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Localization of inorganic and organic mercury in the liver and kidney of *Cyprinus carpio* by autometallography

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Abstract

Mercury bioaccumulation in different tissues largely depends upon metal chemical form and has important implications on tissue-specific toxicity. Then, simple tools for mercury tissue localization are very useful for understanding the toxicity of mercurial compounds in fishes. In the present study, inorganic and organic mercury distribution was investigated in carp *Cyprinus carpio*, utilizing autometallography. Juvenile *C. carpio* were exposed through force-feeding with mercury contaminated pelleted food at doses of 0.5 µg g^{-1} of Hg^{+2} or MeHg twice at 15 days interval, and killed 15 days after the second exposure for sampling. Organic mercury was observed inside hepatocytes and in biliary ducts of liver, whereas inorganic form of mercury was observed only in biliary ducts. In the kidney, mercury was observed mainly in renal tubules, with the predominance of inorganic over organic mercury. The present study demonstrated that autometallography is a useful tool for the tissue localization of two mercurial compounds in the liver and kidney of *C. carpio* after trophic exposure.

Keywords: Autometallography, mercury, liver, kidney, *Cyprinus carpio*.

Localização do mercúrio inorgânico e orgânico em fígado e rim de *Cyprinus carpio* através da autometalografia

Resumo

A bioacumulação de mercúrio em diferentes tecidos depende da forma química do metal e tem implicações importantes na toxicidade de tecidos específicos. Deste modo, ferramentas simples para a localização de mercúrio em tecidos são úteis para a compreensão da toxicidade de compostos mercuriais em peixes. No presente estudo, a distribuição de mercúrio inorgânico e orgânico foi investigada em carpa *Cyprinus carpio*, utilizando autometalografia. Exemplares juvenis de *C. carpio* foram expostos através de alimentação forçada com pellets de ração contaminada com mercúrio em doses de 0.5 µg g^{-1} Hg^{+2} ou MeHg duas vezes em um intervalo de quinze dias, e sacrificados 15 dias após a segunda exposição para amostragem. O mercúrio orgânico foi observado no interior dos hepatócitos e nos ductos biliares. No rim, o mercúrio foi observado principalmente nos túbulos renais, com predominância do mercúrio inorgânico sobre o orgânico. O presente estudo demonstrou que a autometalografia é uma ferramenta útil para a localização tecidual de dois compostos mercuriais em fígado e rim de *C. carpio* após exposição trófica.

Palavras-chave: Autometalografia, mercúrio, fígado, rim, *Cyprinus carpio*.

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INTRODUCTION

Metal contamination of aquatic ecosystems is a serious worldwide environmental problem and mercury (Hg) is among the best-known nonessential toxic metals, occurring in both organic and inorganic forms. However, different mercury compounds have distinct toxicity, tissue bioaccumulation and dose-effect and dose-response relationships (Mason *et al.*, 2002).

Methyl mercury (MeHg) is the main organic form of Hg, produced through methylation of inorganic Hg by bacteria. MeHg bioavailability and bioaccumulation usually exceeds that of inorganic mercury (Laporte *et al.*, 2002; Manson, 2002; Oliveira Ribeiro *et al.*, 2002).

Exposure route and chemical form of mercury affect its tissue-specific distribution. Critical target organs of mercury chloride trophic exposure are kidney, liver, blood, intestinal epithelium and lungs (Berlin & Ulberg, 1987; Nielsen & Andersen, 1989), whereas liver, nervous system, intestinal epithelium, kidney and muscle are common targets of organic mercury (Möller-Madsen & Danscher, 1991; Oliveira Ribeiro *et al.*, 2002; Clarkson, 2003; Mela *et al.*, 2007). Although chemical analyses provided information about tissue distribution, the exact cellular localization of mercury has not been available until the introduction of the autometallographic technique (AMG) (Danscher & Möller-Madsen, 1985; Baatrup & Danscher, 1987; Stoltenberg & Danscher, 2000). The AMG is an auto-induced silver amplification method of some metal atoms and metal molecules in biological sections by applying basic principles of photography (Danscher, 1984). Using AMG, metal atoms, metal sulphides or metals selenides can be visualized under the light and the electron microscopes after silver enhancement as black silver deposits (Danscher & Möller-Madsen, 1985; Mela *et al.*, 2010).

