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Effects of exposure to cadmium in sperm cells of zebrafish, *Danio rerio*

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ABSTRACT

Cadmium is a natural element found in the earth's crust; it is usually associated with other metals, but due to the impacts caused by human activity, its concentration has increased in the aquatic environment. This metal may damage aquatic animal reproduction, decreasing the rate of fertilization of organisms such as fish. Thus, this study aimed to evaluate the *in vitro* toxicity of different concentrations of cadmium (0 (control), 0.5, 5, and 10 μg/L) using sperm cells of model organism zebrafish, *Danio rerio*. Structural parameters, including integrity and fluidity of the plasma membrane, concentration of oxygen species, mitochondrial function and DNA fragmentation were measured by flow cytometry. The following sperm movement parameters were also measured using the computer assisted sperm analysis (CASA) system: motility, time of motility, curvilinear velocity, average path velocity and straight line velocity in μm/s. Significant effects were observed on path speed, straight speed, curvilinear velocity, motility time, progressive and total motility, and plasma and DNA integrity. The results showed that cadmium can negatively affect some reproductive parameters in *D. rerio*, which may reduce the fertility rate of these animals.

1. Introduction

Cadmium (Cd) is a major heavy metal present in the earth's crust and is usually associated with other metals such as zinc, copper and lead. Cadmium bioaccumulates in phytoplankton and complex food webs involving aquatic animals as mollusks, fish and crustaceans [5]. Because it is a pollutant at high concentrations, there are regulatory rules that stipulate safe concentrations of cadmium in water resources. In Brazil, for example, the maximum concentration permitted by CONAMA (National Environmental Council) in fresh water for classes I, II and III (consumption, recreation, irrigation, and protection of aquatic and aquaculture communities) is 0.01 mg/L (10 g/L) [8]. However, in the US and Europe the limits are 1 and 5 mg/L of cadmium, respectively [14,13].

Cadmium's toxicity is related to its ability to replace calcium (Ca^{2+}) in biological reactions, which is due to the similar characteristics of these two elements such as their similar ionic radii. Therefore, the cellular uptake of Cd occurs mainly through Ca^{2+} channels, inhibiting the uptake of Ca^{2+}. Hence, Cd acts as a potent blocker of these channels [26], leading to deleterious effects because Ca^{2+} in critical to many cell signaling pathways [25].

The mechanisms of Cd-dependent reproductive toxicity have been reported in *in vivo* and *in vitro* studies. This metal can decrease testosterone levels in rats [27], significantly affecting the testicular tissue by changing the hematotesticular barrier [6]. It can also reduce sperm concentration and motility in rice fish, *Oryziaslatipes*, and zebrafish, *Danio rerio* [9], increase lipid peroxidation in carp, *Cyprinus carpio* (Tale Cinier et al., 1998), and affect sperm maturation in common snook, *Centropomus undecimalis* [1].

Aquatic organisms are commonly used for toxicological studies of various contaminants because they are affected both directly through contact with contaminated water and indirectly through their diet, whether it includes plants, invertebrates, or various species of smaller fish [3]. Thus, fish may reflect contamination in other organisms and trophic levels within the aquatic ecosystem

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and are an important part of the diets of mammals and waterfowls [20]. Zebrafish, *Danio rerio*, has been used extensively as an experimental model because of its advantageous features such as its small size, which allows easy handling, and its rapid absorption of substances added directly to the water [34].

D. rerio uses external fertilization like most teleost fish, the male and female gametes are released into the aquatic environment and they need to come in contact for fertilization to occur [9]. Thus, while the fish itself has defenses against toxicants that can cause reproductive effects, the release of spermatozoa into the water exposes the gamete directly to pollutants such as cadmium. The spermatozoa have a poor antioxidant defense system and are highly prone to oxidative stress induced by pollutants [36]. Furthermore, zebrafish spermatozoa do not contain metallothionein, which has been identified in other fish sperm cells and reduces the toxicity of Cd (Tale Cinier et al., 1998). Therefore, experiments to assess the direct exposure of spermatozoa to cadmium that closely mimic the reproductive biology of teleost fish exposed to cadmium in nature are environmentally relevant. This study aimed to analyze the effects of different concentrations of cadmium on various *in vitro* parameters of *Danio rerio* sperm cells.

