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Cryoprotective effect of phosphorous-containing phenolic anti-oxidant for the cryopreservation of beluga sperm

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ABSTRACT

A cryoprotective effect of an addition of a new synthetic antioxidant – a representative of phosphorus-containing sterically hindered phenols is presented. The efficiency of the compound was shown to exceed the effect of lipid-soluble antioxidants butylated hydroxytoluene (BHT) and trolox in the conditions of cryopreservation of beluga sperm in the presence of the modified Stein's medium. It was shown that the level of carbonyl oxidation by-products, which can react with thiobarbituric acid (TBARS), in beluga sperm was inversely proportional to the motility time of sperm cells. The fertility of beluga sperm increased 2 times upon the addition of phosphorus-containing phenol to a modified Stein's medium. The prospects of the new antioxidant application to improve cryoresistance of beluga sperm in the conditions of cryopreservation for its efficient protection from the peroxidation processes are discussed.

Introduction

At recent decades, there was noticeable exhaustion of sturgeon species reserves, as can be seen from the data of official statistics. The decline of these species of fish, some of which are rare and being under threat of disappearance, was accelerated under the action of anthropogenic impact, which is significantly different from evolutionary factors. Beluga (*Huso huso* Linnaeus, 1758), as the largest and most long-living species of sturgeon, is now one of the vulnerable species. It is listed in the Red book of the International Union for Conservation of Nature (IUCN), the Azov subspecies of beluga is listed in the «Red book of the Russian Federation» (2001) [24,43]. In connection with current situation the conservation of this species of sturgeon is a priority task. Cryopreservation is a promising way for preservation of rare and endangered species of sturgeon. For these purposes the fish sperm is used, because it can be stored in liquid nitrogen during several decades without change of its productive qualities [9]. Such biological features of sperm cells, as dense packing of genetic material, low water content (which is important in view of cells damage at freezing), low level of vital activity in the stationary conditions make them the most suitable object for freezing [21]. However, the stable high percentage of the viability of spermatozoids after defrosting cannot be guaranteed. This situation is caused by the injury of the majority of the cells during cryopreservation because of intra- and extracellular water crystallization [52], as well as hyper-production of reactive oxygen species (ROS) in sperm [31,4,6]. It is known that the level of cryo-injury of the sperm can be estimated by the intensity of free-radical reactions [33].

Sperm is characterized by a high concentration of polyunsaturated acids. These acids cause sensitivity of the sperm to the harmful effects of free radicals that can speed up the process of lipid peroxidation (LPO) [11,12]. Disposal of ROS is provided by various antioxidants both inside and outside of a cell. In the case, if the concentration of antioxidants in cryoprotective medium in the process of preservation, is insignificant, oxidative injury of important sperm cell structures occurs [44].

We have previously determined that phosphorus-containing sterically hindered phenols possessed inhibiting activity in reactions of liver lipid peroxidation. This finding allowed to propose a way for reduction of the peroxidation level of liver lipid of Russian sturgeon [5], Russian sturgeon sperm [37], fish feed lipids [7], based on addition of (3,5-di-tert-butyl-4-hydroxy phenyl) methylenediphosphonic acid (MDPA). It was shown that MDPA possessed the highest efficiency of antioxidating activity, such activity of this compound exceeded the effect of commonly known antioxidants.

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In this work we discuss the use of MDPA to improve the cryoresistance of beluga sperm cells. Cryoprotective effect of this compound was estimated by its influence on the rate of lipid peroxidation of beluga sperm fragments, on its activity and fertility in the conditions of cryopreservation. We performed a comparison of the efficiency MPDA's antioxidating effect on beluga and Russian sturgeon sperm cells.

Materials and methods

Reagents and solutions

MDPA was synthesized by known methods [41], BHT (2,6-di-tert-butyl-4-methylphenol, 99%), trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%) and all other reagents were purchased from Sigma–Aldrich. Phenolic compounds were dissolved in the modified Stein's cryomedium (130 mM NaCl, 5 mM KCl, 20 mM $NaHCO_3$, 5.5 mM glucose, 12.5% egg yolk, 12.5% DMSO) [39] to make a stock solution with the sperm at a concentration of 0.1 mM.

Sperm collection

Beluga sperm, received from sturgeon hatcheries of the Low Volga: Bertyulsky, Lebyazhyi, Sergiyevsky, was used in the study. Tests were carried out during the period from 2005 to 2013. Every year naturally mature fish (10–12 male fish, weighing 65 kg individually, 18–25 years old) were obtained during peal stage of the spawning season (from the end of March to the middle of May). The sperm was collected by the hypophysial injections method. The LH-RHa (Luteinizing Hormone – Releasing Hormone Ethylamide) was entered to males and females. The dose was calculated per 1 kg of female body weight and it was 4 mg/kg at water temperature 9 °C. Females were exposed to two injections. The first one (10% of total dose) was injected 36 h prior to receiving eggs from females, the second injection (90% of total dose) was made 12 h after the first one. Males were subjected to a single injection of LH-RHa 2–3 h before the second injection of females. The sperm was collected by catheter. The sperm samples were placed on ice and transported to the laboratory.

