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Evolution of cadmium effects in the testis and sperm of the tropical fish *Gymnotus carapo*

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ABSTRACT

The present study investigated the testis and sperm morphology of the tropical fish *Gymnotus carapo* after exposure to increasing $CdCl_2$ concentrations (5–40 μM) for 24 and 96 h. The treatments induced Cd accumulation in the testis and a decrease in the gonadosomatic index from a 10 μM. Cd induced alterations in testis since 24 h; however the extension and severity of damages increased after 96 h in all tested concentrations. Marked variations in the cysts size, proliferation of the interstitial tissue, infiltration of inflammatory cells, necrosis, reduction of germ cells and sperm aggregation was observed in 96 h treated fishes. In this time, there was a complete absence of germ cells in the testis of fish treated with 40 μM. The ultrastructural analysis allowed for the visualization of the initial damages over germ cells, such as the presence of vacuoles in the cytoplasm of spermatogonia, spermatocytes, and spermatids. Exposed fish (20 μM for 24 and 96 h) had alterations in sperm number and morphology. These results are important for establishing a direct correlation between the Cd accumulation and incidence of damages and can help characterize the mechanism of Cd-induced pathogenesis in the male reproductive system.

1. Introduction

The testis morphology consists of germ cells in different stages of differentiation that undergo several processes of division until there is release of mature spermatozoa. The integrity of such cells is essential for developing future generations, affecting the survival of offspring (Friedmann et al., 1996). This arrangement and the frequency of cell division makes this organ highly vulnerable to some pollutants (Bonde, 2010). Morphological investigation in the testis is a relevant aspect of toxicological evaluations because adverse effects in this organ can directly influence several reproductive processes, such as spermatogenesis which may directly affect the fertilization success (Crump and Trudeau, 2009) and the development of the embryonic and postembryonic stages (Sellin and Kolok, 2006).

Cadmium (Cd) is a toxic metal that can induce adverse effects in the male reproductive system of several animal species, including humans (Thompson and Bannigan, 2008). Fish are good models for evaluating the toxic effects of Cd because the aquatic systems are the final receptacle of chemical substances. Further, the consumption of contaminated fish and seafood is one of the main sources of Cd exposure in human populations. Therefore, the study of Cd accumulation and effects in fish can express both the human and ecological health (Ju et al., 2012).

Recent studies have shown the effects of dietary Cd exposure in the testis of red tilapia with degeneration of spermatogenic elements, fibrosis of lobule walls and blood infiltration (El-Refaiy and Eissa, 2013). Disorganization of testicular lobules was also observed in *Puntius sarana* (Kumari and Dutt, 1991) and *Salvelinus fontinalis* (Sangalang and O'Halloran, 1972) after Cd exposure through injection or waterborne administration, respectively. Cd waterborne exposure also induced testicular apoptosis in *Labeo bata* (Das, 1988) and *Gobius niger* (Migliarini et al., 2005). Despite these known effects, the steps involved in the development of Cd-induced pathogenesis in the male reproductive system of fish is not fully described, and the evaluation of the progression of Cd effects in the testis remains unclear. Further, the cellular processes involved in the death of germ cells after Cd exposure has not been fully investigated.

A decrease in the sperm motility, speed and trajectory have also been demonstrated following Cd exposure in different fish

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species (Chyb et al., 2001; Dietrich et al., 2010, 2011; Kime et al., 1996; Lahnsteiner et al., 2004; Li et al., 2010). However, the Cd effect in sperm morphology is not being investigated despite the known importance of this parameter for sperm performance. Therefore, further investigations into the sperm count and morphology following Cd exposure are needed for different fish species because these alterations may directly affect the fertilization success, thereby altering fish populations (Crump and Trudeau, 2009).

Gymnotus carapo (banded knifefish) is a tropical freshwater fish species that has typical carnivorous and sedentary habits and is widely distributed in South and Central America (Albert et al., 2004; Lovejoy et al., 2010). This species, easily maintained under experimental conditions, is largely used in professional and sportive fishery as live bait because it is preferred by higher predator fish species. These characteristics make this organism a useful model for evaluating the toxic effects of pollutants in Brazilian ecosystems (Vergílio et al., 2014).

The present study aimed to investigate the toxic effects of $CdCl_2$ in the testes and sperm of a teleost fish *G. carapo* to elucidate the pathological processes that occur during exposure to a range of Cd concentrations (5–40 μM) and different exposure times (24 and 96 h).