2. Materials and methods

The animals were euthanized by sectioning of the spinal cord, an accepted method, with restrictions according to the Federal Council of Veterinary Medicine (Resolution no. 05/2012 1000) because the use of anesthetics could affect the results of sperm analysis. The methodology used in this study was approved by the Ethics Committee at the Federal University of Pelotas/Rs, Brazil under number 10016. The gonads were withdrawn from 10 reproductive-phase, male zebrafish adults aged 4–6 months by dissection using an abdominal incision. The gonads were placed individually in Eppendorf tubes containing 1.5 mL of Beltsville Thawing Solution (BTS) [37] at pH 7.4 and an osmolarity of 350 mOsm and sectioned to aid the release of the spermatozoa. Sperm motility was assessed to determine motility rate and motility time for the pre-treatment samples (only fresh semen diluted with BTS was assessed before the incubation period) by activation using Milli-Q water; non-motile samples, indicative of sperm death, were excluded. Then, each semen sample was diluted at a 1:1 ratio (v/v) with four different concentrations of cadmium for treatment with final concentrations of 0 (control, BTS only), 0.5, 5.0 and 10 μg/L cadmium, osmolality of 300–320 mOsm/kg.

The samples were incubated at 20 °C for 10 min. This temperature was determined to be acceptable for the survival of zebrafish, which live at temperatures between 18 and 26 °C [18], and was also chosen because higher temperatures, such as 22 and 25 °C, had deleterious effects on sperm survival in previous experiments conducted by our research group (unpublished data).

After the incubation period, sperm analysis was performed to measure total motility; progressive sperm velocity parameters, straight line velocity (VSL) curvilinear velocity (VCL) and average path velocity (VAP); time of motility; fluidity and integrity of the plasma membrane; production of reactive oxygen species; mitochondrial function; and the DNA fragmentation index

2.1. Assessment of sperm motility using computer-aided semen analysis (CASA)

To estimate sperm motility, 1 μL of diluted semen and 4 μL of Milli-Q water containing cadmium (at concentrations of 0.5, 5 and 10 μg/L cadmium) were placed on slides under coverslips and analyzed using CASA [7].

The images generated were reproduced and efficiently and objectively analyzed using the Sperm Class Analyzer software (SCA) to assess overall motility parameters, progressive motility, VSL, VCL and VAP [38]. The time or duration of motility after sperm activation was determined based on the time of complete arrest of the progressive movement of the spermatozoa following the method described by [33]. Each image (n = 10) was analyzed using the standard settings for fish by SCA. Sperm was considered immotile when velocity was <10 m/s. Although SCA simultaneously assessed more than 15 sperm motility end points, for brevity only curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity (VAP) were considered for further analysis, as similar effects were observed for all end points. To determine these velocities, each individual sperm cell (n = at least 1000 sperm/concentrations Cd) was followed throughout the 10 images and a sperm trajectory was calculated.

2.2. Flow cytometry

We used the Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems). To detect the sperm population, non-sperm cells were removed based on the FSC x SSC scatter plots [28,29] and debris was eliminated by staining of the cells with Hoechst 33342 at a concentration of 16.2 μM (Sigma-Aldrich Co., St. Louis, MO, USA), except for those samples used to measure the DNA fragmentation index. A total of 10,000 events per sperm sample with a flow of 200 cells/s were analyzed using the Cytometric Attune Software V2.1 program.

2.3. Integrity of the plasma membrane

To verify the integrity of the plasma membrane, we used 20.0 μM carboxyfluorescein diacetate (DCF), which fluoresces green, and 7.3 μM propidium iodide (PI), which fluoresces red (Sigma-Aldrich Co., St. Louis, MO, USA). The sperm were classified as non-injured (DCF + /IP-) and injured (DCF + /IP+; DCF-/IP+; DCF-/IP-) [19,17].

2.4. Fluidity of the plasma membrane

Plasma membrane fluidity was analyzed using hydrophobic merocyanine 540 dye (M540) at a final concentration of 2.7 μM (Sigma-Aldrich Co., St. Louis, MO, USA) and YO-PRO, which fluoresces green, at a final concentration of 0.1 μM (Invitrogen, Eugene, OR, USA). Only live sperm (YO-PRO negative) were selected and classified into high fluidity cells (high M540 concentration) and low fluidity cells (low M540 concentration) [17].

2.5. Mitochondrial functionality

The fluorescent dyes Rhodamine 123 (13 μM), which fluoresces green, and PI (7.3 μM) (Sigma-Aldrich Co., St. Louis, MO, USA) were used to assess mitochondrial function. Only intact sperm (IP-) were selected and classified into cells with high functionality (high fluorescence, high accumulation of Rhodamine) and low functionality (low fluorescence, low accumulation of Rhodamine) [19].

2.6. DNA fragmentation index

DNA integrity and fragmentation were measured using the sperm chromatin structure assay (SCSA). For this evaluation, 10 μL of semen were mixed with 5 μL TNE (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH 7.2). Then, after a 30 s incubation, 10 μL of Triton (0.1% Triton X-100) (v/v) were added, followed by another 30 s incubation, and finally, the addition of 5 μL acridine orange (Sigma-Aldrich Co., St. Louis, MO, USA). This reaction was then incubated