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Accumulation and Histopathological Effects of Mercury Chloride after Acute Exposure in Tropical Fish *Gymnotus carapo*

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Abstract: The present study evaluated potential Hg bioaccumulation and its morphological effects in different organs of the tropical fish, *Gymnotus carapo*, after a single acute intra-peritoneal exposure (0.6 $\mu g.g^{-1}$) and over progressively longer exposure times (24 h, 48 h, 72 h and 96 h). The Hg accumulation was differential and time dependent for most target organs (testis > liver > gills > muscle). Hg exposure leads the highest accumulation potential in testis since the initial examination point (24 h) until the last (96 h). The liver showed progressive Hg accumulation, presenting its highest levels only at the 96 h exposure point. Hg concentrations in the gills and muscle oscillated over the exposure times; however, the highest values of both organs also occurred in 96 h exposed fish. Histopathological alterations were observed in testis, liver and gills from 24 h of Hg exposure, and the extent of the alterations and their severity increased out to 96 h of exposure. These results shows a correlation between Hg accumulation and the induced morphological damages in different organs along the time in a tropical fish species *G. carapo*, being the histopathology a sensitive technique for the observation of the initial damage from Hg exposure.

Keywords: Hg, histopathology, intra-peritoneal, morphology, organs

INTRODUCTION

Mercury (Hg) is one of the most harmful pollutants due to its high toxicity and persistence in the environment [1]. Once Hg is in aquatic systems, different organisms can accumulate Hg, leading to its biomagnification through the food web [2]. Studies examining metal accumulation in fish tissues are important because these organisms represent the main human contamination pathway [3, 4, 5].

Studies of tissues and organs dealing with metal uptake, accumulation, biotransformation, and excretion are fundamental to increase the understanding of the effects of chemicals in fish [2].Information on the effects of Hg distribution and accumulation in temperate freshwater fish species have been intensely reported, but data on tropical fish and effects on different fish tissues are still scarce [6, 7]. Moreover, there are no studies dealing with the steps associated with metal toxicology including accumulation and damage in the different target organs. *Gymnotus carapo* (banded knife fish) is a widely distributed South American tropical freshwater fish with sedentary and omnivorous habits [8]. This species is a favorite prey of higher predator species and is easily maintained under experimental conditions. Accordingly, it is an interesting model for the evaluation of the toxic effects of pollutants in Brazilian ecosystems, especially because little is known about metal distribution in this species.

Bioassays have proven to be valuable tools to characterize the toxic action of chemicals in different target organs [9]. Under well-defined exposure levels, they allow the quantification of metals in organs together with an analysis of the effects in cells and tissues. This information gives an overall evaluation of the health status of fish and can help to predict comparative effects of metals, such as Hg, in natural aquatic systems [10]. Studies of Hg uptake in fish show relatively slow absorption and low bioavailability of this metal when exposure occurs through water or food; these effects result from a significant and variable elimination of Hg [11]. According to [10], approximately 60% of Hg in water is lost after 96 h. Consequently, an improved understanding of the relationship between dose and time in Hg accumulation and distribution could be more accurately evaluated using an intra-peritoneal exposure route. Intra-peritoneal exposure offers a bioavailability of 100% of the metal to the studied organs. This results from the great absorptive surface area of the peritoneal cavity from which substances are quickly absorbed into the circulation. These characteristics make possible a better portrayal of the distribution and persistence of metals in fish.

The objective of the present work was to evaluate Hg's potential for bioaccumulation and its effects on the morphology of different organs in the tropical fish

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species, *Gymnotus carapo*. To achieve this objective, analyzes were conducted of Hg concentrations in testis, liver, gills, and muscle over progressive exposure periods, as well as the histological effects of higher bioaccumulation in certain organs. This study aimed to enhance the knowledge of Hg tissue damage after acute exposure in tropical fish.