Currently, there are a number of studies describing the distribution of mercury in mammal tissues (Hansen & Danscher, 1997; Pedersen *et al.*, 1999), but few in fishes (Baatrup & Danscher, 1987; Mela *et al.*, 2010). Fish are considered good indicators of environmental contamination because they are aquatic organisms that bioaccumulate several classes of pollutants in their tissues from water and food sources. In particular, the common carp *C. carpio* is an economically important fish spread worldwide in cultures in Asia, Europe and Latin America (Aydın & Köprücü, 2005) and is easily maintained in laboratory (Gengiz, 2006).

Upon entering cells, mercury can interact with several biomolecules such as glutathione and protein sulphydryl groups present in antioxidant, DNA repair and metal homeostasis proteins, altering their normal physiological activity. Metallothioneins, for example, are low molecular mass, cysteine-rich metal-binding polypeptides that production can be induced by mercury exposure (Nordberg & Nordberg, 2000; Yoshida *et al.*, 1999) and occur in a large number of evolutionarily diverse organisms, including fishes (George & Langston, 1994). Metallothioneins have an important role in essential metal homeostasis and in sequestering and detoxication of non-essential metals (Nordberg, 1998), as evidenced by the overexpression of these proteins in the target organs due to metal exposure, including in fish *Cyprinus carpio* (Ariyoshi *et al.*, 1990).

Data about the toxic mechanisms of mercury and its pharmacokinetics are abundant though still incomplete, and distribution studies based on methods to detect minute mercury traces can help understanding mercury toxicity and provide cues for the diversity of toxic effects reported in the literature. In the present study, AMG was utilized to visualize deposits of mercury in kidney and liver sections of *C. carpio* exposed through feed to mercury chloride or methyl mercury. Metallothionein concentration was also assayed in the liver and kidney of fishes in order to verify whether those doses of inorganic and organic Hg altered metallothionein levels.

It is the first comprehensive investigation aiming to determine the localization of two forms of mercury with different chemical and toxicological properties in the liver and kidney of *C. carpio* after trophic exposure.

MATERIAL AND METHODS

Mercury exposure conditions

A number of eighteen juvenile *Cyprinus carpio* (body weight: 92.12 ± 7.34 g w.w.; standard length: 19.36 ± 0.48 cm) were randomly placed in three tanks containing 100 L of dechlorinated tap water and maintained during ten days for acclimation under controlled aeration, temperature (21 ± 2°C), photoperiod (12 h light: 12 h dark), pelleted food supply (three times a week) and cleaning procedures (replacement of one-third water volume twice a week). After acclimation, *C. carpio* were exposed through force-feeding with mercury contaminated pelleted food, one group of fish (n=6) was exposed to 0.5 µg g^{-1} of Hg^{+2}, a second group (n=6) to 0.5 µg g^{-1} of MeHg and a third group (n=6) was not exposed (control). In all cases exposure was performed twice (0 and 15 days) through anesthetized fish (0.02% MS222, ethyl-ester-3-aminobenzoic acid in water) with a solution containing mercury (100-240 µL, according to the body mass of the fish) or the vehicle solution (HCl at 0.1 moles L^{-1}). On day thirty, fish were anesthetized and killed by section of spinal cord, and liver and the posterior kidney were collected for subsequent analysis. All procedures were performed according to the NIH Specify guidelines and Federal University of Parana commission for studies involving human or animal subjects (http://www.bio.ufpr.br/ceea/html/index.html).