2. Materials and methods

2.1. Fish contamination

The present study was conducted in accordance with the ethics Committee of the State University of Northern of Rio de Janeiro – Universidade Estadual do Norte Fluminense. The *Gymnotus carapo* specimens ($n = 80$) used in the present study were all males at the same sexual maturity stage, defined by correlation between the total length and body weight (length: 36.8 ± 6.0 cm/weight: 205.8 ± 59.9 g) as described by (Barbieri and Barbieri, 1984) The fish obtained from Cima Lake, which is in the northern region of Rio de Janeiro state (21°46′ S and 41°31′ W) were maintained for 24 h in a continuous flux aquarium system for acclimation. Following this time, the fish contamination was performed by intra-peritoneal injection (0.1 mL) with progressive $CdCl_2$ concentrations (5 μM, 10 μM, 20 μM, 30 μM, and 40 μM) for 24 or 96 h. For the control group, the fish were injected with phosphate buffered saline solution (pH 7.2). This range of doses allowed for the evaluation of damages in the testis, from no apparent effect until the occurrence of severe alterations, but with no induction of fish death. Intraperitoneal exposure offers a total bioavailability of the metal to the studied organ. This results from the great absorptive surface area of the peritoneal cavity from which substances are quickly absorbed into the circulation. Consequently, an improved understanding of the relationship between dose and time in Cd accumulation and distribution could be more accurately evaluated using an intraperitoneal exposure route. The fish ($n = 72$) were separated in six groups with 12 fishes each; there was one group for each concentration tested and a control group. Of the six fish used to evaluate the effects in each exposure time, three were used for the histological analysis and the other three for Cd chemical quantification. Following the period of 24 or 96 h, the fish were measured, weighed and dissected to obtain testes that were processed for histological preparations or frozen (−20 °C) for subsequent analysis of Cd accumulation.

2.2. Cadmium analysis in testis

The procedure for Cd extraction in nitric acid followed that proposed by Páez-Osuna et al. (1995). The extracts were analyzed by atomic emission spectrophotometry with induced coupled plasma (ICP-AES, Varian, Liberty II model). The method's limit of detection ($0.02\ \mu g\ kg^{-1}$) was calculated according to Skoog and Leary (1992).

2.3. Gonadosomatic index

The gonadosomatic index was determined using the following formula (Devlaming et al., 1982): GSI = (gonadal weight/total body weight) × 100.

2.4. Testis morphology

Four samples (approximately $5\ mm^3$) of testis were collected from the fishes designed for histological evaluations. As *Gymnotus carapo* testes are paired and oval organs, two samples were taken from each pair. This represents the sampling of almost the entire organ. The preparation of testis for the morphological analysis included 24 h of fixation in 10% buffered formalin, dehydration in graded series of ethanol and embedding in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) for examination by light microscopy.

2.5. Lesion index

Morphological damages were measured according to the injury index described by Bernet et al. (1999), where testis observations were classified in three severity factors (minimal, moderate and marked pathological importance) (Vergílio et al., 2014). The injury indexes were obtained after the application of a mathematical equation established for each group of lesions: (LI): $LI = \Sigma rp\ \Sigma alt\ (a \times w)$, where rp = reaction pattern, alt = alteration, a = score value of the alteration and w = importance factor.

2.6. Ultrastructural analysis of the testis

Samples of testis (approximately $1\ mm^3$) were also fixed in 4% formaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer. These samples were then post-fixed in 1% osmium tetroxide and 0.8% potassium ferricyanide (1:1), dehydrated with acetone and embedded in Epon®. Ultra-thin slices (70 nm) sectioned with an ultramicrotome (Reichercuts Leica) were contrasted with uranyl acetate and lead citrate for observation in a Zeiss TEM 900 transmission electron microscope.

2.7. Sampling and analysis of sperm morphology

For evaluation of Cd effects in sperm, the testis from control fish ($n = 4$) and those exposed to Cd (20 μM for 96 h) ($n = 4$) were minced with anatomic scissors in 2 mL of 0.1 M cacodylate buffer (pH 7.2) for 5 min. After dilution, the sperm count was determined with a hemocytometer using phase contrast microscopy at ×400 magnification. For analysis sperm morphology, the seminal fluid from control and contaminated fishes (20 μM for 96 h) were fixed in fixative solution (4% formaldehyde, 2.5% glutaraldehyde, 5% sucrose in 0.1 M cacodylate buffer, pH 7.2), attached to a coverslip with poly-L-lysine, post-fixed in osmium tetroxide (1%), dehydrated in ethanol, critical-point dried, and sputtered with gold. The samples were observed in Zeiss Evo 40 scanning electron microscope at 15 kV, employing secondary electrons.

2.8. Statistical analysis

Quantitative data expressed as the means and standard errors represent experimental replicates. Statistical significance was determined using GraphPad Prism v.4 software (GraphPad Software, Inc. CA, USA). One-way ANOVA followed by Tukey test was