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The Effect of Tin Compounds on the Lipid Peroxidation Level of Russian Sturgeon Fresh and Cryopreserved Sperm

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The effect of organotin compounds (OTs) on the accumulation of the lipid peroxidation (LPO) carbonyl by-products, which react with thiobarbituric acid (TBARS) in fresh and cryopreserved sperm of Russian sturgeon, was studied. It was found that incubation (1 hour) of Russian sturgeon sperm with OTs (CH_3SnCl_3, $(CH_3)_2SnCl_2$, $(CH_3)_3SnCl$, $(n\text{-}C_4H_9)_2SnCl_2$, $(n\text{-}C_4H_9)_3SnCl$, $(C_6H_5)_2SnCl_2$, $(C_6H_5)_3SnCl$) in concentration 0.1 mM led to the promotion of the accumulation of TBARS in native semen. Dimethyl- (DMT) and diphenyltin dichlorides (DPT) exhibited the greatest promoting activity, and the LPO level of both native and cryopreserved sperm of Russian sturgeon, including those in modified Stein's cryomedium, increased in the presence of these compounds. It was found that Russian sturgeon's cryopreserved sperm had lower sensitivity to the promotion of sperm LPO by DMT and DPT compared with the native sperm. The protective effect of Stein's cryomedium decreased in the presence of the studied OTs. The results suggest that accumulation of OTs by gonad of fish is another stress factor affecting the cell productivity in the cryopreservation process.

Keywords: Russian sturgeon fresh and cryopreserved sperm, organotin compounds, lipid peroxidation, cryomedium.

Introduction

Russian sturgeon (*Acipenser gueldenstaedtii Brandt, 1833*) is among those commercially precious sturgeon species in the Caspian Sea, the natural populations of which have drastically declined in the last decade. One of the most significant factors influencing the population of sturgeons in the Caspian Sea is contamination of water and sediment by various pollutants, which are disrupting for the migration and reproduction of the sturgeons (Basu and Janz, 2013).

Due to their benthic nature, sturgeons are very sensitive to contaminants (Miandare et al., 2016) including organotin compounds (OTs), which are highly toxic persistent organometallic xenobiotics in the environment. At the same time, OTs are widely used in several commercial applications. The extensive use of trisubstituted butyl and phenyl derivatives of tin as biocides in antifouling systems on ships has led to an unprecedented contamination of the marine environment at a global scale. Methyltins are probably produced by biomethylation of the commercial OTs in the environment (Bridou et al., 2018). Thus, OTs are habitual components of aquatic ecosystems. These OTs effectively accumulate in molluscs (Antizar-Ladislao, 2008), which are the preferred foodstuff of sturgeons.

Elevated levels of heavy metals and persistent organic pollutants in water, sediments, bottom organisms and some fish species (Mirnategh et al., 2018), including sturgeons of the Caspian Sea (Kajiwara et al., 2008), were considered in some studies. However, not much is known about the status of OTs contamination in the sturgeons from the Caspian Sea.

Despite the fact that since 2008 the use of tributyl tin compounds as a biocidal additive in anti-fouling coatings was prohibited in the EU due to the accumulation of these compounds by bottom sediments, they continue to enter water bodies (Chen et al., 2019). OTs can be easily absorbed into the tissues of aquatic animals with bioconcentration factors of 10^2–10^4 (Chen et al., 2017) and are considerably persistent, including persistence in hydrobiont gonads (Hu et al., 2009). The reproductive system of aquatic organisms is the most sensitive to the toxic effects of xenobiotics. Spermatozoa, including cryopreserved sperm, are used for the ecotoxicological evaluation of aquatic environment (Fabbrocini et al., 2013). Despite the sturgeon's endangered status in the Caspian Sea, there are only a few studies dealing with relative sensitivity of sturgeons to OTs (Graceli et al., 2013). Literature data indicate the relative stability of sturgeon sperm (Siberian sturgeon and starlet) to the negative effects of such heavy metals as mercury and cadmium (Dietrich et al., 2012). It was found that at presence of the OTs the mobility of hydrobionts sperm reduced (Shim et al., 2006) and its conservation also was inferior (Li, 2001); however, this problem has not been studied in view of the tasks of cryobiology.

