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Hematologic and hepatic responses of the freshwater fish *Hoplias malabaricus* after saxitoxin exposure

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ABSTRACT

The bioaccumulation of saxitoxins (STX) in the trophic chain, mainly in freshwater, are not completely known. This work aimed to elucidate the effects of STX on *Hoplias malabaricus* through trophic bioassay. The fish were fed once every five days with *Astyanax* sp. before being subjected to an intraperitoneal inoculation with the lysate of *Cylindrospermopsis raciborskii* culture containing 97% STX and 3% by neosaxitoxin and gonyautoxin during 20 days. The animal's liver was assessed using biomarkers as activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPx), and concentrations of reduced glutathione (GSH) and lipoperoxidation (LPO) and protein carbonylation (PCO). In the blood was analyzed the genotoxic and hematological parameters. The hepatosomatic index and the relative condition factor did not show a significant difference between the exposed and control groups. The values of mean corpuscular hemoglobin concentration and mean corpuscular hemoglobin increased in the STX group. The hepatic tissue from both groups exhibited a typical pattern that have been already described for most teleost fish. The results suggested the generation of reactive oxygen species, with increased activity of GPx and concentrations of LPO and GSH; whereas the specific activity of SOD decreased. However, no changes were observed in the CAT, PCO, and DNA damage. Although the STX effects are known as neurotoxic, this cyanotoxin caused liver biochemical alterations that can be considered ecologically relevant.

1. Introduction

The anthropogenic pressure that is closely related to the nutrient input from point sources (usually sewage discharge) as well from diffuse sources (agriculture and other industrial activities) in the drainage basin is certainly one of the main causes of cyanobacteria mass occurrence. The presence of potentially toxic cyanobacteria in water supply reservoirs has been described in many countries in the world (Garcia Nieto et al., 2011) and leads to concerns regarding the risk for human health. In Brazil, cyanobacterial blooms are an important issue, as the deaths of human beings that are caused by water contaminated with cyanotoxins were already reported in this country (Azevedo et al., 2002).

Saxitoxin (STX) is a water-soluble neurotoxin that binds to the voltage-dependent sodium channels in excitatory

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cells. This binding blocks the inward Na^+ current while leaving the outward K^+ current unaffected (Cestelle and Catterall, 2000), ultimately leading to hyperpolarization of the cell. In addition, the Na^+-channel blockage may alter the selective permeability of the membrane and change the flow of ions, leading to damage to the cellular homeostasis (Silva et al., 2011).

Fish are particularly sensitive to water contamination, and pollutants may impair many physiological and biochemical processes when assimilated by fish tissue. When abnormal or xenobiotic-induced reactive oxygen species (ROS) production exceeds the endogenous protection, damage to cellular components can be often observed. This process is known as oxidative stress (Oakes and Van der Kraag, 2003). The antioxidant defense system includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione S-transferase (GST), and other low-molecular-weight scavengers such as reduced glutathione (GSH). The liver is not the target organ to the STX, but it plays a key role in most metabolic processes, especially detoxification and, consequently, in the formation of free radicals. It is known that the STX causes oxidative stress in the brain (Silva et al., 2011), but this has not yet been investigated in the liver of *Hoplias malabaricus* after trophic exposure.

The freshwater fish species *H. malabaricus* exhibits a large ecological plasticity, with a wide distribution in Brazilian rivers and reservoirs (Hensley and Moody, 1975) and occupies high trophic levels.

The aim of the present work was to evaluate oxidative stress, as well as the hematological, morphologic, and genotoxic effects of STX in the liver of *H. malabaricus* after subchronic and trophic exposure using biomarkers of environmental contamination.