MATERIAL AND METHODS

Fish contamination strategy

G. caparo specimens (n=48) were obtained from Cima Lake, in northern Rio de Janeiro state (21º 46' S and 41º 31' W). According to [12, 13], this region has low levels of metal pollution in sediments and biota. Fish exposure was performed by intra-peritoneal injection of a $HgCl_2$ solution (0.6 $\mu g.g^{-1}$), followed by progressive exposure times (24 h, 48 h, 72 h and 96 h). The fish control group received an injection with phosphate buffered saline solution (pH 7.2). Only a small volume (0.1 mL) of Hg solution was injected in the peritoneal cavity of the fishes. All fish used in this study were of similar size (length: 30.5 ± 1.2 cm and weight: 114.3 ±0.9 g) (Table 1) to avoid differences among the exposures.

Table 1. Number, average weight (g) and average length (cm) of *G. carapo* specimens used in each contamination experiment. The standard derivations follow the weight and length data.

Exposure time	Control	Hg exposed	Weight (g)	Lenght (cm)
24h	6	6	115.2 ± 4.9	29.8 ± 3.3
48h	6	6	114.4 ± 4.0	30.1 ± 2.6
72h	6	6	113.1 ± 3.9	32.3 ± 2.9
96h	6	6	114.6 ± 4.6	30.0 ± 3.6
total	24	24	114.3 ± 0.9	30.5 ± 1.2

From the eight specimens selected for each experiment, four fishes (one for each exposure time: 24 h, 48 h, 72 h and 96 h) were kept as a control group and the other four were exposed to $HgCl_2$. The above procedure was repeated six consecutive times. After each exposure, the specimens were measured, weighed and dissected to obtain the testis, liver, gills and muscle that were processed for histological analysis or were frozen (-20°C) for subsequent analysis of total Hg accumulation.

Chemical extraction method

The procedure for total Hg extraction followed the methodology described by [14]. Samples of testis, liver, gills and muscle from control and treated fishes were digested with an acid mixture (H_2SO_4:HNO_3, 1:1), followed by addition of $KMnO_4$ solution (5%). Excess $KMnO_4$ was then reduced by the addition of NH_2OHCl solution (12%). For analysis, $SnCl_2$ solution was added, allowing the Hg quantitation. Blanks were run after every 10 samples to ensure analytical control of the procedure. The accuracy of the methodology was confirmed by the use of certified reference material – DORM – 1 (muscle tissue of *Squalus acanthias*) - prepared by the "Marine Analytical Chemistry Standards Programs", Canada; 98.92% recovery was obtained. The method's detection limit, calculated according to [15] was 20 $\mu g.kg^{-1}$.

Instrumentation

The Hg concentrations were determined by CV-ICP (Varian, Liberty II model) with a cold vapor accessory (VGA-77).

Tissue Morphology

Samples of testis, liver and gills from the control and treated fishes were fixed by immersion in 10% buffered formalin for 24 hours. The samples were then dehydrated in a graded series of alcohol, cleared in xylene and embedded in paraffin. The samples were sectioned (5 µm) and stained with hematoxylin and eosin (H&E) for morphological examination by light microscopy. Digital images were obtained using an Axioplan microscope equipped with a Cannon Power Shot camera A610/620, employing 10x, 20x and 40x objectives.

Statistical analyses

All the concentrations presented in the graphics of the present study are averages and their standard deviations. Significant differences were determined with Graph-prism v.4 software (GraphPad Software, Inc. CA, USA). The data of Hg concentrations in organs were analyzed using two-way analysis of variance followed by the Bonferroni post-test. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

The total Hg concentrations were determined in each organ after 24 h, 48 h, 72 h and 96 h. For the Hg bioaccumulation potential, the organ concentrations of all fishes used in the present study (at all exposure times) were considered (Figs. 1 and 2). The Hg concentrations for all organs in exposed fishes (0.6 $\mu g.g^{-1}$ $HgCl_2$) were higher than observed for the control group. The Hg accumulation pattern was testis > liver > gills > muscle (Fig. 1).

