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Bioaccumulation of metals and induction of metallothioneins in selected tissues of common carp (*Cyprinus carpio L.*) co-exposed to cadmium, mercury and lead

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The concentrations of mercury (Hg), cadmium (Cd) and lead (Pb) at various exposure periods were determined in the gill, kidney, liver and muscle of common carp (*Cyprinus carpio L.*) co-exposed to 1.0 μg ml^{-1} each of Cd^{2+}, Hg^{2+} and Pb^{2+} for up to 10 days. Metallothionein fractions (MTs) in these organs were characterized using the hyphenated technique of size-exclusion chromatography (SEC) and inductively coupled plasma mass spectrometry (ICP-MS). After 10 days of exposure, maximum toxic metal concentrations of Hg, Cd and Pb were 10.7 (gill), 0.145 (kidney) and 0.112 μg g$^{-1}_{\text{dry weight}}$ (gill), respectively. The pattern of accumulation of Hg and Pb was in the order gill > kidney > liver > muscle. In the case of Cd, accumulation was in the order kidney > gill > liver > muscle. Cd and Hg binding MTs were significantly induced in the gill, kidney and liver of all the exposure groups in comparison with the control group ($p < 0.05$), and the amounts of them increased with the longer exposure time. Despite the higher intracellular Hg concentration and the stronger Hg–SH binding affinity, the amount of Cd-binding MTs was much higher than that of Hg-binding MTs. The results indicate that MT synthesis in these organs was clearly metal-specific. MTs in gill may be used as a bio-marker to detect the metal pollution caused by Hg and Cd. Zinc and copper binding MTs in the organs of the exposed fish were also increased. This may be due to the MTs' important role in the homeostatic regulation of essential metals and their protective role against the acute toxicity of non-essential metals. Even though there was considerable accumulation of lead in the organs of the exposed fish, Pb-binding MT synthesis was non-significant. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: carp (*Cyprinus carpio L.*); multi-metal binding metallothioneins; size-exclusion chromatography (SEC); inductively coupled plasma mass spectrometry (ICP-MS)

INTRODUCTION

Toxic metals including cadmium (Cd), mercury (Hg) and lead (Pb) are well-known environmental contaminants that are mostly industrial or anthropogenic in origin, and exert significant chronic toxicity in vertebrates.[1] Accumulations and bio-magnifications of toxic metals in tissues of aquatic organisms have recently received considerable attention,[2,3] and bio-indicators such as fish are useful tools to assess the availability of these pollutants. Toxic metals can enter the body of fish through different routes, such as gill, skin and digestive tract. However, the accumulation and distribution patterns of toxic metals in fish tissues are dependent on their uptake and elimination rates.[4]

Metallothioneins (MTs) constitute an important metal-binding proteins with low molecular weight (6–7 kDa) and

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high cysteine content (>30%). They are known to be inducible by heavy metals and are able to bind with metals in fish tissues. MTs are reported to play important roles in the detoxification of toxic metals and in the maintenance of homeostasis of essential metals like zinc (Zn) and copper (Cu), and may be used as a biomarker to assess the toxicity of toxic metals.[5]

Responses of fish to single toxic metals exposures are well documented. However, relatively limited studies have been conducted to analyze the bioaccumulation of toxic metals and induction of MTs in fish tissues in the co-exposure mode of waterborne toxic metals. In the present investigation, attempts have been made to analyze the patterns of accumulation of Cd, Hg and Pb in different tissues, i.e. gill, liver, kidney and muscle of common carp (*Cyprinus carpio L.*) exposed to a mixture of these metals. Efforts have also been made to detect multi-metal binding MTs using hyphenated analytical technique of size-exclusion chromatography (SEC) and inductively coupled plasma mass spectrometry (ICP-MS).