2. Material and methods

2.1. Experimental design

Ten individuals per group (experimental STX and control groups) of *H. malabaricus* (mean weight 110.02 ± 19.00 g) were fed once every five days with *Astyanax* sp. before being submitted to intraperitoneal inoculation with the lysate of *Cylindrospermopsis raciborskii* culture (T3) containing 97% STX and 3% by neosaxitoxin and gonyautoxin. The culture was carried out at University of Rio de Janeiro, Rio de Janeiro, RJ. In the STX group, the used dose was 0.08 μg/100 g of *H. malabaricus*, total of four doses. This chosen dose is below that acceptable for human ingestion by Food and Agriculture Organization (FAO) of the United Nations (Chorus and Bartram, 1999). In the control group was administered 0.9% NaCl, as vehicle. After 20 days, the animals were anesthetized and killed by medullar section. The blood was collected to genotoxic assay and to the hematological parameters. The body weight and the length were used to the calculation of the hepatosomatic index and the condition factor. The liver was collected for morphological and for biochemical analysis, such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), reduced glutathione (GSH), lipoperoxidation (LPO) and protein carbonylation (PCO).

2.2. Hepatosomatic index and condition factor

The hepatosomatic index (HSI) represents the percentile organ weight related to the fish total weight: $HSI = Wl \times 100/Wt$; where Wl represents the liver weight and Wt the total weight. The length–weight relationship was expressed by the equation $Wt = aLb$, where Wt represents the total body weight (g) and L the total length (cm) of the fish and the constants a and b were estimated by linear regression: $W = \log a + b \times \log L$. These data were employed in calculating condition factor (Kn) (Le Cren, 1951).

2.3. Hematological biomarkers

The blood was collected from the caudal vein using heparinized syringes. Hematocrit (Ht) was determined by the microhematocrit centrifugation technique at 12,000 rpm for 5 min and the hematocrit values (%) were read immediately. Hemoglobin (Hb) was determined by cyanomethahemoglobin method (Collier, 1944) and the results expressed in g dL^{-1}. The red blood cell count (RBC) was determined optically with a Neubauer chamber using Formol-citrate solution and reported as the number of cells.μL^{-1} of blood. Mean corpuscular volume (MCV = fL), mean corpuscular hemoglobin (MCH = g dL^{-1}) and mean corpuscular hemoglobin concentration (MCHC = g dL^{-1}) were computed from the Ht, Hb and RBC values (Wintrobe, 1934).

2.4. Biochemical biomarkers

Samples of liver were homogenized in phosphate buffer (0.1 M, pH 7.5) and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatants were used to estimate the activities of the enzymes SOD, CAT, GST, GPx, and to estimate the concentrations of GSH, LPO and products of PCO.

The activity of SOD was assayed by measuring its ability to inhibit the reduction of nitroblue tetrazolium (NBT), which was determined by the method described by Crouch et al. (1981). CAT activity was measured at 240 nm on the basis of the method described by Aebi (1984). GST activity was measured at 340 nm by the method described by Keen et al. (1976), GPx activity was measured at 340 nm (Sies et al., 1979), and GSH concentration was measured at 415 nm (Sedlak and Lindsay, 1968).

The analysis of LPO was carried out using the ferrous oxidation – xylenol orange assay at 570 nm (Jiang et al., 1992), PCO analysis was conducted at 360 nm by derivatization of the protein carbonyl groups with 2,4-dinitr ophenol hydrazine to yield dinitrophenyl hydrazones (Levine et al., 1994; Quinlan and Gutteridge, 2000). Protein concentration was determined using Bradford's method (1976), with bovine serum albumin as the standard.

2.5. Genotoxic biomarker

The comet assay was performed with peripheral blood (erythrocytes) as described by Singh et al. (1988),

modified by Ferraro et al. (2004). Briefly, 10 μL of the homogenized blood in fetal bovine serum was diluted in 120 μL of low melting agarose (LMA) and placed on a slide covered by normal agarose. The slides placed in lysis solution (lysis stock solution: NaCl (2.5 M), ethylenediaminetetraacetic acid (EDTA; 100 mM), tris (hydroxymethyl)amino methane (Tris; 10 mM), NaOH (0.8%), and N-lauroyl sarcosinate (1%); working lysis solution: Triton X-100 (1%), dimethyl sulfoxide (DMSO) (10% in lysis stock solution) for 24 h at 4 °C. In the following step, the slides were first immersed in a solution of NaOH (10 N) and EDTA (200 mM), pH > 13, for 20 min to cause DNA denaturation and were subjected to electrophoresis at 300 mA/25 V for 25 min. After neutralization in 0.4 M Tris, pH 7.5, and fixation in ethanol for 10 min, the slides were stained with 0.02 g/mL ethidium bromide, and the DNA strand breaks were scored using a Leica DMLS2 epifluorescence microscope at a magnification of 400×. For each liver slide, 100 cells were visually analyzed by the method of Collins et al. (1997) and scored visually as belonging to one of five classes—from undamaged (0) to maximally damaged (4)—predefined with reference to the tail intensity. The score of the comets for a group could range from 0 (completely undamaged = 100 cells × 0) to 400 (maximum damage = 100 cells × 4).