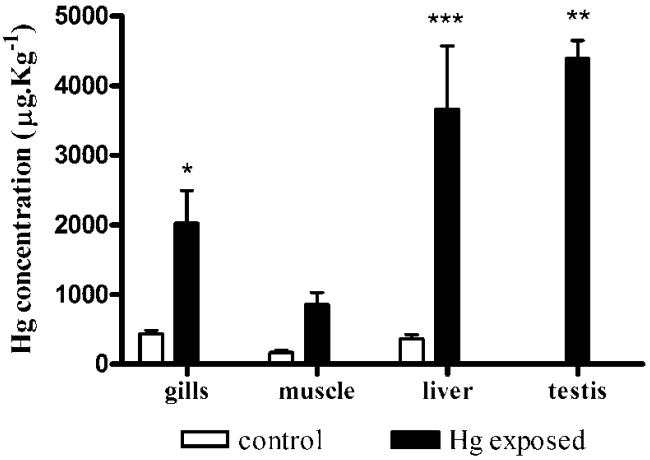

Fig 1. Hg total concentrations (µg.Kg^{-1}) in gills, muscle, liver, and testis of control and Hg-exposed (0.6 µg.g^{-1}) *G. carapo* fish. This graph presents the average of Hg concentrations in organs of fishes exposed to Hg for different exposure times (24 h, 48 h, 72 h and 96 h). The control bar for testis is not shown because it was below the limit of method detection. The statistical analyses used control testis assigned values of the detection limit (20 µg.Kg^{-1}). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Therefore, the present study shows that even when the Hg is available over the same period for organ accumulation from the peritoneal cavity, the accumulation is differential (testis > liver > gills > muscle) and time dependent for each of the target organs. This is the result of the distinct metabolism and blood supplies of the examined organs (testis, liver, gills and muscle). It should also be noted that the blood is the main transport medium of metals in most organisms [16].

Testis and liver showed the highest Hg levels, with concentrations reaching up to 10 times higher than the values measured for the control group. The testis Hg concentrations of the control group were lower than the detection limit of the method (<20 µg.kg^{-1}). Gills and muscle of exposed fishes also showed increased Hg concentrations when compared to a control group, reaching up to four and five times the control specimen's concentrations, respectively (Fig. 1).

To evaluate Hg accumulation across the time range, Hg concentrations were analyzed in each organ after the distinct exposure times (Fig. 2). Hg accumulation in all the studied organs began with the first 24 h. The testis was the organ that showed the highest Hg accumulation over the first 24 hours and maintained the highest levels during all the following exposure times (Fig. 2). In contrast, liver showed a progressive increase in Hg concentration with exposure time, presenting the highest Hg levels after 96 h of exposure (Fig. 2). The Hg concentrations in gills and muscle showed some oscillation across the exposure times; however, the highest values for both organs also occurred in the fish exposed for 96 h (Fig. 2). These results suggest that even when the Hg was available over the same period, the Hg accumulation is differential and time dependent for all the target organs. The only exception was the testis, which did not show a clear accumulation trend (Fig.2).

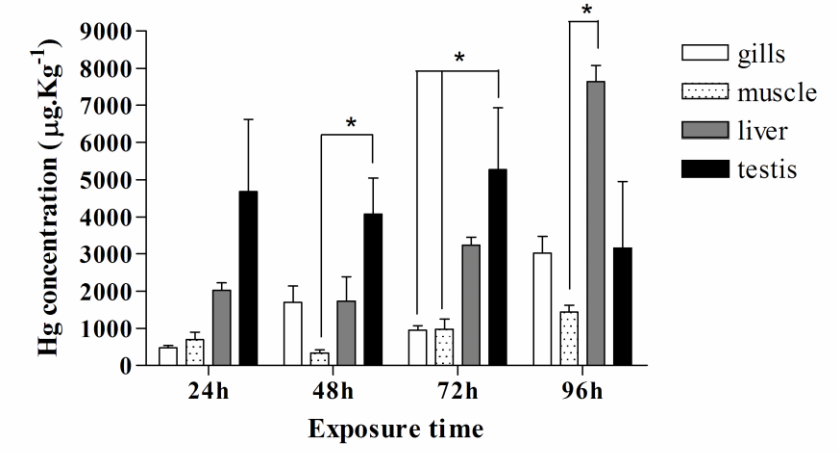

Fig 2. Hg total concentrations (µg.Kg^{-1}) in gills, muscle, and liver of *G. carapo* specimens exposed to Hg (0.6 µg.g^{-1}) for different times. Livers showed a progressive increase in Hg concentration; this pattern

was not clearly observed for the other organs. Despite having the highest bioaccumulation pattern, the testis had a slight decrease in Hg levels at 96 h. The gills did not show an increasing tendency due to a decrease in levels after 72 h of exposure. For muscle, the concentrations were similar for the different exposure times with a decrease in levels after 48 h. * $p<0.05$.

