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Cadmium affects the mitochondrial viability and the acid soluble thiols concentration in liver, kidney, heart and gills of *Ancistrus brevifilis* (Eigenmann, 1920)

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Abstract
The freshwater fish *Ancistrus brevifilis,* which is found in Venezuelan rivers, is considered a potential sentinel fish in ecotoxicological studies. The cadmium (Cd) effect on the mitochondrial viability (MV) and acid soluble thiols levels (AST) in *A. brevifilis* tissues (liver, kidney, heart, and gill) was evaluated. Forty-two fish with similar sizes and weights were randomly selected, of which 7 fish (with their respective replicate) were exposed for 7 and 30 days to a Cd sublethal concentration (0.1 $mg.l^{-1}$). We determined the MV through a Janus Green B colorimetric assay and we obtained the concentration of AST by Ellman's method. Mitochondrial viability decreased in fish exposed to Cd for 30 days with the liver being the most affected tissue. We also detected a significant decrease in AST levels was in fishes exposed to Cd for 7 days in liver and kidney tissues; these results suggests that AST levels are elevated in some tissues may act as cytoprotective and adaptive alternative mechanism related to the ROS detoxification, maintenance redox status and mitochondrial viability. Organ-specifics variations were observed in both assays. We conclude that the Cd exposure effect on AST levels and MV, vary across fish tissues and is related to the exposure duration, the molecule dynamics in different tissues, the organism and environmental conditions.
Keywords: *Ancistrus brevifilis*, Cadmium, Soluble thiols, Janus Green B, Mitochondrial viability.

Introduction

In Venezuela, there has been a disturbing increase in the ambient concentrations of heavy metals in watersheds (Bifano and Mogollón, 1995; Hermoso and Márquez, 2005; Corona, 2013; Mora *et al*., 2013). In particular, cadmium (Cd) has been reported in moderate concentrations (Hermoso and Márquez, 2005; Salazar, 2009). Cd is known as cytotoxic and immunotoxic metal and is potentially carcinogenic (Salazar *et al*., 2009). As a result of the increased levels of Cd in the aquatic environment, the bioaccumulation of the metal in organisms has enhanced, especially in fishes (Souid *et al*., 2013, Salazar-Lugo *et al*., 2014, Perera *et al*., 2015). The accumulation of Cd in tissues has been reported from: *Aglyptodactylus laticeps* (Marcano and Troconis, 2001), *Hoplias malabaricus, Prochilodus reticulatus* (Vanegas, 2003), *Colossoma macropomum* (Hernández, 2005), *Centropomusun decimalis* (Márquez *et al*., 2008). The liver, kidney and gills are the target organs of metal uptake (Perera *et al*., 2015; Sabullah *et al*., 2015). The heart, is an aerobic organ rich in mitochondria and several studies have suggested that the heart is very sensitive to Cd toxicity (Wang *et al*., 2004; Soares *et al.*, 2008; Akpakpan and Akpanyung, 2014).

In cells, the mitochondria is a target for Cd. There are numerous reports in fish documenting the effect of Cd on these organelles including ATP synthesis depression, free radicals generation, lipid peroxidation, and mitochondrial membrane depolarization (Sokolova, 2004; Atli and Canli, 2008; Padmini and Usha, 2011). To counter the oxidative damage derived from Cd accumulation in different tissues, organisms increase their antioxidant molecules, such as metallothioneins (MT), glutathione (GSH), and other molecules rich in thiol groups. These molecules constitute the first defense line of the cell against oxidative damage (Salazar *et al*., 2009; Sevcikova *et al*., 2011; Dorts *et al*., 2012; Hatem *et al*., 2014).

The Guaraguara (*Ancistrus* sp.), is a native fish widely distributed in freshwater ecosystems of northeastern Venezuela, the Amazon River and other rivers in South America. It is the fourth most common fish species, in relative abundance, in the Manzanares River, Venezuela (Ruiz *et al*., 2005). It is a nocturnal benthic fish, which lives in the bottom of rivers and feeds mainly on algae and invertebrates. These traits lead to an increased uptake of pollutants and make *A. brevifilis* a sentinel organism to detect pollution in these ecosystems (Lárez, 2011).