Drastic declines in sturgeon natural populations have led to sturgeons classifying as an endangered breed. Thus, sturgeon aquaculture has gained importance over the past years. The exposure of fish to industrial and agrochemical origin pollutant-containing water is one of the factors affecting the quality of fish sperm in aquaculture. OTs can enter fish sperm during artificial fish farming due to their accumulation in the germ cells of spawners, caught in natural water bodies polluted with toxicants (Okoro et al., 2011). Artificial fish food, used in sturgeon industrial breeding, may also contain OTs (Saïdi et al., 2013). In addition, possible leaching of dimethyl-, dibutyltin dichlorides used in aquaculture polyvinyl chlorine (PVC) and chlorinated polyvinyl chloride (CPVC) plastics used as stabilisers of polymers (Matthews, 1996) should be taken into account. Although OTs are leached out in very low concentrations, it is very probable that they accumulate in a lipid-rich sperm of fish. Taking into account that an increase of lipid peroxidation intensity (LPO) may be one of the mechanisms of a negative effect of OTs on hydrobiont sperm, the effect of organotin compounds on the LPO level in Russian sturgeon fresh and cryopreserved sperm was investigated in this study.

Methods

Reagents and solutions

Organotin compounds (CH_3SnCl_3, $(CH_3)_2SnCl_2$, $(CH_3)_3SnCl$, $(n\text{-}C_4H_9)_2SnCl_2$, $(n\text{-}C_4H_9)_3SnCl$, $(C_6H_5)_2SnCl_2$, $(C_6H_5)_3SnCl$) and all other reagents were purchased

from Sigma–Aldrich. In this work, 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) (Osipova et al., 2014) was used.

Sperm collection

Russian sturgeon sperm was received from Bertyulsky, Lebyazhyi and Sergiyevsky sturgeon hatcheries of the Low Volga. Tests were carried out during the period from 2016 to 2018. Every year naturally mature fish (8–10 male fish, each weighing 15–18 kg (17.0 ± 0.9 kg), 10–12 years old) were obtained during the period of the spawning migration (from the middle of April). Spermiation in males was induced by a single injection of surfagon (Mosagrogen LTD, Russia). The dose was calculated per 1.0 kg of male body weight and amounted to 5.0 mg/kg at water temperature 12°C. Sperm was collected by a catheter. Sperm samples were placed on ice and transported to the laboratory.

Incubation of semen with organotins

The CH_3SnCl_3, $(CH_3)_2SnCl_2$, $(CH_3)_3SnCl$, $(n\text{-}C_4H_9)_2SnCl_2$, $(n\text{-}C_4H_9)_3SnCl$, $(C_6H_5)_2SnCl_2$, $(C_6H_5)_3SnCl$ were diluted in the sturgeon semen. The resulting mixture was incubated for 1 h at 5°C.

General procedure for sperm freezing and thawing

Sperm cryopreservation was carried out according to the methods of Tsvetkova et al. (Tsvetkova et al., 1997). The diluted with the cryoprotective medium sperm was distributed in labelled 1.5 mL Eppendorf tubes and placed in a refrigerator for 40 min for equilibration (Kopeyka et al., 1981). The ratio of sperm and cryomedium was 1:1. After equilibration, deep freezing was performed in three stages in a programmable freezing box with an electronic thermometer: from 5°C to –15°C with the rate 2–5°C/min (freezing time 2–5 min); from –15°C to –70°C with the 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 38–40°C.

Determination of the accumulation level of TBARS in Russian sturgeon sperm

The intensity of sperm lipoperoxidation was assessed by the accumulation of carbonyl oxidation by-products, which react with thiobarbituric acid (TBARS), using the traditional method as described previously (Osipova et al., 2017; Polovinkina et al., 2019). The content of TBARS was expressed as nanomoles per 10^9 cells. In experiments of TBARS determination before cryopreservation (without cryomedium), 1 mL of sperm was used. In experiments of TBARS determination with cryomedium before and after cryopreservation, the diluted sperm, the quantity of which was equivalent to 1 mL of undiluted sperm, was applied.

a before cryopreservation without cryomedium

To 156 mL of 1.2% solution of KCl at 0–4°C, 8 mL of sturgeon sperm without the addition of tin compounds (control) or with the addition of tested OTs were added. The resulting mixture was incubated for 1 h at 5°C, and the 2 mL probes of a mixture were taken into the plastic tubes (4 mL) for centrifugation. 0.1 mL of 2.6 mM solution of ascorbic acid, 0.1 mL of 40 mM Mohr's salt, and 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 and then were centrifuged for 10 min at 3,000 g. On the next step, 2 mL of supernatant were transferred to clean tubes, 1 mL of 0.8% solution of thiobarbituric acid was added, and the tubes were placed into a boiling water bath for 10 min and then were cooled to the room temperature (25°C). After cooling, 1.0 mL portions of chloroform were added to the tubes to obtain the transparent solutions and these probes were centrifuged at 3,000 g 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:

$$X=(E \times 3 \times 3.2)/(0.156 \times 2)$$

where X (nmol) is the quantity of TBARS in native sperm; E is 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 organotin compounds supplements on TBARS accumulation in the sperm species diluted by the modified Stein's cryomedium was studied before cryopreservation during incubation of the compound

at room temperature for 1 h and after cryopreservation for 3 days at a temperature equal to –196°C.