The first 24 h seems to reflect the beginning of Hg entrance. Because Hg introduction occurred via the intra-peritoneal cavity, the testis and liver, organs located in the visceral cavity, presented the highest Hg levels. The 48, 72 and 96 h exposure times most likely reflect Hg distribution by blood circulation through the organs. This is reflected in the gills and muscle, which presented a trend of increasing Hg levels with time. In gills, the higher levels, in longer exposure times, represent the Hg transport to gills, in order to be excreted. The muscle is not part of gastrointestinal absorption, therefore the muscle concentrations increased during the exposure time as due to Hg distribution through blood circulation.

To demonstrate the toxic effects of the Hg concentrations, histological observations were performed on the *G. carapo* organs. In this perspective, the morphology of testis, liver and gills were examined over the distinct exposure times (Figs. 3-5). The control and Hg-exposed fishes were healthy according to the external condition of their gills, eyes, scales, and organs. However, morphological analysis of the testis, liver and gills revealed alterations after 24 h of Hg exposure, which increased in severity over the 96 h of exposure.

The testis showed the highest Hg accumulation rate, presenting morphological alterations after 24 h of exposure onward. The untreated testicular tissue of *G. carapo* presented a characteristic organization with many spermatogenic cysts surrounded by interstitial tissue (Fig. 3A). Germ cells were present at different stages of differentiation, from undergoing cell division up to the formation of spermatozoa inside the cysts (SZ) (Fig. 3A). These observations agree with the features described in the literature by [17].

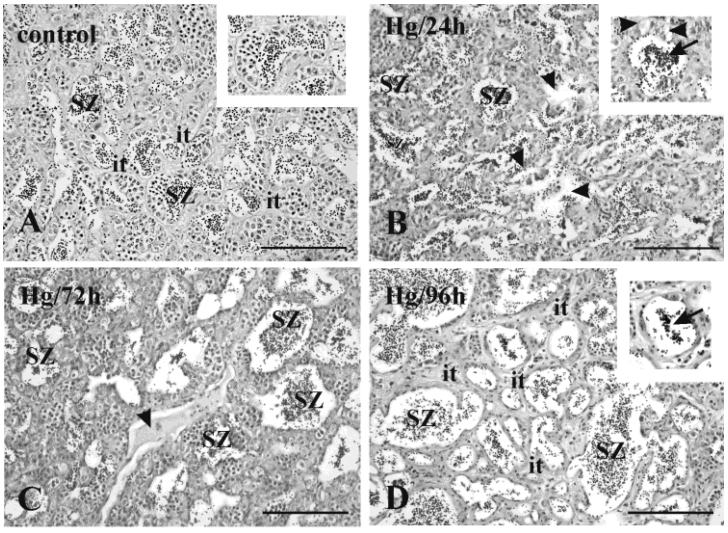

Fig 3. Light microscopy of hematoxylin and eosin (H&E) stained testis of control and Hg-exposed (0.6 $\mu g.g^{-1}$ for 24 h and 96 h) *G. carapo* fishes. (A) shows the characteristic organization of cysts surrounded by interstitial tissue (it). Germ cells typically undergo a number of cell divisions until spermatozoa (SZ) formation occurs inside the cysts. (B) shows disorganization of the cysts' arrangement (arrowheads), vacuolization of germ cells (inset, arrowheads) and sperm aggregation (inset, arrow), following 24 h of Hg exposure. (C) shows fibrosis in testicular tissue (arrowhead). (D) demonstrates the reduction of germ cells (inset), marked variations in cyst size and sperm aggregation (inset, arrow) after 96 h of Hg treatment. it= interstitial tissue, SZ= spermatozoa. Scale bar: 100 µm (x 200).

After 24 h of Hg exposure, there was a disorganization of the arrangement of the cysts (Fig. 3B, arrowheads), a vacuolization of germ cells (Fig. 3B, inset, arrowheads) and aggregation of the sperm (Fig. 3B, inset, arrow). More severe damage was observed after 72 h and 96 h of Hg treatment including fibrosis (Fig. 3C, arrowhead), a reduction in germ cells (Fig. 3C, inset), an increase in the cyst size (Fig. 3D) and sperm aggregation (Fig. 3D, inset, arrow).