The biomonitoring of freshwater fish species can be used to assess the consequences of chemical pollution by heavy metals in aquatic environments. These ecotoxicological studies are needed to increase

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our understanding of the impact humans have on the environment. The objective of this study was to evaluate the effects of Cd exposure on the mitochondrial viability (MV) and acid soluble thiols levels (AST) in *A. brevifilis*.

Materials and Methods

Fish maintenance

We collected *A. breviflis* specimens (n=84) from the Manzanares River, Yaque sector (10°12'17.58'' N and 63°53'21.08'' W). (11.39±1.69 m and 12.21±3.01 g) were used. All fish handling and maintenance procedures were done according to bioethics code of FONACIT (2011). We transported fish in black bags with aerated water; to the Immunotoxicity Protein Laboratory, Universidad de Oriente, Sucre State. We maintained the fish for 15 days in holding tanks with continuously aerated and flowing de-chlorinated tap water at a constant temperature of 27±3 ºC. We fed fish *ad libitum* with fish food, protein content 45%. In order to simulate the natural conditions these fish live in, we reduced artificial illumination by surrounding the aquariums with black bags. We maintained the pH at 7.7±0.25, temperatures (27±3 ºC), oxygen level at 3.8 $mg.l^{-1}$, and total hardness at 106 $mg.l^{-1}$ throughout all experiments.

Lethal mean concentration (LC_{50}) determinations

We determined the LC_{50} of Cd at 96 using a static test with water exchange every 24 h and daily feeding as previously described (Peltier and Weber, 1985), but with a few modifications. We exposed fishes, in groups of four (with replicates), to 0.01, 0.5, 5, 10, 20, and 30 $mg.l^{-1}$ Cd for 96 h in 12 l plastic aquariums. In addition, we included a control group, which was kept in water free of Cd. We recorded mortality was at 12, 24, 36, and 96 h post exposure to Cd. We calculated the LC_{50} using the Logic method (Weber, 1993) using LC_{50} software. *A. brevifilis* LC_{50} was 11.81 $mg.l^{-1}$, 0.1 $mg.l^{-1}$ Cd from $CdCl_2$ was selected for sublethal assay (<1 % LC_{50} 96 h).

Bioassay with 0.1 $mg.l^{-1}$ Cd

We tested the Cd levels in *A. brevifilis* tissues prior to any sub lethal experiments. The Cd concentrations were 0.19 (±0.005 $mg.g^{-1}$) which are under the permitted limits for fish (Osman and Kloas, 2010).

We randomly selected 42 fishes for the assay. We made sure all fish were a similar weight and size. We exposed seven groups of fishes to sublethal 0.1 $mg.l^{-1}$ Cd for 7 and 30 days. We included 14 fishes as a control unexposed to Cd. We continuously aerated each 40 l aquarium and we maintained the same physical, and chemical characteristics of the water as those for the laboratory acclimation. The aquarium water was renewed daily to maintain the sublethal Cd concentrations. Control fish were maintained under the same conditions in water devoid of detectable Cd. Fishes were fed before adding Cd to the water (every 24 h). We anesthetized the fishes with cool water and euthanized them by ventral dissection 7 and 30 days post Cd exposure. We collected the liver, kidney, heart and gills were and stored them at -20 °C for biochemical test.

Mitochondrial viability with Janus Green B (JG-B) assay

We used a previously described protocol to measure mitochondrial fraction (Saz and Lescure, 1969). Briefly, we minced 0.05 g of tissue with small scissors in 1 ml of cold mitochondrial buffer (0.24 $mg.l^{-1}$ sucrose, 0.0005 $mg.l^{-1}$ EDTA and 0.15 % BSA pH 7.4). After mincing, we gently homogenized the tissue in a glass homogenizer and then centrifuged at 100 x g for 10 min at 4 °C to remove nuclei, unbroken cells, and other non-subcellular tissue. We then filtered the supernatant through glass wool and centrifuged it at 7800 x g for 30 min at 4 °C. We then re-suspended the dark packed lower layer (heavy mitochondrial fraction) in mitochondrial buffer and again centrifuged at 7800 x g for 30 min. We verified the mitochondrial integrity fraction by NADH extinction at 340 nm.