b before cryopreservation and with cryomedium

1 mL of semen without the addition of tin compounds (control) or with the addition of tested OTs diluted with cryomedium (1:1) was mixed with 19.5 mL of cooled 1.2% KCl solution. 0.1 mL of 2.6 mM solution of ascorbic acid and 0.1 mL of 40 mM solution of Mohr's salt, 1 mL of 40% solution of trichloroacetic acid were added to 2 mL of the mixture. The subsequent procedure was identical to the described in the previous case.

c after cryopreservation with cryomedium

The sperm without the addition of OTs (control) or with the addition of the tested organotin compound at 0.1mM concentration diluted by cryomedium (1:1) was frozen and thawed as described in *General procedure for sperm freezing and thawing*. After thawing, the sperm (2 mL) in a quantity equivalent to 1 mL of undiluted sperm was placed in tubes, then 0.1 mL of 2.6 mM solution of ascorbic acid, 0.1 mL of 40 mM solution of Mohr's salt, and 1 mL of 40% solution of trichloroacetic acid were added. The subsequent procedure was identical to the described in the section *a*.

Statistical analysis

The statistical analysis was performed using Statistica for Windows, Version 9.0 (StatSoft, Inc.), and the data were presented as mean ± SD. All experiments were repeated three times. TBARS concentrations in experiment were analysed using an unpaired Student's *t* test. Statistical significance was set up at $p < 0.05$.

Results and Discussion

Fish spermatozoa are sensitive to damage by ROS, since they contain large amounts of highly unsaturated fatty acids – substrates for reactive oxygen species (ROS), but possess limited endogenous antioxidant protection (Poli et al., 2004). In spermatozoa, ROS are generated endogenously as a by-product of normal aerobic metabolism, but they may also arise from reactions with exogenous sources, such as environmental pollutants which can both depress the antioxidants capacity to remove oxyradicals or enhance the intracellular ROS formation (Regoli and Giuliani, 2014). LPO is a biomarker of oxidative stress. The results of Li et al. (2010b) suggested that LPO could be a more sensitive indicator for evaluating oxidative stress of fish spermatozoa compared with protein oxidation.

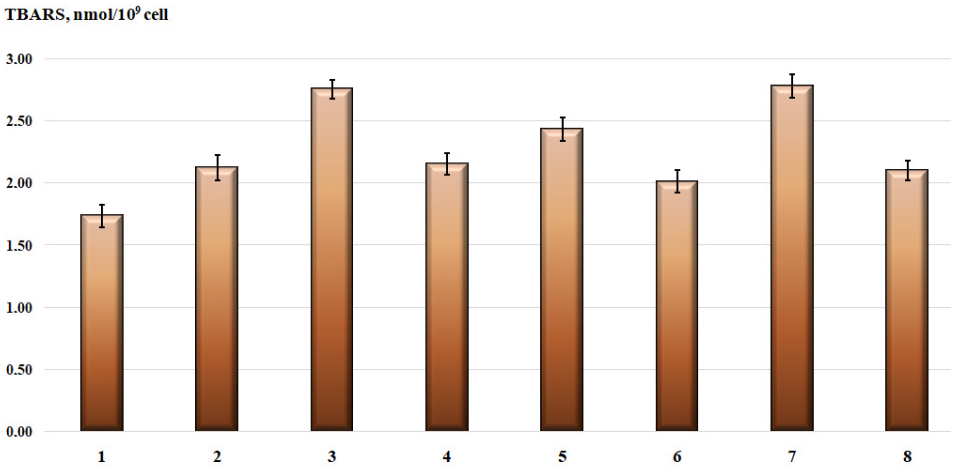
In the present work, it was found that incubation (1 hour) of Russian sturgeons sperm with OTs (CH_3SnCl_3, $(CH_3)_2SnCl_2$, $(CH_3)_3SnCl$, $(n\text{-}C_4H_9)_2SnCl_2$, $(n\text{-}C_4H_9)_3SnCl$, $(C_6H_5)_2SnCl_2$, $(C_6H_5)_3SnCl$) in 0.1 mM concentration led to the promotion of TBARS accumulation in native semen (Fig. 1). The promotion effect of OTs upon oxidation of oleic ((Z)-9-octadecenoic) acid (Petrosyan et al., 2002), sturgeon liver lipids (Antonova et al., 2008), lipids of fish feed (Osipova et al., 2017) by O_2 has been previously found. Most of the carbonyl by-products derived from lipid peroxidation were toxic, since they could easily diffuse through membranes and could covalently modify important biomolecules far from their unmodified state (Negre-Salvayre et al., 2008).