The testis highest Hg accumulation potential occurred since this organ is located in the visceral cavity, where the Hg became immediately available after intra-peritoneal injection. The testis suffered progressive morphological alterations, which reflected the elevated Hg concentrations. This result is important because such damages can induce male reproductive dysfunction and impairment of both gonadal development and growth, thereby affecting fertilization success and offspring survival [18, 19]

The liver showed progressive accumulation of Hg over the exposure times and presented higher levels after 96 h of exposure. This observation is most likely related to the important role that liver has in the metabolism and excretion of toxic substances [20]; it is capable of transforming harmful compounds into

metabolites, which are excreted directly into bile for continued detoxification [21]. Accordingly, this organ is frequently used in monitoring studies [22]. Moreover, the blood that perfuses the liver comes from two sources: the major part comes from the portal vein that drains the gastrointestinal tract and the remainder comes from the hepatic artery. Therefore, as the exposure to Hg occurred from the peritoneal cavity rather than a contaminated diet, the liver showed a gradual increase in Hg concentration. This fact also explains the high accumulation in testis over the first 24 hours of exposure.

However, morphological alterations in liver were observed after 24 h, when the lowest Hg levels were measured. The untreated liver showed typical compact structure (Fig. 4A), where hepatocytes presented a characteristic cytoplasmic distribution and nuclear morphology (Fig. 4A, inset). The 24 h Hg treatment induced disorganization of hepatic tissue (Fig. 4B), with changes in hepatocyte cytoplasmic and nuclear morphology (Fig. 4B, inset). The 72 h and 96 h treatments displayed congestion of blood vessels (Fig. 4D, arrows). Areas with severe degradation of the liver parenchyma (Fig. 4C, 4D, arrowheads) were also observed, usually in close proximity to the blood circulation (Fig. 4C).

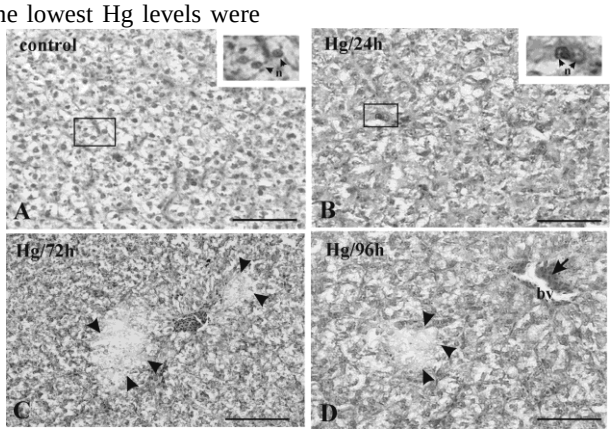

Fig 4. Light microscopy of hematoxylin and eosin (H&E) stained hepatic tissue of *G. carapo*. (A) demonstrates the compact hepatic parenchyma and the normal appe arance (inset) of hepatocytes in control fishes. (B) - (D) show the severe disorganization of hepatic tissue in $HgCl_2$ treated fishes (0.6 $\mu g.g^{-1}$). In (B), the inset shows the changes in the nuclear morphology of hepatocytes after a 24 h Hg-exposure. (C) and (D) demonstrate congestion of blood vessels (arrow) and an area with severe degradation of the liver parenchyma (arrowheads) after Hg exposure for 72 h and 96 h, respectively. n= nucleus. bv=blood vessel. Scale bar: 100 µm (x 200).

These Hg-induced alterations agree with other studies of Hg contamination in fishes [2, 6, 7]. Some of the observed changes seen in this study have also been reported after contamination with other metals [23, 24, 25, 26, 27, 28]. This trend likely indicates that the liver is a sensitive organ for the evaluation of damage after pollutant exposure [29]. However, the histological changes seen in the liver are not metal specific but are generally associated with the response of hepatocytes to toxicants [20].