2.6. Histopathological biomarker

Liver samples were preserved in Alfac fixative solution (ethanol 80%; formaldehyde 40% and glacial acetic acid 5%) for 16 h, dehydrated in a graded series of ethanol baths, and embedded in Paraplast Plus resin (Sigma®). Sections (3–5 μm) were stained in hematoxylin/eosin (Woods and Ellis, 1994) and observed in Zeiss Axiophot photomicroscope. A liver lesion index was determined according to the method established by Bernet et al. (1999), and described in Mela et al. (2007). Free melano-macrophages (MMs) and melano-macrophages centers (MMCs) were evaluated according to Rabitto et al. (2005).

2.7. Statistical analysis

The normality test preceded data analysis. The biological parameters were analyzed using the unpaired Students'*t*-test. The comet assay results were analyzed using the Mann–Whitney test. All tests were regarded as statistically significant when $p < 0.05$.

3. Results

The HSI and the Kn did not exhibit significant differences ($p > 0.05$) between the groups. The HSI was 0.59% ± 0.08 in the control group and 0.50 ± 0.09 in the experimental group.

The hematological parameters were not altered by the STX, only the MCH and MCHC values increased ($p < 0.05$) in the group with STX (Table 1).

In the fish liver, the specific activity of SOD decreased in the STX group in relation to the control group ($p < 0.05$); whereas the specific activity of GPx and the GSH concentration increased in the STX group ($p < 0.05$). In addition, the LPO process, expressed as the concentration of hydroperoxides, increased in the STX group ($p < 0.05$) (Fig. 1). Therefore, no changes occurred in the PCO process, expressed as the concentration of dinitrophenyl hydrazones, GST and CAT activities (data not shown).

Table 1
Hematological parameters of *H. malabaricus* of the control and saxitoxin groups. Hematocrit (Ht), red blood cell count (RBC), hemoglobin concentration (Hb), mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC). The values are expressed as mean ± standard error.

Parameter/Treatment	Control	Saxitoxin
Ht (%)	22.50 ± 6.00	23.19 ± 4.36
Hb (g dL^{-1})	5.21 ± 1.38	6.20 ± 1.46
RBC (erythrocytes μL^{-1})	1.61 ± 0.44	1.58 ± 0.24
MCV (fL)	141.13 ± 17.57	146.72 ± 6.54
MCH (g dL^{-1})	32.60 ± 1.40	39.15 ± 4.80*
MCHC (g dL^{-1})	23.31 ± 2.14	26.63 ± 2.50*

* Indicates difference statistically significant between the treatments.

The hepatic tissue of *H. malabaricus* from the control and exposed groups exhibited a typical pattern already described for most teleost fish: a very homogeneous hepatic tissue with sinusoids and polyhedral hepatocytes arranged in cords presenting spherical nuclei (Fig. 2A). The presence of melano-macrophages centers (MMC) was also observed in the experimental group than in the control group. In *H. malabaricus*, the MMC present granular or heterogeneous pigmented material (from yellow to dark brown) when stained with hematoxylin/eosin (Fig. 2B). The morphological lesions observed (necrosis and leukocyte infiltration) in the liver of *H. malabaricus* were not significant.

The DNA damage in the control group was not observed in the blood cells of the fish exposed to STX ($p > 0.05$).