EXPERIMENTAL

Apparatus and reagents

The ICP-MS instrument used was a HP4500 (Agilent, USA). The sample introduction system included a glass double-path spray chamber fitted with a concentric nebulizer. Nickel sampler and skimmer cones were used for all experiments. HPLC was a HP Model 1100 system equipped with a UV diode array detector (DAD) (Agilent, USA). Injection was performed using a model 7725 injection valve with a 20 μl injection loop (Rheodyne, CA, USA). The combination of SEC-ICP-MS was accomplished by connecting the outlet of the UV detector of HPLC into the inlet of ICP nebulizer using a Teflon tubing (0.5 mm, i.d.). The instrumental conditions of hyphenated technique were similar to our previous report.[6] The commercial SEC column was G3000PWxl (7.8 mm i.d. × 300 mm, 6 μm; TSK-gel, Japan). The column was calibrated with bovine albumin (66000 Da), rabbit liver MT-2 (6800 Da), cobalamin (1355.4 Da) and cysteine (121.15 Da) as exclusion volume markers. A 30 mmol l^{-1} buffer used for HPLC was prepared with Tris(hydroxymethyl) aminomethane (Shanghai, China) containing 5 mmol l^{-1} of 1,4-dithiothreitol (DTT), and the pH was adjusted to 7.8 with 6 mol l^{-1} HCl (GR grade).

The microwave-assisted digestion oven used to digest all of the samples was a MK-III fitted with an optical fiber pressure controlling system (manufactured by the Institute of Xinco Microwave Technology, Shanghai, China).

MT standards (MT − 1 : MT − 2 = 1 : 1), from rabbit liver induced with cadmium and zinc were purchased from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany) and Lugu Biotechnology Corporation Ltd (Hunan, China), respectively. Cadmium chloride (A.R.) and mercury chloride (A.R.) were the products of Beijing Hongxing Chemical Plant (Beijing, China), and lead nitrate (A.R.) was purchased from Shanghai Jinshan Chemical Plant (Shanghai, China). The 65% HNO_3 (product of Beijing Reagent Factory, Beijing, China) was a high purity grade. All the other chemicals used were AR grade. An ultra-pure water system (0.055 μS cm^{-1}) (TKA-Genpure, Germany) was used throughout.

Bio-assay and sampling

Twenty-five healthy carp weighing 50 ± 10 g (about 15 cm length) were purchased from the Aquaculture Farm of Xiamen, China. Fish were kept in two tanks (1.3 × 0.3 × 0.8 m) at 20 ± 5 °C and 12 : 12 h light : dark cycle. After being tamed for a week for acclimation, 10 fish randomly selected were kept in one tank with tap water, and referred to as the control group. Another 15 fish kept in another tank were exposed to tap water containing 1.0 μg ml^{-1} of Cd^{2+}, Hg^{2+} and Pb^{2+} (in the form of cadmium chloride, mercury chloride and lead nitrate) for 2, 5 and 10 days, and five of the fish were randomly sampled at each sampling period and named groups I–III, respectively. In order to counterbalance the metal concentrations in the tank, two-thirds of water in the tank was renewed every 48 h by adding fresh water containing the same concentrations of the three toxic metals. All fish in both tanks were fed with commercial pellet feed floating on water surface at a ration of 1% of their body weight every other day. Air bubbles were continuously pumped into water in the tanks to promote soluble oxygen throughout the experiments. The sampled fish were subjected to total extraction of blood from the pericardium antrum. Then the fish were sacrificed and the gill, liver, kidney and muscle were dissected. The dissected tissues were washed with ultra-pure water, cleaned, dried on filter paper, weighed and stored at −20 °C.

Tissue preparations

Stored tissues were thawed and homogenized with buffer (30 mmol l^{-1} Tris–HCl, pH 7.8 containing 5 mmol l^{-1} DTT; 1 : 3, w/v) in a glass homogenizer. The homogenized extract was transferred to 15 ml tubes and centrifuged at 6000 rpm 20 min with a Sigma 2–16 centrifuge (Sartorius, Germany). The supernatant was heated at 85 °C for 10 min, and then cooled in ice-cold water for 5 min. It was then centrifuged again at 15000 rpm for 10 min. The supernatant collected in an Eppendorf tube was stored at −20 °C for further SEC separation.

Heavy metal measurement

For metal quantification, the frozen samples were lyophilized directly for 24 h to obtain the dry weights. The dried tissues were digested and analyzed as the method described in our previous report.[5]

Analysis procedures of MTs

A sample of 20 μl of supernatant was introduced to the SEC column and eluted with Tris–HCl buffer (pH 7.8) at a flow rate of 1.0 ml min^{-1}. The eluent from the SEC column was

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first monitored with a DAD detector at wavelength of 225 nm, then fed directly into ICP-MS in which the signals of ^{202}Hg, ^{111}Cd, ^{208}Pb, ^{66}Zn and ^{63}Cu were monitored simultaneously. Zn and Cu originating from the tissues were closely related to the synthesis of metal-binding MTs in the exposure period. The signals of Zn and Cu were also recorded.

