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The *in vitro* effect of nonylphenol, propranolol, and diethylstilbestrol on quality parameters and oxidative stress in sterlet (*Acipenser ruthenus*) spermatozoa

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ABSTRACT

The sturgeon is a highly endangered fish mostly due to over-fishing, habitat destruction, and water pollution. Nonylphenol (NP), propranolol (PN), and diethylstilbestrol (DES) are multifunctional xenobiotic compounds used in a variety of commercial and industrial products. The mechanism by which these xenobiotic compounds interfere with fish reproduction is not fully elucidated. This study assessed the effect of NP, PN, and DES on motility parameters, membrane integrity, and oxidative/antioxidant status in sterlet *Acispenser ruthenus* spermatozoa. Spermatozoa were incubated with several concentrations of target substances for 1 h. Motility rate and velocity of spermatozoa decreased in the presence of xenobiotics in a dose-dependent manner compared with controls. A significant decrease in membrane integrity was recorded with exposure to 5 μM of NP, 25 μM of PN, and 50 μM of DES. After 1 h exposure at higher tested concentrations NP (5–25 μM), PN (25–100 μM), and DES (50–200 μM), oxidative stress was apparent, as reflected by significantly higher levels of protein and lipid oxidation and significantly greater superoxide dismutase activity. The results demonstrated that NP, PN, and DES can induce reactive oxygen species stress in fish spermatozoa, which could impair sperm quality and the antioxidant defence system and decrease the percentage of intact sperm cells.

1. Introduction

Most fish populations are exposed to a wide variety of man-made chemicals at concentrations that are not directly toxic. Nevertheless, at sublethal concentrations, exposure can induce harmful effects and potentially reduce populations. In recent years, there has been increasing concern about the potential health effects of xenobiotics, since these compounds have been associated with reproductive dysfunction in a variety of wildlife (Kime, 1999). Reports have shown that xenobiotics can cause disruption of the reproductive endocrine system (Arukwe et al., 1997) or directly affect gamete development and viability as a result of cytotoxicity (Kime, 1999) or by altering the hormonal environment during gamete development (Giesy and Snyder, 1998).

Nonylphenol (NP), propranolol (PN), and diethylstilbestrol (DES) are multifunctional xenobiotic compounds used in a variety of commercial and industrial products. Detected environmental concentration for NP ranges from 0.7 ng L^{-1} to 15 μg L^{-1} (Petrovic et al., 2002), for PN from 0.59 to1.9 μg L^{-1} (Owen et al., 2007) and for DES from 0.98 to 51.6 ng g^{-1} (Lei et al., 2009). During the past decade, concern over their widespread usage has increased because of toxicity to both marine and freshwater species and their ability to induce estrogenic responses. Estrogenic effects of NP have been observed at 10–20 μg L^{-1} (Carlisle et al., 2009), of PN at 0.5 μg L^{-1} (Huggett et al., 2002), and of DES at 2–10 ng L^{-1} (Zha et al., 2008). Propranolol is a non-selective beta blocker, blocking the action of epinephrine and norepinephrine on both β1- and β2-adrenergic receptors. Nonylphenol and DES are endocrine disrupting chemicals (EDCs) that can act as xenoestrogens, modulating the endocrine pathways *via* a receptor-mediated process (Feng et al., 2011; WHO/UNEP, 2013). The presence of estrogen receptors (ERs) and beta-adrenergic receptors on the spermatozoon membrane has been reported in mammals (Saberwal et al., 2002; Adeoya-Osiquwa et al., 2006), but there is no evidence for these receptors in fish spermatozoa. Therefore, it is necessary to observe direct effects of xenobiotics on fish spermatozoa to gain a better understanding of molecular mechanisms of their toxicity.

Sperm quality, defined as those traits of sperm that determine its capacity to fertilize eggs, is crucial for aquaculture purposes and must be monitored in fish farming to predict male reproductive success. Sturgeon spermatozoa are immotile in the testis and acquire the potential for motility after contact with hypoosmotic medium (fresh

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water). Water pollution can damage sperm, affecting their viability (Kime, 1999). Sperm of most fish species can be affected by exposure to a wide variety of manmade compounds released into water. Spermatozoon head possess membranes containing polyunsaturated fatty acids, which are highly susceptible to oxidative damage (Fabrik et al., 2008). When the production of reactive oxygen species is excessive, the gamete's limited defences are rapidly overwhelmed, and oxidative damage induces lipid peroxidation (LPO), with a resulting loss of motility and fertilizing potential (Aitken et al., 1998; Shaliutina-Kolesova et al., 2014). Therefore, assessment of motility parameters of sperm may be a sensitive and accurate bio-indicator of aquatic pollution (Li et al., 2010).

The sturgeon is among the world's most valuable wildlife resources. These northern hemisphere fishes can be found in large river systems, lakes, coastal waters, and inland seas throughout Eurasia and North America (Birstein and DeSalle, 1998). Most of the world's sturgeon populations have undergone significant decline, mainly due to overfishing, habitat destruction, and pollution (Pikitch et al., 2005). It is logistically difficult and costly to conduct toxicity evaluations on broodstock (Tashjian et al., 2006), therefore, in this study, we used an *in vitro* sperm assay with sterlet *Acipenser ruthenus* as a model to investigate potential adverse effects of the xenobiotics nonylphenol, propranolol, and diethylstilbestrol.

The main objectives were to explore effects of short-term (1 h) *in vitro* exposure to NP, PN, and DES on quality parameters and oxidative stress in spermatozoa of the sterlet *Acipenser ruthenus* by assessing spermatozoon motility, velocity, and membrane integrity and analysing oxidative stress indices, including lipid oxidation, protein carbonylation, and superoxide dismutase activity.