The promotion of the fish sperm LPO may be caused by the fact that the interaction of OTs with the first comparatively stable products of LPO (ROOH, R`OOR) breaks the Sn–C bond (Davies, 1997) and active alkyl radicals formed (Petrosyan et al., 2002). It was shown that in the case of $(n\text{-}C_4H_9)_3SnCl$ direct chemical combination of organometallic xenobiotics with the first ROS, i.e., superoxide, which was mainly generated via autoxidation reactions or oxygen-dependent enzymatic reactions in aerobic cell, radicals also could form (Rivera et al., 1992).

The production of ROS in the presence of $(n\text{-}C_4H_9)_2SnCl_2$ and $(C_6H_5)_3SnCl$ has been reported (Chantong et al., 2014; De Castro et al., 2018). Literature data indicate that the activity of the antioxidant enzyme superoxide dismutase decreased under the action of $(n\text{-}C_4H_9)_2SnCl_2$ (Weber et al., 1995). It should be also taken into account that sperm is particularly rich in mitochondria, which may predispose these cells to enhanced effects of OTs, because the mitochondria is the major cellular location for ROS generation upon stress (Doherty and Irwi, 2011). According to the data obtained in this work, the degree of promotion of sperm LPO by toxicants decreases in the row:

$(C_6H_5)_2SnCl_2 > (CH_3)_2SnCl_2 > (n\text{-}C_4H_9)_2SnCl_2 > (CH_3)_3SnCl > CH_3SnCl_3 > (C_6H_5)_3SnCl > (n\text{-}C_4H_9)_3SnCl$

Fig. 1. *The effects of 1 h exposure of Russian sturgeon fresh sperm to organotins on TBARS level in sperm in vitro*



Different levels of LPO of sturgeon sperm in the presence of OTs can be associated with different capacity of germ cells to accumulate toxicants. It is known, that Chinese sturgeons have a greater capacity to accumulate $(C_6H_5)_3SnCl$ relative to $(n\text{-}C_4H_9)_3SnCl$ than other fish species (Hu et al., 2009).

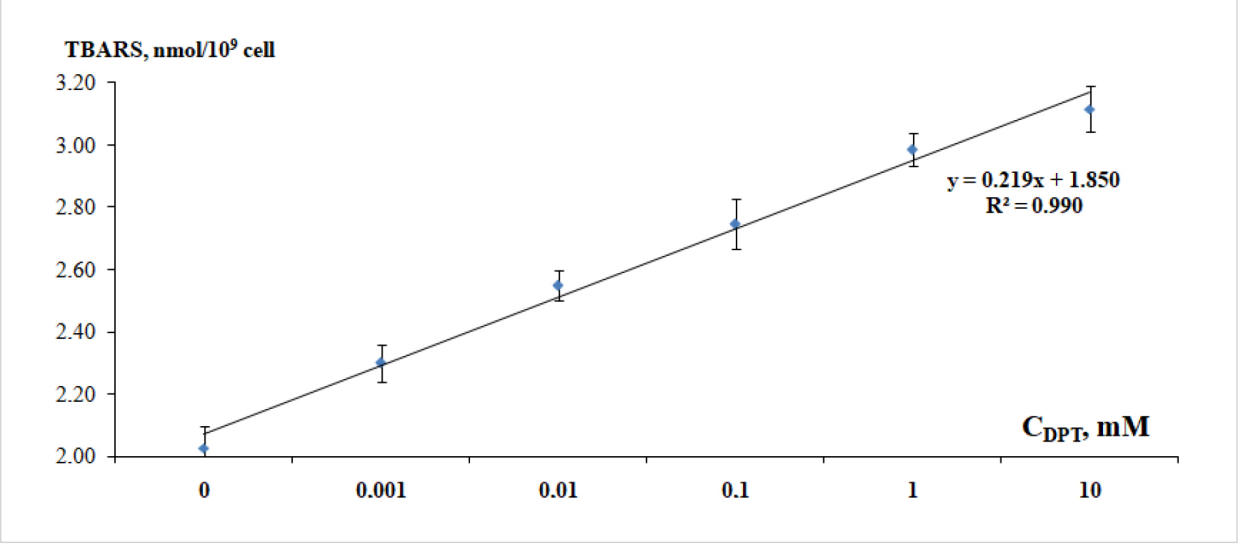
According to the data obtained, the degree of LPO promotion in Russian sturgeon sperm in the presence of $(n\text{-}C_4H_9)_3SnCl$, one of the most toxic xenobiotics in the natural waters, is lower than in the presence of $(n\text{-}C_4H_9)_2SnCl_2$. It has been reported that $(n\text{-}C_4H_9)_3SnCl$, $(C_6H_5)_3SnCl$ had a high complex toxic effect on molluscs, and even at OTs concentrations in water as low as 1.0 ng/L, it could produce endocrine disrupting effects, inducing imposex in female molluscs (de Araújo et al., 2018).