Statistical analysis

Statistical analysis was performed using SPSS 10.0 software. Differences were analyzed by a one-way ANOVA with *post hoc* multiple comparisons and two-way ANOVA with GLM multivariate method.

RESULTS AND DISCUSSION

Distribution of toxic metals in fish tissues

Concentrations of Cd, Hg and Pb in the various tissues determined with ICP-MS are shown in Table 1.

The distribution patterns of Hg, Cd and Pb in the tissues of exposed groups indicate that all three toxic metals accumulate significantly in these tissues when compared with the control one in which the toxic metals are below detection limits. However, the pattern of accumulation varies from metal to metal in various tissues at different exposure periods. For example, the concentrations of three toxic metals in group II and III increased significantly in comparison with group I at $p < 0.05$ for all organs, but the concentrations of Cd (in the gills and kidneys), Hg (in the livers) and Pb (in the gills and livers) in group III did not significantly increase (some even decreased) $p < 0.05$) in comparison with that of group II. It seems that the accumulations of toxic metals in the tissues have saturation levels. The accumulation of mercury in the same tissues of each exposed groups was significantly higher than that of cadmium and lead at $p < 0.05$, such as the gills and kidneys. On the other hand, the pattern of accumulation varies from organ to organ for the same metal. For example, mercury concentrations in the gill and kidney were significantly higher when compared with other organs at $p < 0.05$ for the same group, but there was no significant differences between the gills and kidneys. The observed maximum toxic metal concentrations were 10.7 $\mu g\ g^{-1}$ Hg in gill (after 10 days), 0.145 $\mu g\ g^{-1}$ Cd in kidney (after 10 days) and 0.123 $\mu g\ g^{-1}$ Pb in gill (after 5 days), respectively. The orders of Hg and Pb accumulation in group III were gill > kidney > liver > muscle. In the case of Cd, the order of accumulation in group III was kidney > gill > liver > muscle. The concentrations of Cd and Pb in the muscles of all exposed groups were well below the detection limits. However, the concentration of Hg in the muscles was much higher than that of the control group, and increased with the longer exposure time. The results indicate that Hg is more easily accumulated in all tissues than Cd and Pb when carp is co-exposed to these toxic metals. For example, Hg content in the gills of group III was 78.7 and 95.5 times higher than that of Cd and Pb. The results also suggest the lower rate of accumulation of Pb in these tissues in comparison with Hg and Cd under the same exposure conditions.

Many of the available reports deal with experiments conducted to investigate the effect of one or double waterborne metal exposures. According to Hollis *et al.*,[7] kidneys accumulated the maximum concentration of Cd, followed by gills, liver and the whole body in the case of juvenile *Oncorhynchus mykiss* exposed to 0.3 $\mu g\ ml^{-1}$ Cd for 30 days. De Smet *et al.*[8] found the order of accumulation of Cd in *C. carpio* exposed to 0.06–0.79 $\mu g\ ml^{-1}$ of Cd for 29 days

Table 1. Toxic metal contents in tissues of each fish groups ($\mu g\ g^{-1}_{dry\ weight}$)

Tissues	Toxic metals	Control group	Group I	Group II	Group III
Muscle	Cd	nd	nd	nd	nd
	Hg	nd[a]	$0.020 \pm 0.007^{a,h}$	$0.36 \pm 0.07^{b,h}$	$0.76 \pm 0.06^{c,h}$
	Pb	nd[a]	nd	nd	nd
Gill	Cd	nd[a]	$0.011 \pm 0.001^{a,f}$	$0.18 \pm 0.01^{b,f}$	$0.14 \pm 0.00^{c,f}$
	Hg	nd[a]	$1.6 \pm 0.1^{b,e,g}$	$7.9 \pm 0.0^{c,e,g}$	$11 \pm 1^{d,e,g}$
	Pb	nd[a]	$0.007 \pm 0.005^{a,f}$	$0.12 \pm 0.01^{b,f}$	$0.11 \pm 0.00^{b,f}$
Liver	Cd	nd[a]	0.018 ± 0.003^{b}	0.023 ± 0.002^{c}	0.05 ± 0.00^{d}
	Hg	nd[a]	$0.074 \pm 0.002^{a,h}$	$2.0 \pm 0.1^{b,h}$	$1.3 \pm 0.1^{c,h}$
	Pb	nd[a]	0.0010 ± 0.0004^{a}	0.0031 ± 0.0008^{b}	0.0042 ± 0.0003^{b}
Kidney	Cd	nd[a]	$0.028 \pm 0.000^{b,f}$	$0.13 \pm 0.01^{c,f}$	$0.14 \pm 0.01^{c,f}$
	Hg	nd[a]	$0.12 \pm 0.01^{a,e,g}$	$4.4 \pm 0.2^{b,e,g}$	$6.0 \pm 0.3^{c,e,g}$
	Pb	nd[a]	$0.0026 \pm 0.0001^{a,f}$	$0.0071 \pm 0.0003^{b,f}$	$0.013 \pm 0.004^{c,f}$

