Biochemical and Mitochondrial Changes Induced by Cd, Fe and Zn in *Limnodrillus hoffmeisteri*

Cambios Bioquímicos y Mitocondriales Inducidos por Cd, Fe y Zn en *Limnodrillus hoffmeisteri*

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SUMMARY: The effects of sublethal concentrations of cadmium (0.64 µg/L), iron (0.043 mg/L) and zinc (0.31 mg/L) and a mixture of these metals on succinate dehydrogenase (SD) and alkaline phosphatase (AP) activity and on structural changes in the mitochondria of epithelium cells of the digestive tract were examined in the oligochaete *Limnodrillus hoffmeisteri* after 96 h of exposure in artificial sediments. SD activity was significantly inhibited, particularly in treatments with Cd alone (92.57%), while AP increased its activity with Cd alone (73.23%). However, when this metal was mixed with Fe and Zn, the inhibition of SD activity was lower (67.82%) than with Cd alone, showing an antagonistic effect and AP increased its activity (73.26%). Mitochondria were structurally damaged by exposure to Cd alone. However, in the metal mixtures, the toxic effects may exert interactive effects eliciting a less structural damage in the mitochondria of epithelium cells of the digestive tract than when Cd is alone.

KEY WORDS: Heavy metals; Mixture; Alkaline phosphatase; Succinate dehydrogenase activity; *Limnodrillus hoffmeisteri*; Epithelium cells; Mitochondria.

INTRODUCTION

Oligochaetes such as *Limnodrillus hoffmeisteri* are organisms of the freshwater benthos which are usually exposed to toxicants in water and sediment. These organisms are thought to have an excellent potential for evaluation of metal toxicity because of their short life cycle and high sensitivity to these metals (Bouché *et al.*, 2000; Martínez-Tabche *et al.*, 2001).

Numerous studies have focused on the effect of heavy metals on aquatic organisms, since the former may elicit diverse pathologies, and even death, in a large number of these organisms (Renella *et al.*, 2003). These toxicants may enter aquatic systems accidentally or deliberately, through lixiviation as well as industrial and domestic wastewater discharges (Giambérini & Cajaraville, 2005; Pyatt *et al.*, 2005; Pyle *et al.*, 2005; Gust & Fleeger, 2006). As contaminants are not found in isolation in aquatic systems, their joint actions, and effects on the aquatic biota have

motivated the interest of research workers. Results of toxic response evaluation reveal that toxicity levels are lower in response to metal mixtures than alone (Antonio *et al.*, 2002; Castañé *et al.*, 2003; Vijver *et al.*, 2005).

For most aquatic organisms, exposure to metals at above threshold concentrations may be extremely toxic. The most important metals, toxicologically, are Hg, Cr, Cd, Ni and Zn, since these elements usually enter the cell through the same transport systems as other physiologically important metal cations such as Ca, Mg, Cu and Zn (Camusso *et al.*, 2000; Castañé *et al.*; Pyle *et al.*; Gust & Fleeger).

Cadmium has no biological function, it is one of the most toxic metals and its presence in the environment has been on the rise (Giambérini & Cajaraville; Pyatt *et al.*; Gust & Fleeger). Once it has been absorbed, primarily through the gut, it affects the cell processes associated with the

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transport and metabolism of essential metals such as Ca, Cu, Fe and Zn, thus affecting mitochondrial function (Kantola *et al.*, 2000; Papanikolaou & Pantopoulos, 2005). In addition, these metals affect the activity of iron-dependent mitochondrial enzymes such as succinate dehydrogenase (SD) and NADH-dehydrogenase. Reduction or induction of these activities alters energy metabolism and cell respiration, thereby significantly decreasing the cell ATP content (Martínez-Tabche *et al.*, 2000; Nicolau *et al.*, 2004).

Phosphorus compounds play a central role in anabolic and catabolic pathways and in the energy conversion of the cell via transfer of energy-rich phosphoanhydride bonds. Esterified phosphates may be released from organic phosphates through the action of cellular and extracellular alkaline phosphatases by catalyzing the hydrolysis of phosphate ester bonds (Sharma & Mallick, 2004).