4. Discussion

Fish hepatosomatic index did not change and can indicate that both the dose and the number of the doses of the neurotoxin did not affect the health of *H. malabaricus*. Therefore, the HSI values were low when compared with other animals of the same species (2.32 ± 0.40) (Rios et al., 2006) and with other species as matrinxã, *Brycon amazonicus* (1.13 ± 0.16). The liver histopathological analysis did not exhibit any damage that can affect the HSI. Ernst et al. (2007) report that *Coregonus lavaretus* exposed to cyanotoxin, mainly microcistin, expressed a low condition factor. These fishes also probably presented low HSI due to the histopathological alteration in the liver detected during the analysis. In other fish species such as *Gasterosteus aculeatus* L., *Clupea harengus* L. and *Salmo salar* L. were also found histopathological alterations in the liver and kidney due to the ingestion of contaminated food with nodularin in the Baltic Sea (Sipiä et al., 2007). It is possible that the exposure time was not enough to cause damage in the liver.

Concern of the hematological parameters only the MCH and MCHC values of *H. malabaricus* in the STX group increased ($p < 0.05$) compared with the control group.

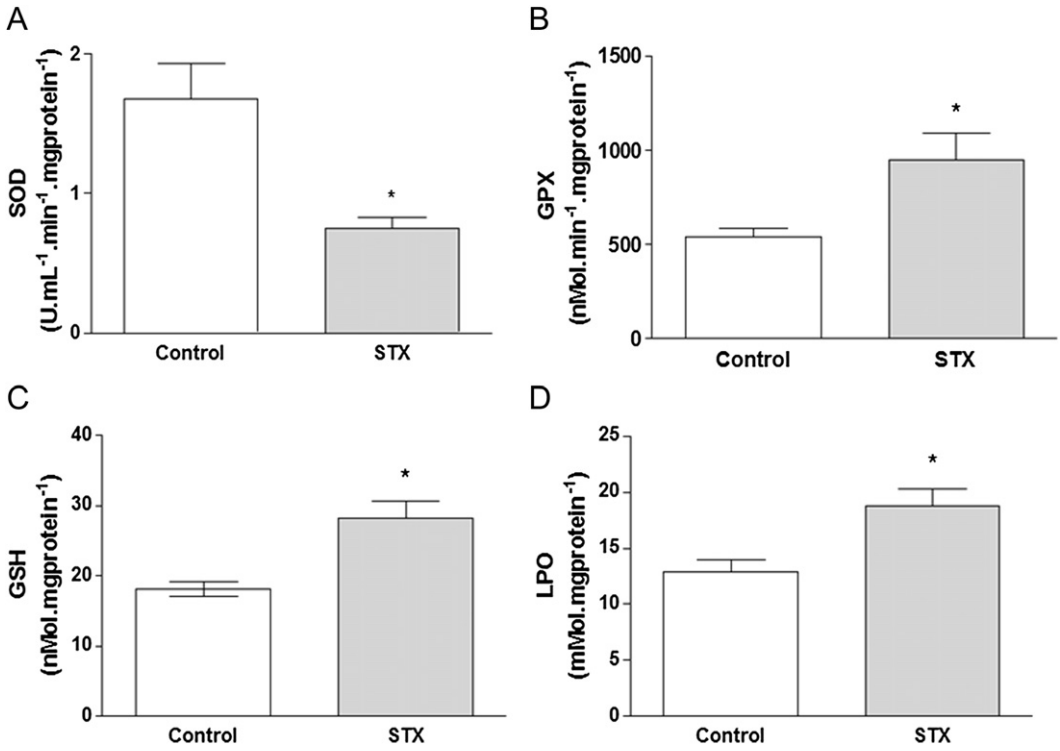

Fig. 1. Biochemical biomarkers evaluated in *H. malabaricus* exposed to the STX extract: (A) specific activity of superoxide dismutase (SOD); (B) specific activity of glutathione peroxidase (GPx); (C) Concentration of reduced glutathione (GSH). (D) Concentration of hydroperoxides (LPO). The results are expressed as mean values ± standard error. * indicates statistically significant differences ($p < 0.05$).

Probably, a high demand of oxygen to the tissues was necessary due to STX exposure. Therefore, studies conducted on the action of cyanotoxins (more specifically, saxitoxin) about both the physiology and the health condition of the fish were not found in the scientific literature.

