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Histopathological Effects of Mercury on Male Gonad and Sperm of Tropical Fish *Gymnotus carapo in vitro*

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Abstract. Hg is toxic metal mostly due to adverse effects on structure and function of tissues and organs in humans and animals. Male reproductive systems of fish species are also sensitive to Hg action. However, the histological alterations in tropical fish testis are less known and little information is available concerning the underlying mechanisms of metal pathogenesis in reproductive functions. Further investigations dealing with Hg direct effects on tissue and organs of tropical species are a need. The present study investigated $HgCl_2$ toxic effects in testes and sperms of tropical fish *Gymnotus carapo*. The histopathology, germ cell structure and number were analysed to elucidate the pathological process during exposure to increasing metal concentrations (1 µM - 30 µM). Fishes exposed to 20µM and 30µM reached testicular Hg concentrations of 5.1 $\mu g.g^{-1}$ and 5.2 $\mu g.g^{-1}$, respectively. No significant alterations in gonadosomatic index (GSI) occurred between control, and Hg exposed fishes. Untreated fishes showed characteristic organization of testicular tissue, with germ epithelium organized in cysts where spermatogenesis occurs. Germ cells and spermatozoa are seen an inner the cysts. $HgCl_2$ induced severe damages characterized by complete disorganization of seminiferous lobules, proliferation of interstitial tissue, congestion of blood vessels, reduction of germ cells and sperm aggregation. Exposed fishes showed a decrease in the sperm's number. Initial reduction of a sperm's number (36,8%) was observed after 20 µM/24h treatment and subsequent decrease (48,7%) was obse rved after 20µM/96h. Hg (20µM) also altered sperm morphology in 24h and 96h where sperm head abnormalities were present. In conclusion, the present study showed $HgCl_2$ progressive damages in testicular tissue, sperm count and morphology of tropical species *Gymnotus carapo.* The effects in testicular tissues were observed since low Hg concentrations. These results are important to establish a direct correlation between the mercury accumulation and severity of lesions.

Key words: Heavy metal, Hg effects, morphology, spermatozoa, testes

Introduction

Mercury (Hg) is a toxic environment pollutant that induces several adverse effects in many tissues and organs of humans and animals. Male reproductive system is also sensitive to Hg (Boujbiha *et al.,* 200). In fishes, Hg can inhibit gametogenesis, induce testicular atrophy, and impair individual reproduction. Despite some of Hg-induced damages are known, there is still less information about the metal accumulation on tissue and its relationship with ultrastructure disorganization of fish testis, especially for tropical species.

Sperms are also useful in assessing the Hg impacts on male reproductive system. In rodents, acute contamination decreased reproductive quality of gametes, where sperm morphology, count, motility and viability were affected. Further investigations in such parameters are needed for different fish species since they may dramatically decrease sperm performance in aquatic environment affecting fertilization success and alter fish populations.

Gymnotus carapo (tuvira) is a tropical freshwater

fish widely distributed in South American. It easily maintained in experimental conditions, being an interesting model to evaluate toxic effects of pollutants in Brazilian ecosystems.

In the present study, the $HgCl_2$ toxic effects in testes and sperms of a teleost *Gymnotus carapo* were observed, elucidating the pathological process during in vitro exposure to increasing concentrations (1 µM - 30 µM).

Materials and Methods

Fish contamination

Gymnotus carapo specimens (n=116) used in the present study were all male in same sexual maturity stage, obtained from Cima Lake, northern of Rio de Janeiro state (21º 46' S e 41º 31' W). Heavy metal's distribution in sediments and biota of Cima Lake has already been described and characterized as area with low levels of metal pollution (Ferreira *et al.,* 2003).

For each $HgCl_2$ contamination, 6 male adult fish were selected, four exposed to $HgCl_2$ concentrations (5 µM/10 µM/ 20 µM/ 30µM) and two kept as the control group. The fishes were exposed at different exposure times (24 h/48 h/72 h/96 h) and control fishes were always dissected in 24h and 96h of exposure. In order to increase the sampling for chemical and histological analysis the procedure was repeated consecutive times for each time/concentration tested.

Contamination was achieved by intraperitoneal injection of $HgCl_2$ solution while the control group was injected with phosphate buffer solution. To avoid differences in treatment, all fish used for this study were of similar size (length: 32 ±1 cm/ weight: 125.8 ±17.8g). After each exposure time, the specimens were measured, weighed and dissected to obtain testis for further analysis.