Autometallography and Light microscopy

Liver and kidney samples were fixed in fixative solution (3% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h at 4°C and rinsed in buffer (0.1 M sodium cacodylate buffer, 2% NaCl, pH 7.4). Then, the samples were dehydrated in graded series of ethanol and embedded in Paraplast Plus resin®. Sections of five micrometers were obtained

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in microtome. Then, autometallography was performed according to Danscher & Møller-Madsen (1985). Prior to the AMG development, all tissue sections were coated with 0.5% of gelatin solution by dipping the slides in a. The slides, placed in jars located in a 26°C warm water bath, were AMG developed for 60 min. The process was stopped by replacing the AMG developer with 5% sodium thiosulphate solution for 10 min and the slides were rinsed with tap water at 40°C to remove the gelatin. Finally the sections were counterstained with Hematoxylin and Eosin, mounted in Entellan and observed under the Leica® DME light microscope.

Determination of Metallothionein concentration

Liver and posterior kidney were collected, frozen and maintained at -76°C prior to metallothionein determination. For the assay, samples were homogenized in ice-cold buffer (0.5 mM de phenylmethylsulphonylfluoride (PMSF), 0.01% de β-mercaptoethanol, 20 mM Tris–HCl, 500 mM sucrose, pH 8.6), and centrifuged at 15000 g for 40 min at 0°C. Then, 300 µL of supernatant (buffer for the blank) and 342 µL of ethanol-chloroform (13:1; at -20°C) were mixed and centrifuged at 6000 g for 10 min, 0°C. A volume of 490 µL of the new supernatant was mixed with 1502 µL of ethanol-HCl (45:1; at -20°C) and kept at - 20°C during 1 h. After this period, the tubes were centrifuged (6000 g for 10 min and 0°C) and the pelleted proteins were suspended with 50 µL of 250 mM NaCl solution. Next, 50 µL of EDTA solution (4 mM EDTA-2Na, 1 M HCl) and 1000 µL of Ellman solution (2 mM DTNB (5,5'-dithio-bis(2-nitrobenzoic acid), 0.2 M sodium phosphate, 2 M NaCl) were added to the tubes mixed and centrifuged (3000 g for 5 min) for absorbance determination at 412 nm. Sulfhydryl content was determined by comparison to a glutathione (GSH) curve, and metallothionein concentration was calculated considering the metallothionein cysteine content described for mussels of 30% (Viarengo *et al.,* 1997 with minor modifications).

Chemicals

Paraplast, MS222 (ethyl-ester-3-aminobenzoic acid), gelatin and chloroform were purchase from Sigma-Aldrich (MO, USA). β-Mercaptoethanol and phenylmethylsulphonylfluoride (PSMF) were from Fluka (MO, USA). Entellan, ethanol, formol, acetic acid, sodium cacodylate and glutaraldehyde were purchased from Merck (North American, S.A.). Hematoxylin, eosin, sodium thiosulphate, sodium phosphate, DTNB (5,5'-dithio-bis(2-nitrobenzoic acid), (EDTA) thylenediamine tetraacetic acid, gum arabic, sodium citrate, hydroquinone and silver lactate were purchased from VETEC (Duque Caxias, RJ, BR). HCL, NaCl, tris-HCl was obtained from Cristalia Laboratories (SP, BR).

RESULTS

No mortality, signs of mobility changes and skin lesions were observed in the control and experimental groups throughout the experiment.