A significant increase in GPx activity in the fish from the STX group indicates that the antioxidant pathway is stimulated, and it is also involved in the metabolization of hydrogen peroxide (Zhang et al., 2004; Maran et al., 2009). Thus, the activation of GPx may indicate a response to compensate the lack of increase in CAT activity. The GSH levels also increased and can be an adaptive mechanism by means of an increased synthesis. Protective and adaptive roles of GSH against oxidative stress-induced toxicity are well established in aquatic animals (Regoli and Principato, 1995; Otto and Moon, 1995).

Glutathione, the major non-protein thiol of cells, is involved in the cellular defense against the toxic action of oxyradicals. This low-molecular-mass thiol can be easily oxidized and serve as a sink for free radicals and other reactive species (Hermes Lima, 2004). Variations in cellular glutathione content are considered indicators of the degree and duration of exposure to oxidant pollutants in fish. The reduction in SOD activity may be related to the production of oxidants. It is known that there is a complex pathway of interaction among the enzymes involved in the animal's antioxidant system and that the activity of one enzyme influences the activity of other enzymes. An excess of hydrogen peroxide may reduce SOD activity, whereas the superoxide anion may be responsible for decreased CAT activity (Bagnyukova et al., 2006). In the present work, no changes in CAT activity were observed. The correlation between the activities of both enzymes is not even observed in biomarkers assays. In an experiment conducted with *Carassius auratus*, an increase of SOD in the liver was observed after metal exposure and a decrease of CAT activity (Shi et al., 2005).

When not neutralized, ROS can react with membrane lipids (Ahmad et al., 2000), producing lipid peroxidation, which is considered one of the main consequences of oxidative stress and cell death (Hermes Lima, 2004). In this work, the occurrence of lipid peroxidation indicated that the STX can cause membrane damage in the liver. Therefore, this damage was not able to cause DNA damage in the liver. In the brain of *H. malabaricus*, we observed genotoxicity that can lead to neurodegeneration (Silva et al., 2011).

In conclusion, the results found in this work suggest that STX can cause oxidative stress and membrane damage in the liver of *H. malabaricus.* Moreover, a further study conducted with different doses of STX is highly recommended, because it is a freshwater fish that is widely consumed in Brazil and could represent an important vehicle of STX transfer to humans when exposed to cyanobacterial blooms.

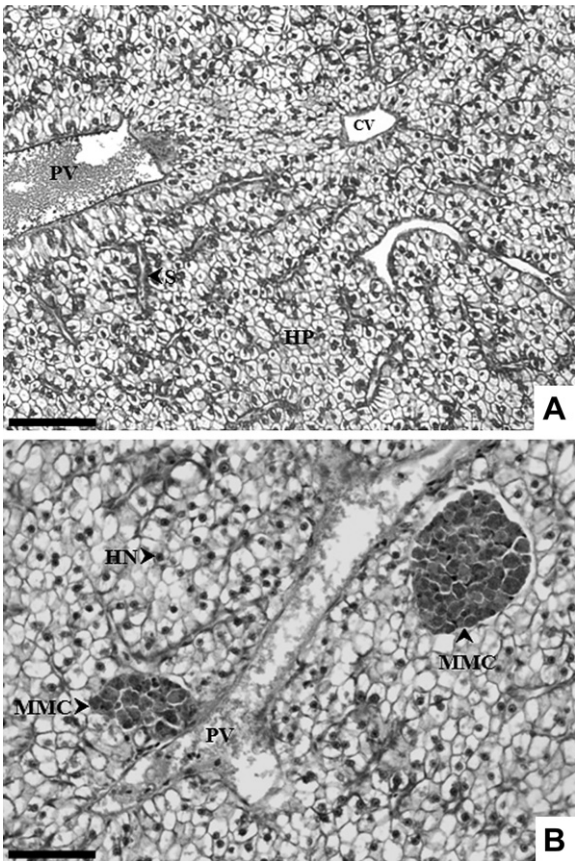

Fig. 2. Cross-section of liver of *H. malabaricus* stained with hematoxylin/eosin. (A) Individual from control group showing the central vein (CV), portal vein (PV), sinusoid vase (S) and hepatic parenchyma (HP). Scale bar = 50 μm. (B) Individual from exposed group showing the melano-macrophage centers (MMC), hepatocytes nucleus (HN) and portal vein (PV). Scale bar = 100 μm.

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Conflict of interest

I have no conflict of interest.

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