* Values are expressed as: mean ± SD ($n = 3$);
** nd: below detection limit;
*** a∼d: Significant differences ($p < 0.01$) of metal content in the same tissue in different exposure time analyzed by one-way ANOVA; e∼f: Significant differences ($p < 0.05$) of Hg contents compared with Cd and Pb in the same tissue analyzed by two-way ANOVA; g∼h: Significant differences ($p < 0.05$) of Hg contents in the gills compared with other tissues in the same group analyzed by two-way ANOVA.

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to be kidney > liver > gill. According to De Conto Cinier *et al.*,[9] in *C. carpio* exposed to 450 μg l^{-1} of Cd for 140 days, the Cd uptake varied from organs to organs and, when the storage limits of liver and kidney were reached, Cd started accumulating in muscle. A similar phenomenon was also observed in the present experiment.

Significant accumulation in tissues of fish exposed to waterborne Hg^{2+} is well known. For example, Elia *et al.*[10] found that the order of accumulation of mercury in freshwater *Ictalurus melas* exposed directly to 35–140 μg l^{-1} of Hg for 10 days was gill > kidney > liver > muscle. The accumulation pattern of Hg was to great extent in agreement with the present results. Another interesting result is that the accumulation of one metal in fish co-exposed to more than one metal has been reported to be affected by the coexisting metals. For example, the accumulation of Pb was greatly affected by Cd, which resulted in the decrease in the gills and increase in the liver of *Carassius auratus* co-exposed to 0.5 μg ml^{-1} of Cd and Pb for 10 days.[11] However, similar results were not observed in the present experiment, in which accumulation of Pb in the gill was much higher than that in the liver and kidney.

It can be concluded that metal accumulation in the tissues of fish may be influenced by many factors, such as the species of exposure metal ion, metal concentration and exposure time. In addition, temperature, salinity, hardness and pH of water would affect the accumulation levels of metal.[12]

Chromatograms of multi-metal binding MTs by on-line SEC-ICP-MS

Chromatographic profiles of the multi-metal binding MT fractions detected by ICP-MS in the gill of *C. carpio* belonging to group III and the control groups are given in Fig. 1(a, b). In the corresponding UV chromatogram (Fig. 2), the MT fractions' peak is observed at the retention time (t_R) 6.83 min. The absence of a peak in the UV spectrum at 280 nm may be due to the lack of aromatic amino acids in the molecules.[13] The standard MTs also show chromatographic profiles similar to the experimental samples except for a small difference in retention time. Induction with different metals in different organs may be the reason for this difference.[6]

The chromatographic profiles of the MTs in the gill of the fish belonging to experimental group III [Fig. 1(a)] indicate that Cd and Hg binding MTs are significantly induced when compared with the control group. Interestingly, the amount of Cd-binding MT is 2 times higher than the Hg-binding MT. However, it is contradictory to the rate of metal accumulation (Table 1). This contradiction indicates that Cd accumulated in the gills is easy to bind with MTs which play a significant role of detoxification to this toxic metal. It has been well validated that the affinity of Hg binding with MTs in the organism of vertebrate animals is much stronger than that of Cd.[14] The high concentration of Hg in gill is hypothesized to induce a large amount of Hg-binding MT for detoxification. However, the synthesis of Hg-binding MT in the gill is not proportional to the rate of accumulation of Hg. The reason