Alkaline phosphatase metalloenzyme (AP) is a homodimeric complex with two Zn(II) ions and a single Mg (II) ion within the active site of each subunit. The Zn(II) ions most likely possess a catalytic role in rendering phosphate monoesters susceptible to hydrolysis (Wyckoff, 1987; Coleman, 1992). As Cd presents strong chemical similarities to Zn, according to their atomic structure and chemical behavior it is a common matter to find them together in nature. These similar characteristics explain their toxicity, as long as Cd is able to use the same transport mechanism as the body uses to introduce the essential metals (Peijnenburg *et al.*, 1999; Sharma & Mallick). Furthermore, Navarro *et al.* (1999) found that high levels of AP indicated cellular damage, as long as phosphatase removes phosphates from membrane phospholipids.

SD, an enzyme in the inner membrane of the mitochondrion, catalyzes oxidation of succinate to fumarate via the Krebs cycle. It is the only enzyme linking the Krebs cycle anatomically and physiologically to the respiratory chain and oxidative phosphorylation (Singer *et al.*, 1963a, 1963b). Because SD is an iron-dependent enzyme, several authors have suggested that the mechanism through which Cd elicits its toxicity is by iron replacement, and therefore causes alterations to the respiratory chain at the electron transport level (Cummings *et al.*, 2000; Barbouti *et al.*, 2001). Due to the significance of damage elicited by heavy metals at the mitochondrion level, Bizarro *et al.* (2003) studied potential damage to mouse Sertoli cells after exposure to concentrations of 0.01 M Pb, 0.006 M Cd and a Cd-Pb mixture at these same concentrations. They found that at the ultrastructural level the metal mixture was responsible for the most severe mitochondrial changes, as compared to those produced by separate exposure to Pb or Cd alone.

The aim of this study was to evaluate the toxicity of sublethal concentrations of Cd, Fe and Zn separately and as a mixture of these metals on SD and AP activity, and by means of an ultrastructural study to determine the changes occurring in mitochondria of epithelial cells of the digestive tract of *L. hoffmeisteri*.

MATERIAL AND METHOD

Culture and maintenance of test specimens. *Limnodrillus hoffmeisteri* were collected in their natural habitat in Lake Texcoco (State of México, México) and acclimated for 15 days in the laboratory on clean sandy sediment (0.4 mm mean particle size). Synthetic water in a 1:4 ratio of sediment to water was added to the 2-L plastic containers before placing the specimens. A constant recirculation system was maintained as follows: pH 7.0, constant aeration, natural light/dark photoperiod (12/12 h), temperature 22 ± 2 °C, and food provided *ad libitum* (Grajeda y Ortega *et al.*, 2008). All animals were treated ethically according to the Norma Oficial Mexicana (NOM-062-ZOO-1999).

Intoxication of specimens. Specimens of *L. hoffmeisteri* were exposed to Cd, Fe and Zn separately and in mixture form at sublethal concentrations, taking into consideration the 96-h 1/10 LC50 of each metal obtained in a previous study (Grajeda y Ortega *et al.*). To this end, 250 g of clean sandy sediment and 2 L of metal solution were placed in polystyrene containers according to the following test scheme: 0.64 mg/L of Cd, 0.03155 mg/L of Fe, 0.3102 mg/L of Zn, a Cd-Fe-Zn mixture at the same concentrations, and no metals were added to the control. The systems were shaken mechanically for 2 h when chemical equilibrium was assumed to be reached, and 10 g of specimens approximately 2.5 cm in length were placed in each. Systems were maintained in constant recirculation at 20 ± 2 °C. The organisms were not fed and a natural light/dark cycle was maintained. All assays were performed in triplicate.

After 96 h of exposure, 1.0 g (wet wt.) of *L. hoffmeisteri* was taken and homogenized. The homogenate was centrifuged at 7000g for 10 min at 4 °C. The sediment was discarded and total protein content was determined in the supernatant by the Bradford method (Bradford, 1976). SD activity was evaluated using the spectrophotometric method described by Singer *et al.* (1963a, 1963b). AP activity was evaluated using the spectrophotometric method described by Berger & Rudolph (1963).