We used specialized mitochondrial staining, JG-B, for the mitochondrial viability assay (Mohammadi and Ghazi, 2007). We suspended the mitochondrial suspension (1 mg protein) with JG-B (1 $mg.l^{-1}$) in 1:1 proportion. We prepared the blank solution the same way, but without the addition of mitochondrial suspension. Each sample was then measured via spectrophotometry at 607 nm. Mitochondrial viability (% MV) was calculated using the formula:

$$\%\ \text{mitocondrial toxicity} = 1 - \frac{\text{Mean absorbance of toxicant}}{\text{Mean absorbance of negative control}} \times 100\%$$

% VM = 100 % - % mitochondrial toxicity

Acid soluble thiols (AST) determination

We determined the level of acid soluble thiols (AST) as previously described (Sedlak and Lindsay 1968), with the proper modifications. We homogenized a 0.1 g of tissue with 0.9 ml Tris EDTA buffer (30 $mmol.l^{-1}$ Tris HCl, 3 $mmol.l^{-1}$ EDTA, pH 8.9). We then centrifuged the homogenate at 1500 rpm for 5 min at 4 °C. We then mixed 200 µl of supernatant with 0.5 g sulfosalicylic acid, placed in the freezer for 15 min, and then centrifuged at 7000 rpm for 10 min to precipitate proteins. We measure the absorbance of the reaction mixture (200 µl supernatant, 800 µl Tris HCl buffer pH 8.9 and 80 µl 5.5'-dithiobis-2-nitrobenzoic acid - DTNB) at a wavelength of 412 nm. We preformed the calibration curve from 100 $\mu mol.l^{-1}$ glutathione reduced (GSH) as standard and expressed the results in μmol^{-1} –SH/ml.

Statistical analysis

All values were expressed as mean±standard error (SEM). Statistical difference in % MV and AST levels (for each tissue evaluated during 7 and 30 days) were determined by Student's t-test (Ts). If the assumptions required for this test were not met, we used the nonparametric Mann-Whitney test. Additionally, we performed a Pearson Correlation analysis to associate % MV and AST levels in each *A. brevifilis* tissue tested. We analyzed all data using SPSS v15 (IBM Corporation, Armonk, New York, USA).

Results

During the last days of the Cd bioassay (7-30 days post exposure), we observed *A. brevifilis* displaying hyperactive aggressive behaviour in conjunction with damage on the skin and scales loss. This behavior was not observed in the control fish.

Mitochondrial viability and AST levels in A. brevifilis tissues

Liver

We detected a decrease in MV of hepatocytes by Cd exposure (P≤0.05), and the lowest values were observed in fish exposed for 30 d (Fig. 1a). We also observed a significant difference (P≤0.01) in AST distribution in the liver between exposed and unexposed fishes in both treatment groups. We detected lower AST concentrations in fishes in the 7d Cd exposed group compared to the controls and higher concentrations in the 30d Cd exposed group when compared to the controls (Fig. 1b).

Kidney

In the kidney we observed a decrease in MV in the fishes exposed to Cd. The lowest values were observed in fishes exposed to Cd for 30 d (Fig. 2a). We also detected a significant decrease in AST concentrations in 7d Cd exposed fishes (P≤0.001); however we did not detect any significant differences in the fishes exposed to Cd for 30 d (Fig. 2b).

Heart

We did not detect any difference in heart MV as a result of Cd exposure (Fig. 3a); however AST levels in this tissue decreased significantly (P≤0.001) in Cd exposed fish both at in both exposure groups (Fig. 3b).