Hg chemical determination in the testes

For mercury detection, testis samples from control and contaminated fishes followed strong acid digestion according to methodology described by Bastos *et al.* (1998). All the Hg determinations were performed by spectroscopy of atomic emission method using the equipment ICP-AES (Varian, Liberty II models) with cold vapor accessory (VGA-77). The method limit detection was calculated according to Skoog and Leary (1992) as being of 0.23 $\mu g.g^{-1}$.

Analysis of testis morphology

Samples of control and Hg exposed testis were fixed in 10% neutrally buffered formalin for 24 hours. The samples were then dehydrated in a progressive series of alcohol, cleared in xylene, embedded in paraffin. The samples sectioned (5 µm) and stained with hematoxylin and eosin (H&E) for examination by light microscopy. Samples of the testis (approximately 1 mm^3) were also fixed in formaldehyde 4%, glutaraldehyde 2.5%, cacodylate buffer 0.1 M, sucrose 5%, calcium chloride 5 mM and post-fixed (1:1) in osmium teroxide 1% and potassium ferricyanide 0.8%, dehydrated with acetone, embedded in Epon®. Semithin slices (0.4µm) were obtained using an ultramicrotome Reichercuts Leica. Slices stained with toluidine blue (1%) were observed with light microscopy.

Sperm Sampling

Following the damages observed in testis, Hg effects in sperm were evaluated in 20 µM concentration after 24h and 72h. Testis from control (n=4), Hg exposed to 20 µM/24h (n=2) and 20 µM/72h (n=2) were minced with anatomic scissors in 2 mL of cacodylate buffer 0.1M (pH 7.2) for 5 minutes at room temperature. After dilution, the sperm's number was counted in hemocytometer under light microscopy using phase contrast at x400 magnification.

Sperm morphology

Seminal fluid from control and contaminated fishes were fixed in fixative solution (4% formaldehyde, 2.5% glutaraldehyde, 5% sucrose in 0.1 M cacodylate buffer, pH 7.2). Attached in coverslip with poly-L-lysine, post-fixed in osmium tetroxide 1%, dehydrated in ethanol, critical-point dried in CO_2 (BAL-TEC CPD 030 Critical Point Dryer) and sputtered with gold (BAL-TEC SCD 050 Sputter Coater) for observation in Zeiss Evo 40 microscopy scanning electron at 15 kV, employing secondary electrons.

Statistic analysis

All the values are expressed as mean±SD. Significant differences were determined with Graph-prism v.4 Software (GraphPad Software, Inc. CA, USA). Two-way analysis of variance followed by Bonferroni test was performed for Hg concentration's data and one-way analysis of variance followed by Tukey test was used for sperm data. Differences were considered significant when $p < 0.05$.

Results and Discussion

External investigation was performed in each fish before and after the execution of the experiments. Control and Hg treated fishes showed to be healthy according to conditions of their gills, eyes and scales. Internal organs, as liver, kidney and especially the testis did no present macroscopic anatomic alteration.

As described in figure 1, testicular tissue in untreated fishes showed characteristic organization of cysts arrangement where spermatogenesis occurs (Fig 1a). Inner the cysts, germ cells (Fig 1b, c) in different stages of differentiation are distributed as: primary (SPGI) and secondary (SPGII) spermatogonia, primary (SPCI) and secondary (SPCI) spermatocytes and spermatids (SPD).

These cells undergo a number of cell divisions until sperm formation inner the cysts (*arrowheads)* (Fig 1b, c). Between the cysts interstitial tissue (it) is present being composed by Leydig cells, blood/lymphatic vessels and connective tissue (Fig 1b).

Hg induced changes in treated fishes for all concentrations administrated and the effects become more severe with increase of dose/time. Hg treatment induced complete disorganization in cysts arrangement (Fig 2) with congestion of blood vessels (Fig 2b) and proliferation of interstitial tissue (Fig 2b). Severe damages were observed in higher concentrations (20 µM and 30 µM) as reduction of germ cells (Fig 2d), marked variations of cyst size (Fig 2c), interstitial and lobular disintegration (Fig 2d), sperm aggregation (Fig 2c).

Hg chemical analysis revealed that treated fishes with 20µM and 30µM reached highest testicular concentrations of 5.1 $\mu g.g^{-1}$ and 5.2 $\mu g.g^{-1}$, respectively (Table 1). Hg concentrations in control and treated with 5µm, 10 µm were bellow the detection limits of the method (Table 3). These results are in agreement with severe damages observed by histopathological analysis and indicate that even low Hg doses can induce morphological alterations in testis.