Statistical analysis. Results were subjected to an one-way analysis of variance (ANOVA), followed by a Student-

Newman-Keuls test for comparison of means. Differences were considered significant at $p < 0.05$.

Ultrastructural study (Luft, 1961). From the batch of organisms exposed in each treatment system, three specimens per system ranging from 2.0 to 2.5 cm in length were taken and cut into five segments. Each segment was removed and fixed in 2.5% glutaraldehyde in water with a pH of 8 for 48 h, then rinsed with 2.5% sodium bicarbonate and postfixed with osmium tetroxide for 1 h. Dehydration was done in three 10-min shifts with different concentrations of ethanol, followed by two 20-min propylene oxide shifts. Samples were then permeated with 1:1 propylene oxide/epoxy and embedded in Epon 812. Very thin 70-nm sections were obtained and set in mesh copper grids. These were treated with uranyl acetate and lead citrate in order to enhance contrast and were later examined using a transmission electron microscope (Jeol-100SX).

RESULTS

Succinate dehydrogenase activity. In all treatments (i.e., individual metals and the mixture) there was a significant decrease of SD activity (Fig. 1). In specimens treated with Cd alone, this decrease was greater with respect to controls (92.57%) than in those exposed to the metal mixture, which showed 67.82% reduction. In organisms treated with Fe alone, SD activity decreased by 38.6%, while specimens exposed to Zn alone had 49.57% reduction. In all cases, significant differences were found with respect to controls ($p < 0.05$).

Alkaline Phosphatase activity. Results of the AP quantification in *Limnodrillus hoffmeisteri* show an increase of this enzyme activity, in all cases as compared to controls (Fig. 2). Results were as follow: in isolated systems 68.9% with Cd, 52.3% with Fe and 56.6% with Zn, whereas in the mixture 73.26%. In all cases significant differences were found as compared to controls ($p < 0.05$).

Fig. 1. Effects of Cd (1/10 of the LC50), Fe (1/10 of the LC50), Zn (1/10 of the LC50) and a Cd+Fe+Zn mixture at these same concentrations on succinate dehydrogenase activity in *L. hoffmeisteri*. * Significantly different as compared to control at $p < 0.05$.

Fig. 2. Effects of Cd (1/10 of the LC50), Fe (1/10 of the LC50), Zn (1/10 of the LC50) and a Cd+Fe+Zn mixture at these same concentrations on alkaline phosphatase activity in *L. hoffmeisteri*. * Significantly different as compared to control at $p < 0.05$.

Ultrastructural study. Ultrastructural results show that in specimens exposed to Fe and Zn separately, the epithelial cells of the digestive tract display no damage to mitochondria as compared to controls (Figs. 3A, 3B and 3C). However, in specimens treated with Cd, these same cells show significant changes as well as a decrease in the number of mitochondria by comparison to controls, and mitochondrial edema with structural damage was observed in some areas (Figs. 4A and 4B). Moreover, in specimens exposed to the Cd-Fe-Zn mixture, a less structural damage in the mitochondria of epithelium cells of the digestive tract than when Cd is alone severe damage to epithelial cells of the digestive tract was seen and some cells showed structural damage (Fig. 4) as compared to controls.

Fig. 3.
Epithelial cells of the digestive tract of *L. hoffmeisteri*.