Gills

We detected an increase in MV in the gills of *A. brevifilis* in the fishes in the 30 d exposure group (P≤0.01) (Fig. 4a). We also detected a significant decrease of AST concentration in the 30 d exposure group. (P≤0.001) (Fig. 4b).

Correlation between MV and AST levels in A. brevifilis tissues

We detected an inverse association between MV and AST levels (r = -0.968**) in the heart. However, in the gills we observed a positive association between %

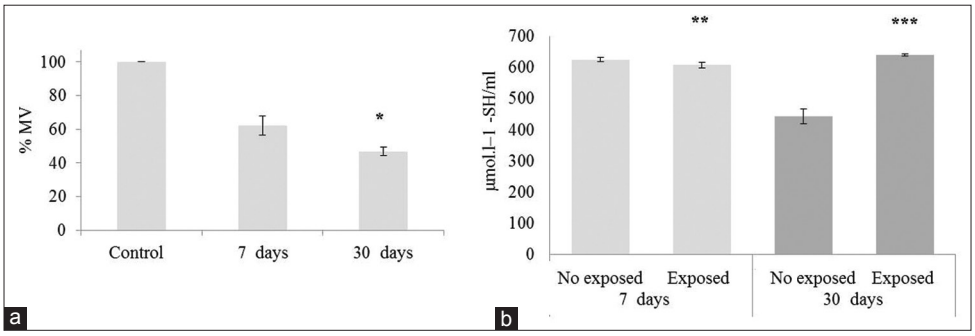

Fig. 1. (a): Mitochondrial viability (%) of *A. brevifilis* liver 7 and 30 days of Cd exposure. (b): AST levels of *A. brevifilis* liver control, and 7 and 30 days Cd exposed. *: significant differences (P<0.05); **: Very significant differences (P<0.01); ***: Highly significant differences (P<0.001).

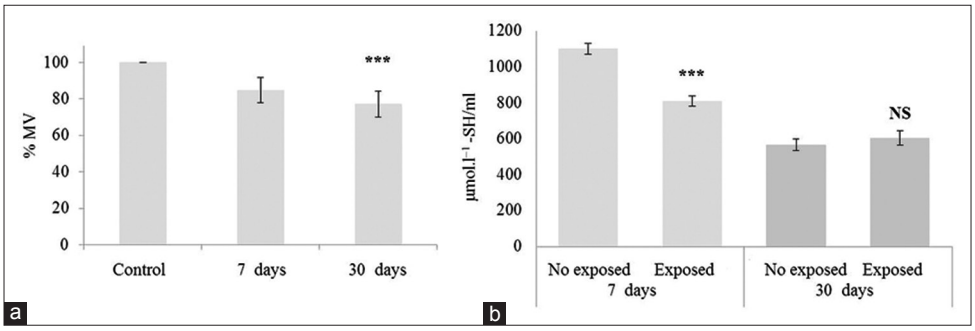

Fig. 2. (a): Mitochondrial viability (%) of *A. brevifilis* kidney 7 and 30 days of Cd exposure. (b): AST levels of *A. brevifilis* kidney control, and 7 and 30 days Cd exposed. NS: Not significant; ***: Highly significant differences (P<0.001).

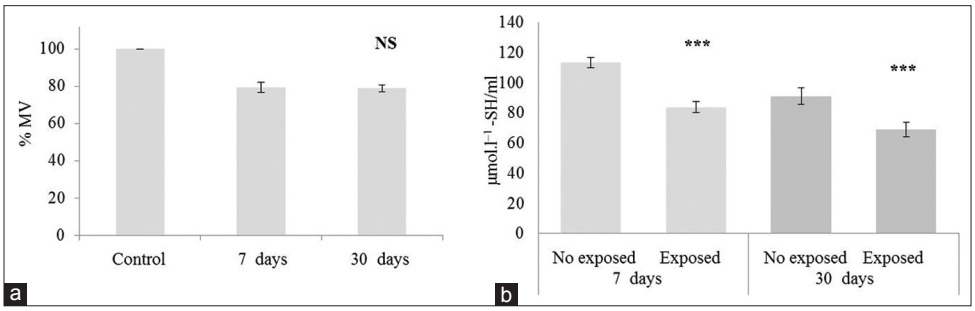

Fig. 3. (a): Mitochondrial viability (%) of *A. brevifilis* heart 7 and 30 days of Cd exposure. (b): AST levels of *A. brevifilis* heart control, and 7 and 30 days Cd exposed. NS: Not significant; ***: Highly significant differences (P<0.001).