2. Materials and methods

All experiments were conducted according to the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany (based on the EU-harmonized Animal Welfare Act of the Czech Republic).

2.1. Fish handling and sperm collection

The breeding and culture of sterlet *Acipenser ruthenus* was carried out at the Genetic Fisheries Center, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice. Six males age 6 years, 0.5–2 kg, were used. Prior to experimentation, fish were held in hatchery tanks with water temperature 15 °C. Spermiation was induced by intramuscular injection of carp pituitary powder dissolved in 0.9% (*w/v*) NaCl solution at 4 mg per kg of body weight. After 24 h, semen was obtained from the urogenital tract using a 5–7 mm plastic catheter connected to a 20 mL plastic syringe. Care was taken to avoid contamination by urine, mucus, faeces, or water. Syringes (one per male) were placed on ice and immediately transported to the laboratory for analyses. Spermatozoon concentrations were examined microscopically (Olympus BX 41) at magnification 20× and estimated using a Burker cell haemocytometer. Mean spermatozoon concentration was $1.26 \pm 0.7 \times 10^9$ mL^{-1}.

2.2. Sperm dilution and exposure

Diethylstilbestrol (DES) [4,4′-(3E)-hex-3-ene-3,4-diyldiphenol; (E)-11,12-Diethyl-4,13-stilbenediol; empirical formula: $C_{18}H_{20}O_2$; MW: 268.35; (≥ 99% (HPLC); Sigma-Aldrich, USA)] and propranolol (PN) [(*RS*)-1-(1-methylethylamino)-3-(1-naphthyloxy)propan-2-ol; empirical formula: $C_{16}H_{21}NO_2$; MW: 259.34; (≥ 99% (TLC) Sigma-Aldrich, USA)] were first dissolved in dimethyl sulphoxide (DMSO) at 200 mM for DES and 100 mM for PN and further diluted with DMSO to obtain stock solutions of 10, 25, and 50 mM for PN and 10, 50, 100 mM for DES. Nonylphenol (NP) [4-(2,4-dimethylheptan-3-yl)phenol; empirical formula: $C_{15}H_{24}O$; MW: 220.35; PESTANAL®, analytical standard; Sigma-Aldrich, USA] was dissolved in ethanol at 100 mM and diluted with ethanol to obtain stock solutions of 1, 5, 10, and 25 mM. For getting the final concentration in μM, stock solutions were diluted with an immobilization medium (IM) (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) at a dilution ratio of 1:1000 to obtain the final concentration of solvents 0.1% (*v/v*) in incubation medium. Stock solutions were prepared daily. The individual sperm samples from six males were centrifuged at 300 × *g* and 4 °C for 30 min to remove seminal plasma and subsequently diluted with IM to obtain spermatozoon densities of 5×10^8 cells mL^{-1}. The sperm sub-samples were separately exposed for 1 h to final concentrations of NP (1, 5, 10, and 25 μM); PN (10, 25, 50, and 100 μM) and DES (10, 50, 100, and 200 μM) at 4 °C. A control group for NP was exposed to IM with 0.1% ethanol, and the control group for PN and DES was exposed to IM with 0.1% of DMSO.

2.3. Spermatozoon motility and velocity recording

The spermatozoa motility parameters were measured as described in our previous works (Linhartova et al., 2013; Gazo et al., 2013). In brief, curvilinear velocity (VCL, μm s^{-1}) and percent of motile spermatozoa (motility, %) were evaluated at 10, 30, and 60 s post-activation under dark-field microscopy (Olympus BX 50, Tokyo, Japan) at × 20 objective. For motility activation sperm was diluted 1:5000 in activation medium (10 mM Tris, 10 mM NaCl, 1 mM $CaCl_2$, pH 8.5). Recordings were made using a video recorder (Sony SVHS, SVO-9500 MDP, Japan). The movements of the spermatozoa heads were analyzed using Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Spermatozoa head positions on five successive frames are assigned different colors: frame 1 red, frames 2–4 green, and frame 5 blue. Those that moved were visible in three colors, while non-moving spermatozoa were white. The percent of motile spermatozoa was calculated from the number of white and red cells. 20 to 40 spermatozoa were counted for each frame. Spermatozoa motility activation and measurement was performed in triplicate for each sample.

2.4. Spermatozoon membrane integrity assessment

To assess membrane integrity, sperm samples were diluted with PBS to a volume of 2 mL and concentration of 10,000 cells/mL. Diluted samples were stained with 5 μL of 5 × SYBR Green (10,000 ×, S9430, Sigma, Singapore) for 5 min and then with 5 μl of 4.8 mM propidium iodide, which penetrates non-viable spermatozoa when the plasma membrane is disrupted, for 30 min. A minimum of 2000 cells were analyzed by CUBE 8 (Partec, Germany) cytometer with flow speed 0.2 μl s^{-1}. The data were processed by CyView 1.3 (Partec, Germany). For each sample, numbers of cells with high and low concentration of propidium iodide were compared to calculate percentage of cells with intact *vs.* damaged membranes.

2.5. Lipid peroxidation and antioxidant enzyme activity

Sperm samples were centrifuged at 5000 × *g* at 4 °C for 10 min. The supernatant was collected and discarded. The spermatozoon pellet was suspended in 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 0.1 mM PMSF to obtain a spermatozoon concentration of 5×10^8 cells mL^{-1} and homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany). A portion of the homogenate was used for measuring thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP), and the remaining was centrifuged at 12000 × *g* for 30 min at 4 °C to obtain the post-mitochondrial supernatant for the antioxidant enzyme activity assay.

The TBARS was measured as described by Lushchak et al. (2005) as