$(n\text{-}C_4H_9)_2SnCl_2$ is a metabolite of $(n\text{-}C_4H_9)_3SnCl$; thus, it is commonly found in tissue after exposure to $(n\text{-}C_4H_9)_3SnCl$. This compound is used as a biocide to treat chickens for tapeworm and, since it is used in PVC production, it will leach into aquatic systems from pipes made of this plastic (Cima et al., 2003). It is considered that $(n\text{-}C_4H_9)_2SnCl_2$ has low toxicity to aquatic organisms. The maximum permissible concentration (MPC) of this fishery waters pollutant is 1.0 μg/L, which is 100 times higher than for $(n\text{-}C_4H_9)_3SnCl$ (The list of fish-farm standards, 1999). According to the results of our study, $(n\text{-}C_4H_9)_2SnCl_2$ has a significant impact on the processes of LPO in Russian sturgeon sperm, increasing the level of accumulation of secondary products of the LPO in sperm by 41%. Thus, the studies carried out in this work, as well as published data on the more significant toxic effects of dibutyltin dichloride on the immune system of fish compared with the effect of $(n\text{-}C_4H_9)_3SnCl$ (O 'Halloran et al., 1998), indicate that potential toxicity of $(n\text{-}C_4H_9)_2SnCl_2$ should be re-evaluated.

The highest promoting activity has been found for dimethyl- (DMT) and diphenyltin (DPT) dichlorides, disubstituted OTs, which, presumably, are the most common organic derivatives of tin in biota, because they are formed during dealkylation of OTs and can also accumulate in tissues with a high level of metabolic processes and increased lipid content (Harino et al., 2007). DPT is a metabolite of $(C_6H_5)_3SnCl$, which, like $(n\text{-}C_4H_9)_3SnCl$, is used in antifouling paints. Direct proportionality between the concentration of toxicant DPT and the level of TBARS in semen was found (Fig. 2).

Cryopreservation conditions may also cause an oxidative stress, since sperm is exposed to cold shock and atmospheric oxygen during cryopreservation, which increases its susceptibility to lipid peroxidation

Fig. 2. *Effect of various concentrations of DPT on the level of TBARS in Russian sturgeon native sperm*



resulting from greater production of ROS (Li et al., 2010a). Although low concentrations of ROS play an important role in sperm physiology (Bansal and Bilaspuri, 2011), high concentrations of ROS cause sperm pathology (Gazo, 2013). LPO is the important factor for the evaluation of sperm quality (Cabrita et al., 2014).

The effect of incubating sperm with toxicants (DMT, DPT) on the level of TBARS in defrosted semen after deep freezing was studied. In order to assess the effect of the cryomedium on the promotion of sperm LPO by tin compounds, experiments were carried out without the addition of a cryomedium and in the presence of modified Stein's cryomedium. We previously carried out research on the impact of the cryomedium composition on the level of LPO of Russian sturgeon sperm at different stages of cryopreservation and it was found that the speed of LPO of the sperm reduced at all stages of cryopreservation, which was more efficient in the modified cryomedium (Osipova et al., 2016).

In our study, the level of TBARS in control increased significantly after 3 days of sperm storage (Table 1), which is in good agreement with literature data (Shaliutina et al., 2013).