A. Control.

B. Treatment with Fe; abundant mitochondria display normal conditions.

C. Treatment with Zn; abundant mitochondria exhibit normal conditions as compared to control ($\Rightarrow$).

Bar = 0.5 μ m

Fig.4. (A) Panoramic view of an epithelial cell of the digestive tract treated with Cd. Fewer mitochondria are observed. (B) Mitochondria showing structural damage are present in some areas ($\Rightarrow$). Bar=0.5mm

Fig. 5. Epithelial cell of the digestive tract treated with the Cd-Fe-Zn mixture. Amorphous structures produced by structural disruption can be seen ($\Rightarrow$). Bar= 0.5 mm

DISCUSSION

In all organisms, mitochondria play a crucial role in regulating cell life and cell death. In these processes, permeability of the mitochondrial membrane can be associated with the release of important molecules, such as ATP and induction of ROS due to incomplete oxygen reduction (Bizarro *et al.*). As has been pointed out by several authors, damage to mitochondria by Cd alone or mixed with other metals is irreversible, since it inhibits several enzymes in these organelles which are involved in cellular respiration and energy production (Antonio *et al.*; del Carmen *et al.*, 2002). Thus, Cd also induces oxidative stress, decreases the activity of antioxidant enzymes and elicits damage at the DNA level (del Carmen *et al.*).

Similarly, Li *et al.* (2003) studied mouse liver cells treated with Cd, finding a decrease in the number of mitochondria and collapse of the mitochondrial membrane, possibly as a result of Cd toxicity, in addition to induction of apoptosis in the mitochondria. Metabolic disorders can elicit a sudden increase in the permeability of the inner membrane of the mitochondrion since opening of protein pores allows osmotic entry of water and low molecular weight molecules. As a result, distention of the mitochondria occurs and the cell undergoes a failure in bioenergy due to ATP depletion, culminating eventually in cell lysis (Renella *et al.*).

In *L. hoffmeisteri*, the results of treatments with either Fe or Zn showed no major changes. Damage to mitochondrial membranes was not observed and the number of mitochondria did not decrease as compared to controls (Figs. 3A, 3B and 3C). However, in treatments with Cd alone and the Cd-Fe-Zn mixture, our observations are in agreement with those of Bizarro *et al.* and Li *et al.*, since this contaminant alone and in mixture form was the one eliciting the most damage in mitochondria, including detachment, membrane loss and destruction of cristae (Figs. 4A, 4B, and 5). Similarly, damage to the mitochondrion by Cd is also linked to reduced cell respiration and distention of mitochondria, possibly due to a decrease in ATP levels. Grajeda y Ortega *et al.* found that ATP decreased by 88.52% in *L. hoffmeisteri* exposed to Cd at concentrations of 6×10^{-3} µg/L. In other studies, Cd has been reported to affect the permeability of the mitochondrial membrane, allowing this metal to enter the mitochondrion via Ca²⁺, inhibiting enzyme complexes, e.g. cytochrome c oxidase in the respiratory chain, as well as induce mitochondrial apoptosis (Bizarro *et al.*; Li *et al.*).

Some enzymes found in mitochondria, such as NADH-dehydrogenase, cytochrome c oxidase and SD, are

associated with proteins known as (Fe-S) bond nonhematic ferroproteins, whose structure contains Fe. SD is an iron-dependent enzyme involved in ATP production via the Krebs cycle and in oxidative phosphorylation. Several studies have shown that SD activity diminishes when the organism is exposed to heavy metals, such as Cd and Zn, through replacement of the iron in its molecule (Renella *et al.*). The results of our study (Fig. 1), show a significant decrease of SD activity in the test specimens of all assayed systems, this decrease being greater in systems exposed to Cd alone than in the form of a mixture. Similarly, other metals with redox potential, such as Fe, are able to induce oxidative stress through production of reactive oxygen species (ROS) (Barboudi *et al.*; Mattson, 2004). ROS increase lipid peroxidation, damaging cell membranes and affecting the biological oxidation processes that give rise to reduced electron transporters, namely NADH. The latter is associated with SD activity and the simultaneous production of energy (Lange *et al.*, 1999). However, Moustafa (2004) and Chung *et al.* (2005) point out the possible role of Zn as an antioxidant, stabilizer and membrane protector, in addition to its protective effect on ROS. It is important to stress the joint action of metal mixtures in aquatic systems, as emphasized by the research of Otitoloju, (2002), who has described different types of interactions. For instance, the antagonistic effect between Zn and Cd, using the mangrove periwinkle *Tympanotonus fuscatus*, in which all tests of Zn-Cd mixtures (4:1, 3:2, 1:1, 2:3 and 4:1) were less toxic than tests with Cd alone. Our results show a similar effect, since SD activity was higher with the Cd-Fe-Zn mixture than with Cd alone. This resulted in reduced toxicity, although SD activity remained much lower than in controls.