Analysis of mercury distribution by autometallography

Unaltered hepatic parenchyma with groups of hepatocytes delimiting sinusoids, some biliary ducts and the hepatic vein (Figs. 1A and 1a) and none mercury deposits were observed in control fish. Conversely, mercury deposits were observed in cells of hepatic biliary ducts after inorganic mercury exposure (Fig. 1B), and inside hepatocyte cytoplasm and epithelial cells of biliary ducts after organic mercury exposure (Fig. 1C), indicating differences in the liver uptake and elimination of different mercury compounds.
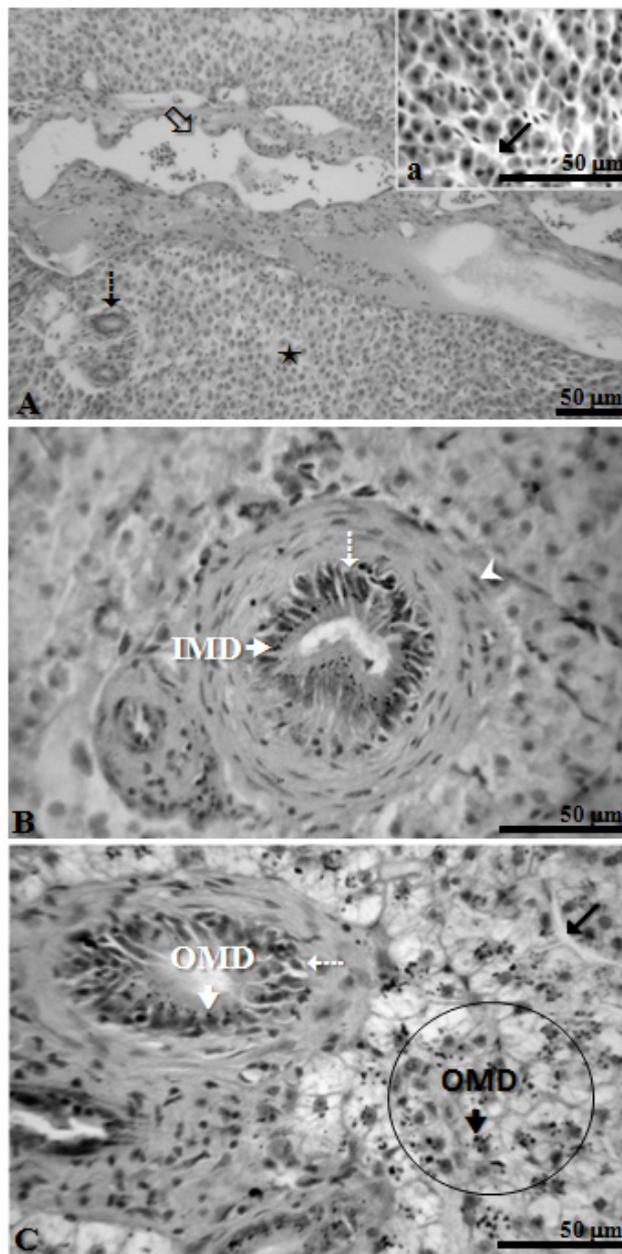

Figure 1 - Autometallography of liver cross-section counterstained with Hematoxylin-eosin. (A) Control group (no mercury was visualized). Hepatic parenchyma (★) with hepatocytes delimiting sinusoids (see detail in "a" (✓)), some biliary ducts (↓) and the hepatic vein (↘). (B) Inorganic mercury deposits (IMD – white arrow) on epithelial cells of biliary ducts (↓) surrounded by connective tissue (◄). (C) Organic mercury deposits inside hepatocyte cytoplasm (OMD – black arrow) and in epithelial cells (OMD – white arrow) of biliary ducts (↓). Sinusoids can be visualized (✓).

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No mercury deposits were observed in the posterior kidney of control fish (Figs. 2A and 2a). Conversely, mercury was visualized mainly in the kidney renal tubules, but not in the glomerulus, with the predominance of inorganic (Fig. 2B) over organic mercury (Fig. 2C) in exposed fish, reflecting kidney preference of inorganic mercury.