The present study also enhances the knowledge about progressive accumulation of $HgCl_2$ and adverse effects in testicular tissue structure. In important point, this study showed that histology damages started at concentrations not detectable by the limit of accumulation method (< detection limit). These results are important to establish a direct correlation between the mercury accumulation and the severity of tissue lesions (Boujbiha *et al*. 2009).

Hg induced severe damages in testis arrangement, affecting the germ cells that are involved in spermatogenesis process. Therefore, investigations in sperms were also performed for overall evaluation of Hg effects in male gonad.

Hg affected sperms count (Fig 3) in a time-dependent matter. Significant reduction (36.8%) of sperms number was observed after treatment with 20 µM/24h (Fig 3). A subsequent reduction (48.7%) was observed in same concentration treatment (20 µM) for 96h (Fig 3).

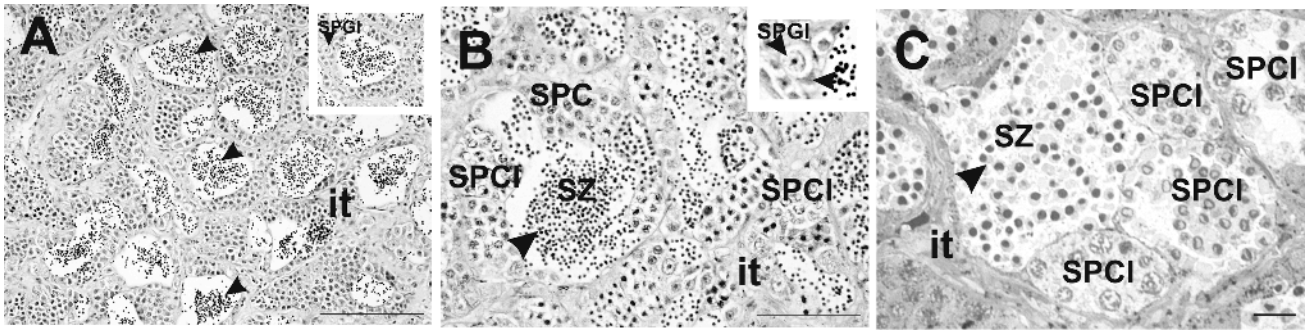

Fig 1. Normal morphology of *Gymnotus carapo* testis. (A) Testicular organization with seminiferous lobes (*inset*) and interstitial tissue (it). (B) and (C) Seminiferous lobes containing germ cells: primary spermatogonia (SGI), primary (SPCI) and secondary (SPCII) spermatocytes and spermatids (SD). Spermatozoids (SZ) are also seen within the lobe's lumen. Scale bar: A: 200µm (100x), B: 100µm (200x), C: 20µm (400x).

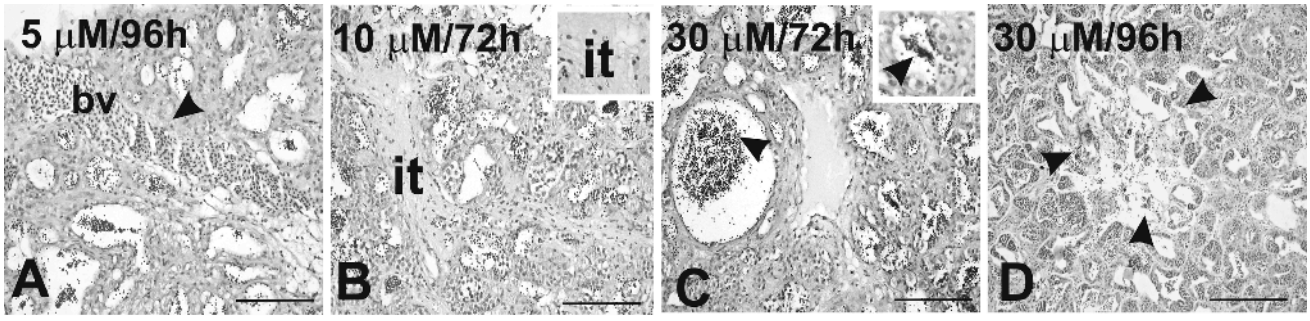

Fig 2. *Gymnotus carapo* testis of $HgCl_2$ exposed fishes. After Hg treatment the fishes showed evident disorganization of testicular arrangement (A - D). (A) shows congestion of blood cells after 5µM/96h treatment. (B) Proliferation of interstitial tissue (it) *(inset)* is evident after 10 µM/72h. (C) shows marked variation in seminiferous lobules size and sperm aggregation after 30 µM/72h. (D) Severe damage to interstitial and lobular tissue is evident after treatment with 30 µM/ 96h. Scale bar: A - D: 200µm.