In the present study, the gills showed increased levels of Hg after 96 h of exposure. Considering that Hg was provided directly to the peritoneal cavity, rather than through water contamination, gill levels reflected Hg distribution through blood circulation and Hg was likely transported to the gills in order to be excreted. However, alterations were observed in this organ at the 24 h exposure point, as observed in the testis and liver. The untreated gills showed a characteristic arrangement of primary and secondary lamellas (Fig. 5A). The gills of the 24 h and 96 h Hg-exposed fishes showed some areas with focal proliferation, occasionally resulting in fusion of adjacent secondary lamellas (arrowheads) (Figs. 5B-C). Epithelial cells also showed vacuolization after 96 h of exposure (Fig. 5C, *arrow*). Other studies examining Hg [2, 7] and others metal contaminants [28] also reported similar alterations to those described in the present study.

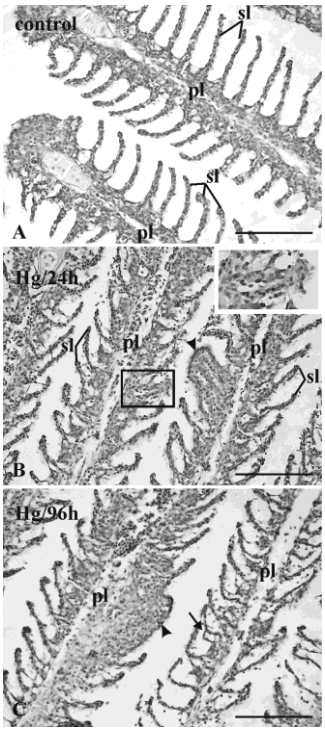

Fig 5. Light microscopy of hematoxylin and eosin (H&E) stained gills of *G. carapo*. In (A), the characteristic arrangement of primary and secondary lamellas in the gills of control fish is demonstrated. (B) and (C) show focal proliferation occasionally resulting in a fusion of adjacent secondary lamellae (arrowheads) in Hg-exposed fish (0.6 µg.g^{-1}) for 24 h and 96 h exposures, respectively. In (C), the arrow shows the vacuolization of epithelial cells in 96 h exposed fish. sl=secondary lamellae, pl=primary lamellae. Scale bar: 100 µm (x 200).

Table 2 summarizes the frequency of Hg alterations observed in testis, liver and gills of *G. caparo* in the different exposure times (24 and 96h). The histopathology was a sensitive technique for the observation of the initial damage from Hg exposure.

Table 2. Pathologies observed for each $HgCl_2$ treatment in testis, liver, and gills. A positive match means that the response was observed in all fishes submitted to each specific exposure time (n=3 fishes for each exposure time).

Alteration/ Exposure time	24h	96h
Testis		
Severe disorganization of the cysts' arrangement	+	+
Vacuolization of germ cells	+	
Reduction of germ cells		+
Sperm aggregation	+	+
Liver		
Disorganization of the hepatic tissue	+	+
Changes in hepatocytes nucleus	+	
Congestion of blood vessels		+
Areas of severe degradation of liver parenchyma		+
Gills		
Areas with focal proliferation	+	+
Fusion of adjacent secondary lamellas	+	+
Vacuolization of epithelial cells		+

Muscle showed the lowest Hg concentrations after Hg exposure, as was also observed by [2, 10]. According to [30], muscle is not in the passage route of Hg after gastrointestinal absorption; however, it seems to act as a site for Hg storage and generally shows the low Hg levels observed in the present study. Consequently, this tissue should be monitored because it is most closely associated with the risks of human Hg contamination by fish consumption [31].

CONCLUSION

The present study demonstrated the comparative Hg bioaccumulation potential of organs of tropical fish *G. caparo* after acute intra-peritoneal Hg exposure. The liver and testis showed high bioaccumulation potential followed by the gills and muscle. The differential of Hg concentration in organs was related to exposure time and the distribution of Hg through blood circulation. Histopathological analysis showed morphological alterations in testis, liver and gills of Hg-exposed fishes revealing differences in the types and severity of lesions according to increases in exposure time. These results are important in establishing a direct correlation between Hg accumulation and morphological damage, and therefore help to characterize the mechanism of Hg-induced pathogenesis. Moreover, these alterations can also be correlated with damages of target organs from fish exposed to natural Hg contamination.

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