Metallothionein in liver and posterior kidney

Metallothionein protein levels in the liver and posterior kidney were not altered by neither mercurial compounds,
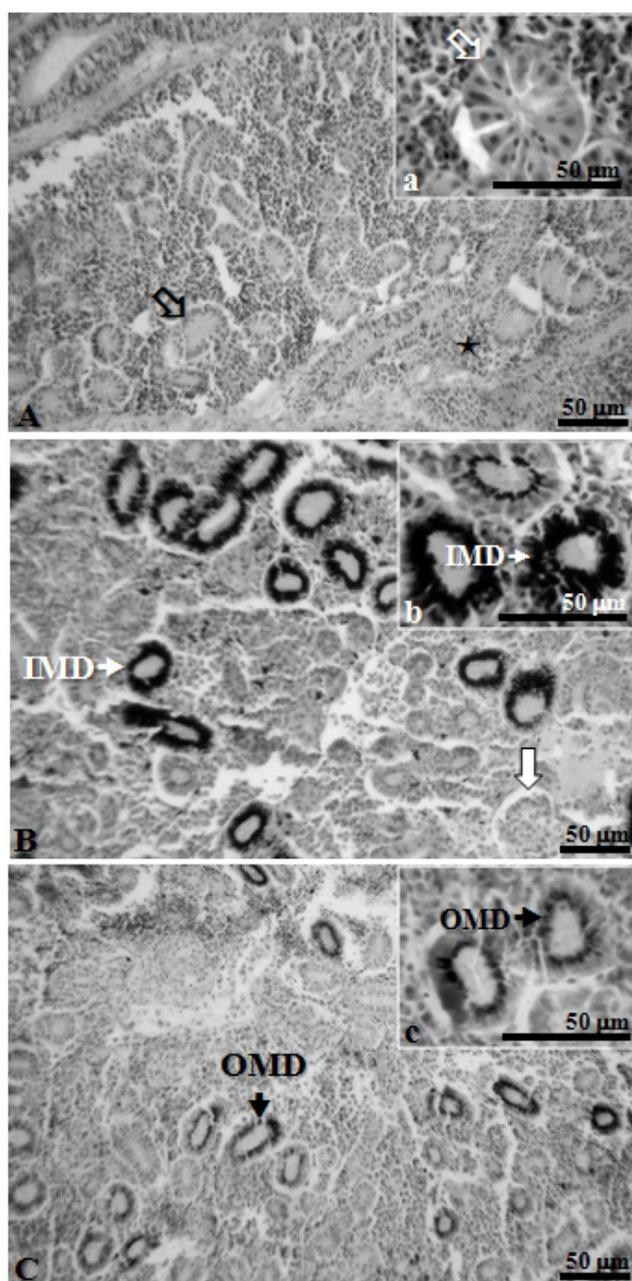

Figure 2 - Autometallography of posterior kidney cross-section counterstained with Hematoxylin-eosin. (A) Control group (no mercury was visualized). Tubules (⇘) (see also detail in "a") and parenchyma (★). (B) Inorganic mercury deposits (IMD – white arrow) almost exclusively in the kidney tubules (see detail in "b"), but not in the glomerulus (⬇). (C) Organic mercury deposits in kidney tubules (OMD – black arrow) (see detail in "c").

even though literature reports the inducing ability of mercury. Metallothionein levels (mean ± SD) was about 17.7± 6.3 µg per mg of proteins in the liver, whereas in posterior kidney metallothionein levels were about 2.5 times lower (7.2 ± 5.6 µg per mg of proteins) (Fig. 3).

DISCUSSION

Autometallography provided new useful information about intracellular distribution of mercury in liver and kidney cells of *C. carpio*. Although it is widely used for determining mercury distribution in mammals (Danscher & Norgaard, 1983; Danscher & Möller-Madsen, 1985; Villegas *et al.,* 1999), data describing liver and kidney mercury localization in fish are scarce (Madsen & Hansen, 1980; Baatrup & Danscher, 1987).

Kidney uptake and accumulation of inorganic mercury are very rapid (Zalups, 1993a) and surpass that of organic mercury (Zalups *et al.,* 1992). Mercury bioaccumulates primarily in the cortex (Zalups, 1993) and in proximal tubules segments of mammal kidneys (Zalups & Barfuss, 1990; Rodier *et al.,* 1988; Zalups, 1991a,b), mostly in endocytic structures and lysosomes (Hulman & Enestrom, 1986). Several findings indicate that a significant mercury fraction in the kidneys of methyl mercury exposed animals is in the inorganic form (Omata *et al.,* 1980; Zalups *et al.,* 1992), suggesting that organic mercury can be converted to inorganic mercury (Dunn & Clarkson, 1980).