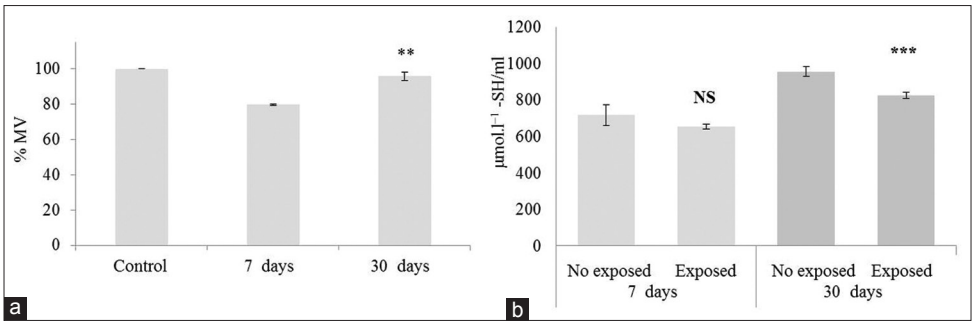

Fig. 4. (a): Mitochondrial viability (%) of *A. brevifilis* gills 7 and 30 days of Cd exposure. (b): AST levels of *A. brevifilis* gills control, and 7 and 30 days Cd exposed. NS: not significant; **: Very significant differences (P<0.01); ***: Highly significant differences (P<0.001).

Table 1. Correlation analysis between mitochondrial viability (%) and AST levels (µmol.l^{-1} –SH/ml) in *A. brevifilis* tissues exposed to Cd.

Tissue	*Pearson correlation (r)*	*p value*
Liver	−0.395^{NS}	0.439
Kidney	0.013^{NS}	0.981
Heart	−0.968**	0.001
Gills	0.933**	0.007

P: Probability; NS: Not significant; **: Very significant p<0.01.

MV and AST levels (r = 0.933**). We did not detect any significant trends with MV and AST in the liver (Table 1).

Discussion

We showed that MV is able to be used as a marker for chronic Cd chronic toxicity in *A. brevifilis*. The liver and kidney are the most sensitive organs to mitochondrial damage generated by chronic metal toxicity, which correlates with their primary function as Cd bioaccumulator tissues (Perera *et al*., 2015). Similar results in mitochondrial Cd toxicity have been reported in a variety of organisms from mammals (Nguyen *et al*., 2015), *Crassotrea virginica* oyster (Sokolova, 2004), fish (Adiele *et al*., 2010, Padmini and Usha, 2011) to plants (Miller *et al*., 1973), suggesting that metal site specific target are conserved throughout distant taxa.

The mitochondria are an essential organelle to ATP generation, involved in other processes such as ROS natural generation, cell death and the ageing processes (Chaiyarit and Thongboonkerd, 2009). Reports suggest that mitochondrial function is highly tuned and prone to heavy metals damage (Adiele *et al*., 2010). The presence of metals such as Cd substantially increases ROS production resulting in lipid peroxidation, mtDNA cleavage and ATP synthesis inhibition which results in mitochondrial damage and apoptosis induction (Cuypers *et al*., 2010). Cadmium may inhibit Wang *et al*. (2004) inhibit complex III CTE (ubiquinone: cytochrome c oxidoreductase) in the liver, heart, and brain mitochondria; in addition, to stimulating ROS production in this complex (Wang *et al*. 2004). This accumulation is probably mediated by Cd binding between semiubiquinone and cytochrome b_{566} of the Q_0 site of cytochrome b complex III, resulting in accumulation of semiubiquinones at the Q_0 site. The semiubiquinones, being unstable, are prone to transfer one electron to molecular oxygen to form superoxide. In *Oncorhynchus mykiss,* Cd accumulation in mitochondria of liver was related to complex III inhibition, causing mitochondrial damage (Adiele *et al*., 2010). It is possible that similar processes could be occurring in *A. brevifilis* liver mitochondria.

