative stress in fish reproductive cells. Thus, our study indicates that, unlike Trolox, chalcone hydroxy derivatives are capable of increasing $O_2^{-\bullet}$-, H_2O_2-utilization activity of sterlet sperm both before and after cryopreservation.

It is known that a sharp increase in SOD activity in a cell without corresponding activation of CAT can lead to the formation of toxic levels of H_2O_2 [26]. In addition to its dismutase activity cytosolic SOD also has peroxidative activity [42]; in the presence of H_2O_2, this enzyme is able to catalyze the formation of the highly aggressive hydroxyl radical ($HO^{\bullet}$). In our study, only when compound **1** was added to the Stein's medium did the increase in SOD-like activity of thawed sterlet sperm (by 1.6 times) exceed the stimulation of CAT-like activity (by 1.1 times). Such an imbalance between the activities of SOD and CAT may be an additional damaging factor.

The work revealed a decrease in $O_2^{-\bullet}$-, and H_2O_2-utilization activity of sterlet sperm after cryopreservation. References and published data [12] also indicate a decrease in the activity of SOD and CAT in the sperm of Russian sturgeon (*Acipenser Gueldenstaedtii* Brandt & Ratzeburg, 1833) during cryopreservation, which is associated with the release of antioxidant enzymes from spermatozoa due to the disruption of the integrity of the plasma membrane during cryopreservation. At the same time, according to Dadras et al. (2022) [2], despite the disruption of the integrity of the plasma membrane of sterlet sperm during cryopreservation, no significant differences were established between the activity of SOD and CAT in sperm and sperm plasma after cryopreservation compared to fresh spermatozoa. According to data obtained during the study of the effect of cryopreservation on the proteome of sterlet sperm and seminal plasma [44], the decrease in the amount of protein in sperm during cryopreservation is due not to protein leakage, but to its degradation.

Sturgeon sperm are especially sensitive to LPO reactions due to the predominance of polyunsaturated fatty acids in membrane phospholipids, which are good substrates for oxidative free radical processes [8, 17].

Previously, the prolonged antioxidant activity of compounds **1–3** was determined in two model systems: oleic acid oxidation and the long-term process of tilapia liver lipid oxidation (72 h) [25]. The greatest efficiency in the first model system is demonstrated by the dihydroxy derivative of chalcone **1**, and in the second model system, by compound **3**, which contains a spatially shielded phenolic fragment that increases the stability of the structure. Compound **3** showed the highest EAA in our study, both before and after cryopreservation (Fig. 2). The structure of the molecule of this compound contains two spatially hindered *tert*-butyl groups that shield the phenolic hydroxyl group, which, when the AO interacts with ROS, ensures the formation of a stable phenoxyl radical that is incapable of continuing the chain reactions of LPO.

The increase in the level of TBARS in sterlet sperm after freezing (by 1.2 times) indicates the development of oxidative stress in the reproductive cells of male fish during cryopreservation, which is consistent with published data [2,34]. The intensification of LPO reactions in sterlet sperm during cryopreservation may be the cause of changes in the lipid composition of thawed fish sperm. Researchers recently reported [2] a decrease in the content of phospholipids, free fatty acids, triglycerides, cholesterol, and its esters in thawed sterlet sperm compared to native ejaculate. Thus, the obtained results indicate the inhibitory effect of all hybrid chalcone derivatives on the process of lipid peroxidation in sterlet sperm both before and after cryopreservation, while Trolox exhibits antioxidant activity only after freezing, and its EAA is lower than that of the chalcone derivatives.

Fish sperm motility is considered the best current biomarker of sperm quality in fish, including sturgeon species [6]. Numerous reports indicate a correlation between fish spermatozoa motility and fertility [7]. The influence of the cryopreservation process on the movement parameters of sturgeon spermatozoa in various cryoprotective media, including those with the addition of antioxidants, has been well established [15].Despite the fact that the motility of sterlet sperm before freezing was 100 %, the current study indicated a significant decrease (more than 97 %) in sperm motility after cryopreservation (Table 3). According to Tsvetkova et al. (1996) [41], the percentage of motile sperm of sterlet after cryopreservation decreased from 68 % to 15 %.

The significant decrease in sperm motility in fish during cryopreservation, along with a less significant decrease in motility time (Table 3) found in our study, may be associated with the “elimination” of poor-quality sperm during cryopreservation [36], damage to proteins associated with sperm motility [44], and damage to mitochondrial ATP synthase in sperm and seminal plasma. ATP synthase catalyzes the synthesis of ATP, and a decrease in its content during cryopreservation may lead to a lack of ATP for sperm movement.

Increasing the motility period of sturgeon species sperm seems to be important because sturgeon fertilization occurs in the external environment, which negatively affects the lifespan of gametes. Therefore, sperm must reach the female gamete as quickly as possible. Additionally, under natural spawning conditions, only a small number of sturgeon species spermatozoa can reach the eggs for fertilization due to dilution of the sperm by water currents and flows [9]. Thus, the long-term motility of sturgeon sperm can be considered an adaptation of the species. In this regard, analysis of the motion activity of sterlet spermatozoa after thawing shows the effectiveness of using new polyfunctional derivatives of chalcones as cryoprotectants. Despite the low percentage of motile sperm in the presence of the studied compounds, the lifespan of spermatozoa increases relative to the control, thus indicating the cryoprotective activity of hydroxy derivatives of chalcones and their ability to increase the cryoresistance of reproductive cells of males of this relict fish species.

Thus, the study established the cryoprotective activity of new chalcone derivatives for sterlet sperm. The greatest protective effect is characteristic of compound **2**, which more effectively increases the cryoresistance of sterlet sperm compared to the well-known antioxidant, Trolox.

5. Conclusions

The study demonstrated the stimulation of sterlet sperm’s ability to utilize ROS ($O_2^{-\bullet}$ and H_2O_2), a decrease in the level of sperm lipid peroxidation, and a beneficial effect on the motility parameters of reproductive cell in the presence of hydroxy derivatives of chalcones. The antioxidant activity of these compounds exceeds the effect of Trolox, which exhibits pro-oxidant properties. The absence of inversion of the antioxidant properties of the compounds, the increase in the efficiency of the antioxidant effect during cryopreservation, and the increase in sperm cryoresistance indicate the prospects of their application in preserving the original quality of sterlet reproductive cells during long-term low-temperature storage.

CRediT authorship contribution statement

V.P. Osipova: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **M.N. Kolyada:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **M.A. Polovinkina:** Methodology, Investigation, Data curation. **A.D. Kolumbet:** Investigation, Data curation. **E.N. Ponomareva:** Methodology, Investigation, Funding acquisition. **A.V. Velikorodov:** Methodology, Investigation.

Conflicts of interest

The authors declare no conflict of interest.

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