Table 1. Mercury (HgT) concentrations ($\mu g.g^{-1}$) in testis.

Treatment\ concentration	5 µM	10 µM	20 µM	30 µM
control	nd [a]	nd [a]	nd [a]	nd [a]
24h	nd [a]	nd [a]	nd [a]	4.6±3.3 [b]
48h	nd [a]	nd [a]	4.7±1.7 [b]	4.1±1.7 [b]
72h	nd [a]	nd [a]	5.1±2.5 [b]	5.2±2.8 [b]
96h	nd [a]	nd [a]	3.6±0.7 [a]	3.2±1.1 [a]

nd: not detected by equipment since concentrations bellow limit of method detection. The letters a, b indicate the indicate groups at means that are significant different at 5% of significance level.

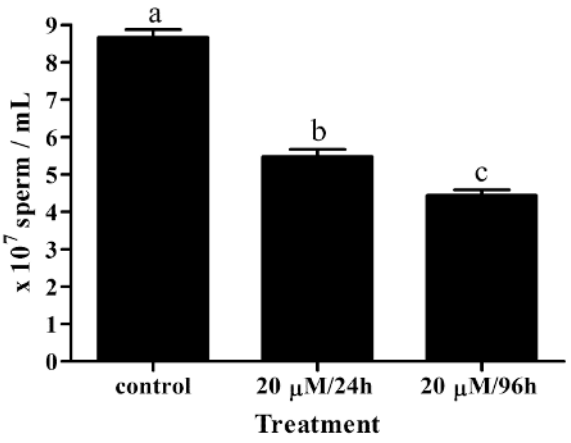

Fig. 3. Effect of mercury on sperm count after 20 µM treatment for different exposure times (24h and 96h). The letters a, b and c indicate that are significant different at 5% of significance level.

Ultrastructural features of sperm cell were drastically altered after Hg exposure (Fig 4). *G. carapo* sperm have an ovoid shape head, midpiece and flagellum (Fig 4a) as observed in control fishes. Both 20µM treatments for 24h and 96h induced changes in sperm head (Fig 4b - d, arrows).

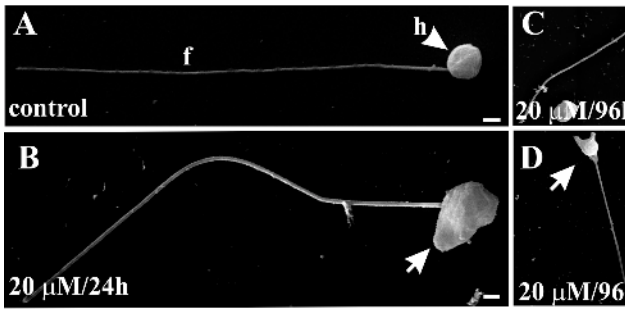

Fig. 4. Changes in morphology of *Gymnotus carapo* spermatozoa after $HgCl_2$ treatment demonstrated by scanning electron microscopy.

Conclusion

The present study showed $HgCl_2$ progressive damages in testicular pathology, sperm count and morphology of tropical fish species *Gymnotus carapo*. These results are important to establish a direct correlation between the Hg accumulation and the severity of lesions since the testis analysis was performed since Hg concentrations bellow limit of method detection until higher doses that induced severe damages. Moreover, this work enhances the data about Hg toxicological effect in fishes from tropical regions.

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References

Bastos WR, Malm O, Pfeiffer WC, Cleary D (1998) Establishment and analytical quality control of laboratories for Hg determination in biological and geological samples in the Amazon, Brazil. Ciência e Cultura 50: 255-260

Boujbiha MA, Hamden K, Guermazi F, Bouslama A, Omezzine A, Kammoun A, Feki AE (2009) Testicular toxicity in mercuric chloride treated rats: Association with oxidative stress. Reprod Toxicol 28: 81-89.

Crump KL, Trudeau VL (2009) Mercury-induced reproductive impairment in fish. Environ Toxicol Chem 28: 895-907.

Ferreira AG, Melo EJT, Carvalho, CEV (2003) Histological aspects of mercury contamination in muscular and hepatic tissues of *Hoplias malabaricus* (Pisces, Erytrinidae) from lakes in the north of Rio de Janeiro State, Brazil. Acta Microscopica 12: 49-54.

Skoog DA, Leary JJ (1992) Principles of Instrumental Analysis. Saunders College Publishing Orlando. Florida.