The present work demonstrated that DMT and DPT (0.1 mM) veraciously increased the level of the LPO

Table 1. *Effect of the DMT and DPT on the TBARS level (nmol/10⁹ sperm) in Russian sturgeon sperm without and in the presence of the modified Stein's medium before and after cryopreservation*

	Before cryopreservation	After cryopreservation
Control	1.50 ± 0.08	2.03 ± 0.08$^{\bullet}$
Cryomedium	1.08 ± 0.07^{b}	1.24 ± 0.08^{a}
DMT + cryomedium	1.63 ± 0.08^{*b}	2.12 ± 0.07^{*c}
DMT	2.03 ± 0.08***	2.42 ± 0.08****
DPT + cryomedium	2.25 ± 0.07^{*b}	2.55 ± 0.08^{*d}
DPT	2.48 ± 0.03*	2.58 ± 0.07**

Control value without additives. The average values for a series of experiments are given.

$^{\bullet}$Differences from the control experiment group before cryopreservation ($p < 0.0001$). Differences from the control experiment group $^{*}(p < 0.00001)$;

$^{**}(p < 0.00005)$; $^{***}(p < 0.0001)$;

$^{****}(p < 0.0005)$.

Differences from the control experiment group without cryomedium $^{a}(p < 0.00001)$; $^{b}(p < 0.0005)$; $^{c}(p < 0.005)$. ^{d}Differences from the control experiment group (DPT after cryopreservation) ($p > 0.05$). The values are expressed as mean ± SD.

derived carbonyl by-products, which react with thiobarbituric acid, in frozen/thawed sperm of Russian sturgeon after prior incubation of the fresh semen in these OTs for 1 h ($p < 0.005$). TBARS levels of frozen/thawed Russian sturgeon sperm would be affected 1 h after the aerobic exposure of fresh semen to DMT and DPT, which indicates a decrease in the sensitivity of Russian sturgeon cryopreserved sperm to the promotion of LPO under the studied disubstituted OTs, especially DPT; therefore this indicator cannot be used as a spermiotoxicity test.

A statistically significant increase in the level of TBARS was found both in the control experiments and in the presence of DMT and DPT in comparison with the native sperm. In the control experiment without Stein's cryomedium and xenobiotics after cryopreservation, the level of LPO increased by 35% ($p < 0.0001$), while in the presence of DMT and DPT, it increased by 20% ($p < 0.00001$) and 4% ($p < 0.01$), respectively, compared with the experiment before cryopreservation. Comparison with the level of TBARS in the control experiment indicates the preservation of the promoting activity of tin compounds in cryopreserved semen. In the presence of DPT or DMT, the level of TBARS in defrosting sperm veraciously increases by 27% ($p < 0.00001$) and 19% ($p < 0.00001$), respectively, relative to control after cryopreservation without cryomedium.

In the control experiment without a toxicant, as well as in the presence of DMT and DPT, the addition of cryomedium to native sperm led to a decrease in TBARS by 28% ($p < 0.00001$), 20% ($p < 0.00001$) and 10% ($p < 0.00001$), respectively. Thus, in the presence of toxicants, the protective effect of Stein's cryomedium is reduced, which, presumably, can be explained by the fact that DMT and DPT can interact with the components of the cryomedium used, for example, with DMSO (the latter is widely used, in particular, in operations with sturgeon sperm). DMSO can capture hydroxyl radical, which has the most injuring effect on the membranes of sperm cells.

With the addition of cryomedium, the level of TBARS in semen defrosted after freezing was significantly reduced in the experiment where no toxicants were added, as well as in the presence of DMT, by 39% ($p < 0.00001$) and 13% ($p < 0.005$), respectively. The decrease in the level of secondary products of LPO of defrosted sperm with the addition of cryomedium in the presence of DPT was doubtful ($p > 0.05$).

Conclusions

Our results suggest that the accumulation of OTs by gonad of fish is the stress factor affecting the cells in the process of cryopreservation. Organotins can induce oxidative stress in sturgeon sperm *in vitro*, which may decrease the quality of gametes, which in turn may affect fertilisation success.

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Перечень рыбохозяйственных нормативов предельно допустимых концентраций (ПДК) и ориентировочно безопасных уровней воздействия (ОБУВ) вредных веществ для воды водных объектов, имеющих рыбохозяйственное значение (The list of fishery standards of maximum permissible concentrations (MPC) and approximately safe exposure levels (TSEL) of harmful substances for water bodies of fisheries importance). М.: «Изд-во ВНИРО», 1999. 211с.

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