Inorganic mercury binds to two GSH, forming GS-Hg-SG complexes, which are enzymatically cleaved in the lumen of the proximal tubule and producing dicysteinyl-Hg complexes (Cys-Hg-Cys), which resemble cystine (Cys-Cys), being absorbed and accumulating in the proximal tubular cells by cystine transporters (Zalups, 2000). This accumulation is responsible for the localization of mercury on proximal tubules of *C. carpio* observed in the current study, particularly for inorganic mercury. Also, mercury-metallothionein conjugates eliminated by the liver can cross the glomerular filtration barrier and so did not accumulated in glomerulus of *C. carpio*.
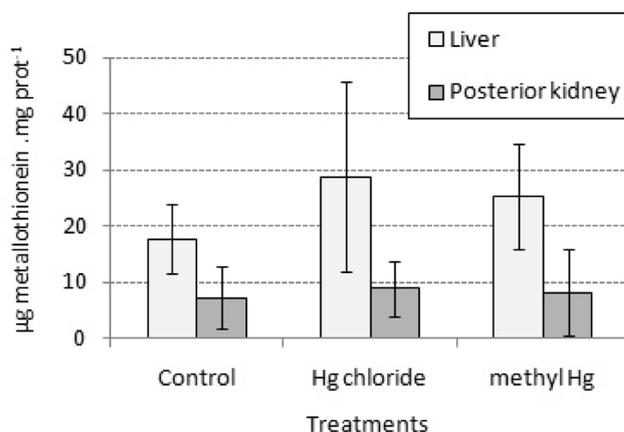

Figure 3 - Metallothionein concentration in liver and kidney of control and exposed fishes to inorganic mercury (Hg chloride) and organic mercury (methyl Hg). Mean (columns) ± standard deviation (SD, bars).

For methyl mercury exposure, formation of GSH conjugates with or without demethylation (i.e., production of MeHg-SG or GS-Hg-SG) might facilitate kidney uptake, although inorganic mercury may still be more available for uptake by kidney in *C. carpio*.

The liver has key importance in toxicological processes, because it is the primary biotransformation site of many xenobiotics. Methyl mercury can easily cross hepatocyte plasma membrane and binds to thiol groups of glutathione, metallothionein or other molecules with different activities, before being eliminated to the bile or blood stream (Ballatori, 1991). In the first case intestine re-absorption (Norseth & Clarkson, 1971) can decrease the capacity of the organism to eliminate mercury, whereas in the latter case, kidney may accumulate the metal. Mercury accumulates mainly in apical domain of hepatocytes, facing the bile canaliculi (Loumbourdis & Danscher, 2004).

After uptake, methylmercury accumulates in hepatocyte lysosomes and can be traced by AMG (Baatrup & Danscher, 1987). According to Alvarado *et al.* (2005), lysosomal metal sequestering is considered a general strategy for reducing metal toxicity. Then, methyl mercury deposits in the cytoplasm of liver and kidney cells of *C. carpio* corroborate data of others authors (Olsson *et al.,* 1998; Baatrup & Danscher 1987).

Based on metallothionein levels and its physiological roles on metal homeostasis, and on distribution and accumulation in *C. carpio* it is more likely that the mercury toxic effects after feeding exposure may be worse in kidney than in liver

The AMG method used in the current study is specific and very sensitive (Hörsted-Bindslev *et al.,* 1997), being extremely useful to demonstrate the inorganic and methyl mercury organ-tropism and tissue localization in *C. carpio*. Furthermore, metallothionein determination was not a good indicator of mercury exposure and bioaccumulation in this species. Finally, methyl mercury and inorganic mercury bioaccumulates preferentially on kidney, whereas hepatocytes bioaccumulates mainly methyl mercury at lowest extent.

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