Motility and duration time of sperm after activation

The percentage of motile sperm cells was estimated using binocular microscope Micmed-5 with video-eyepiece HB-200 (LOMO, Russia) with ×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 sperm of the beluga with 4 and 5 points activity determined according to the Persov scale [38], the concentration of spermatozoa in the sperm (Goryaev camera) was $2.51 \cdot 10^9$ cells/mm^3. Duration time (total period of sperm movement, sec) was defined as the time from the activation to the termination of movement using stopwatch. Motility and duration time of sperm after activation were measured by the same operator for three times.

General procedure for sperm freezing and thawing

Sperm cryopreservation was carried out according to the methods of Tsvetkova et al. [48]. The sperm diluted with the cryoprotective medium was distributed in labeled 1.5 ml Eppendorf tubes and placed in the refrigerator for 40 min for equilibration [26]. The ratio of sperm and cryomedium was 1:1. Freezing temperature was measured with an electronic thermometer. After equilibration deep freezing was performed in three stages: from 5 °C to −15 °C with rate 2–5 °C/min (freezing time 2–5 min); from −15 °C to −70 °C with rate 20–25 °C/min (freezing time about 3 min); deep freezing in liquid nitrogen. Thawing of sperm was performed in a water bath during 30–40 s at a temperature of 38–40 °C. Sperm survival was evaluated using the criteria of the post thaw spermatozoa motility.

Fertility by post-thaw sperm

Fertilization by post-thaw sperm of beluga eggs in vitro was performed by the method of Kokhanskoy (1980) [25] in Petri cup [13,23]. The eggs (from 150 to 180) were placed in Petrie dish. After thawing of sperm samples and calculation of their quantity egg fertilization was carried out. The egg to sperm ratio was 1:2. Incubation was carried out in tanks with flowing water. The sperm in river water (ratio 1:200) was added to provide the high probability of monospermic fertilization. The eggs were stirred vigorously during 2 min. Values are given as mean ± SD, $N = 3$. Mathematic data processing was performed conducted by Student t-test.

Determination of antioxidative activity

The intensity of sperm lipid peroxidation has been assessed by the accumulation of carbonyl oxidation by-products, which react with thiobarbituric acid (TBARS), using the traditional method [47]. The content of TBARS was expressed as nano-moles per 10^9 cells. In experiments of TBARS determination before cryopreservation (without cryomedium) 1 ml of sperm was used, while in experiments of TBARS determination (with cryomedium) after cryopreservation the diluted sperm, quantity of which was equivalent to 1 ml of undiluted sperm, was applied. All experiments were repeated three times.

(1) Before cryopreservation and without cryomedium.

To 156 ml of 1.2% solution of KCl at 0–4 °C 8 ml of sturgeon sperm and 0.1 mM of tested compound (MDPA, BHT, trolox) was added. The resulting mixture was incubated for 48 h at 5 °C, the 2 ml probes of a mixture were taken after 1, 3, 24 and 48 h into the plastic tubes (4 ml) for centrifugation. 0.1 ml of 2.6 mM solution of ascorbic acid and 0.1 ml of 40 μM Mohr's salt, 1 ml of 40% solution trichloroacetic acid were added to each probe. The tubes were placed for 10 min in a water bath at 37 °C, then they were centrifugated for 10 min at 3000g.

On the next step 2 ml of supernatant were transferred to the clean tubes, 1 ml of 0.8% solution of thiobarbituric acid was added, the tubes were placed into a boiling water bath for 10 min and then they were cooled to the room temperature (25 °C). After cooling 1.0 ml portions of chloroform were added to the tubes to obtain receive the transparent solutions and these probes were centrifuged at 3000g for 15 min. Supernatant liquid was collected and extinction of the probe was measured using SF-103 spectrophotometer at 532 nm, the test probe was taken as a standard. The calculation was performed by the formula:

$$X = (E^*3^*3.2)/(0.156^*2)$$

where X (nmol) is the quantity of TBARS in native sperm; E – the extinction factor of the probe; 3.2 ml is the total volume of sperm from the tested fish; 2 ml is the volume of supernatant used for TBARS determination; 3 ml is the total volume of probes; 0.156 is the extinction factor of the 1 nmol TBARS at 532 nm.

The effect of addition of MDPA, BHT, trolox on the level of accumulation of TBARS in beluga sperm in the presence of the modified Stein's cryomedium before cryopreservation during incubation of the compound at room temperature for 3 h and after cryopreservation for 3 days at a temperature equal to −196 °C was studied.

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