Gobe and Crane (2010) reported that kidney mitochondrial toxicity is mediated by similar mechanisms to those reported for liver, including ROS production, ATP altered levels and membrane potential alteration that stimulates apoptosis activation causing nephrotoxicity. Cell morphology studies in different organs of the fish *Dicentrarchus labrax,* showed that the mitochondria in liver, kidney, and gills exposed to Cd were damaged. This damaged was marked by signs of swelling, disappearance of ridges, vacuolation and myeloid bodies formation, classic characteristics of cellular processes caused by high metals action (Giari *et al*., 2007).

In the present study, cardiac mitochondrial viability of Guaraguara was unaffected by Cd exposure. Some studies suggest that heart mitochondria are vulnerable to metal poisoning (Wang *et al*., 2004); however, we found a negative association between MV and AST levels in heart. This result suggests that increment of AST concentration emerges as a compensatory mechanism conferring long-term protection to mitochondria against the Cd oxidative effect. Hatem *et al*. (2014) reported the exist of a protection mechanism against oxidative stress conditions that stimulates GSH accumulation in nucleus and mitochondria.

On the other hand, the variation of AST content in *A. brevifilis* could be an adaptive and antioxidative effect to organ-specific Cd accumulation. Zirong and Shijun (2007) showed that GSH content may increase or decrease in different tissues exposed to Cd, attributable to organ-specific responses also varies among species. In liver tissues from *Labeo rohita,* the GSH content decreased in Cd chronic assay clear that heavy metals cause of oxidative stress (Khalid *et al*., 2015).

Cao *et al*. (2012) reported the long-term GSH increase (20 days exposure) with increase of γ-glutamylcysteine synthetase (γ-GCS) enzyme activity. This is believed to constitute an adaptive compensatory mechanism in the liver to synthesize GSH *de novo* as a way to counteract metal oxidative effect, as observed for *A. brevifilis* liver and the other tissues evaluated. Other studies in *Oreochromis niloticus* report decrease in liver of GSH associated to Cd oxidative effect and its use as a cofactor in glutathione peroxidase and glutathione transferase enzymatic systems (Zirong and Shijun, 2007).

GSH is synthesized in the liver and transported in the blood to organs such as kidney and muscle (Atli and Canli, 2008). In the kidney tissues of *Anguilla anguilla,* a decrease in GSH levels was evidence to prevent ROS regeneration (Ahmad *et al*., 2006). In the kidney tissue of *Paralichthys olivaceus*, it was found that GSH levels decrease in fish exposed to Cd, but was associated with high metal accumulation in these cells, which causes complexes Cd-GSH formation that results on GSH reduction (Cao *et al*., 2010, 2012). While, in *Cyprinus carpio* an increase in GSH levels and antioxidant enzymes in liver and kidney were observed due to their function as high metal accumulation and detoxification organs (Dugmonits *et al*., 2013; Paritha and Deepak, 2015).

We could establish that the AST elevated levels in some tissues act as cytoprotective and adaptive alternative mechanism related to the ROS detoxification, maintenance redox status and mitochondrial viability. Additionally, it is also important to note that the Cd exposure effect on AST levels, vary across fish tissues and is related to the exposure duration, the molecule dynamics in different tissues, the organism, and environmental conditions. Future research should include histopathology studies and an evaluation with other biochemicial markers (e.g. antioxidant enzymes) to help establish tolerance mechanisms of *A. brevifilis* to heavy metal contamination.

Conflict of interest

The Authors declare that there is no conflict of interest.

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