Different studies have mentioned that AP activity is a good indicator of Zn source (Durrieu & Tran-Minh, 2002; Yousef *et al.*, 2002). However, divalent ions as Mg, Ca, Ba, Mn, Co, Ni, Cd are able to activate this enzyme (Chen *et al.*, 2000).

AP in the cellular external membrane plays a main role in the phosphates metabolism, without this enzyme the external membrane is damaged by heavy metals (Durrieu & Tran-Minh). In this study an increase in AP activity (Fig. 2) could be observed, possibly due to the metals induction mainly in the tests where Cd was included, an increase of 68.9% of the enzyme activity was detected, and 73.26% increase in the mixture was found. These results were in accordance with those of Durrieu & Tran-Minh when algae was exposed to Cd and Pb, finding an AP activity increase possibly due to phosphate ingestion by organisms.

Sastry & Gupta (1979) point out the importance of studies on the pathological histology and biochemistry of aquatic organisms, which may show relevant effects as a result of toxicity elicited by metals such as Cd. These authors conducted histopathological studies of the liver and digestive tract of the teleost *Heteropneustes fossilis* treated with 6.8 mg/L of CdCl₂, observing damage to connective tissue of the digestive tract, vacuolation of cytoplasm in hepatocytes, and rupture of the cell membrane leading eventually to hepatocyte degeneration and mitochondrial damage. Renella *et al.* stated that Cd can compete with phosphate and Zn ions, which are part of the enzyme structure of AP. They suggested that it can be one of the mechanisms that produce its toxicity. Furthermore, Vallee (1991) reported the possible direct transference of Zn, Cu and Fe from the metallothioneins to the AP apoenzyme, implying that these proteins could be involved to the regulation or activity of AP and some other enzymes.

CONCLUSIONS

Based on the results obtained in this study, we conclude that Cd showed decreased toxicity when mixed with Fe and Zn. The toxic effects of Cd significantly altered SD and AP levels in *L. hoffmeisteri*, as well as elicited damage and reduced the number of mitochondria in epithelial cells of the digestive tract.

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RESUMEN: Se estudió el efecto de las concentraciones subletales de Cd (0,64 µg/L), Fe (0,043 mg/L) y Zn (0,31 mg/L) en forma aislada y en mezcla sobre la actividad de la succinato deshidrogenasa (SD) y la fosfatasa alcalina (AP) en las mitocondrias de las células epiteliales del tracto digestivo en el oligoqueto *Limnodrillus hoffmeisteri* después de 96 h de exposición en sedimentos artificiales. La SD se inhibió significativamente, particularmente en los tratamientos con Cd en forma aislada (92,57%), mientras que la AP se incrementó con Cd en forma aislada (73,23%). Sin embargo, cuando este metal se mezcló con Fe y Zn, la inhibición de la SD fue menor (67,82%) que con Cd en forma aislada, lo que mostró un efecto antagonístico y la AP incrementó su actividad (73,23%). Sin embargo, cuando este metal estaba en mezcla con Fe y Zn, la inhibición de la SD fue menor (67,82%) que con Cd en forma aislada, mostrando un efecto antagonístico y un incremento en la actividad de la AP (73,26%). Las mitocondrias fueron dañadas estructuralmente por exposición al Cd en forma aislada. Sin embargo, con los metales en mezcla, los efectos tóxicos pudieron ejercer efectos interactivos provocando un menor daño estructural en la mitocondria de las células del epitelio del tracto digestivo que cuando el Cd estaba en forma aislada.

PALABRAS CLAVE: Metales pesados; Mezcla; Fosfatasa alcalina; Actividad succinato deshidrogenasa; *Limnodrillus hoffmeisteri*; Células epiteliales; Mitocondrias.

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