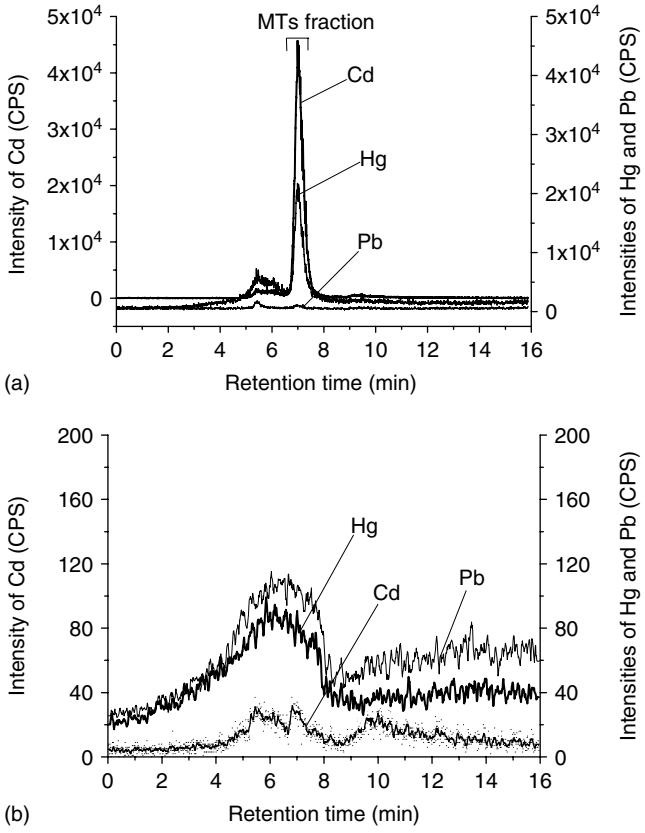

Figure 1. Chromatographic profiles of Cd, Hg and Pb-binding MT fractions in the gills of *C. carpio.*(a) Experimental group III; (b) control group.

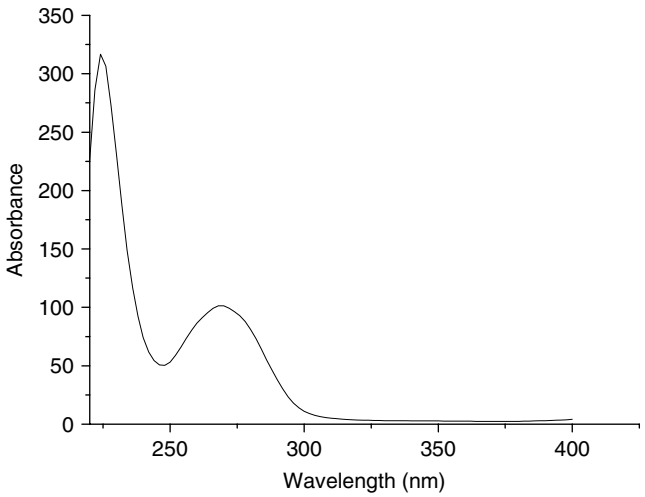

Figure 2. Spectrum of MT fractions in the gills of *C. carpio* in experimental group III.

may be interpreted that the detoxification role played in the organism is not only inducing MTs. Actually, the Hg-binding proteins (such as MTs, stress proteins, etc.) and non-proteins

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(such as glutathione, etc.) may be simultaneously operated in the detoxification against the metal. For example, the protection of cells against Hg toxicity could be attributed to the conjugations by Hg–SH such as metallothionein and glutathione.[15] For another example, it is common that mercury in organs is easy to bind with the high molecular weight fractions. But most of the mercury-binding proteins would have been lost at the heat denaturation step (85 °C for 10 min) during the sample preparation. However, cadmium in tissues is one of the only metals that nearly exclusively partitions to the MT fraction that is one of the only proteins that can survive the heat denaturation step. Therefore, only partial Hg in the gill exists in the form of Hg–MTs, and the remaining Hg is redistributed in the tissue and bound with other molecules. It can be concluded that metal-binding MTs synthesis in fish gill is clearly metal-specific.

Few of Pb-binding MTs are synthesized in the gill of fish in group III [Fig. 1(a)], although Pb is significantly accumulated (Table 1). It may be inferred that the combination of Pb with MTs through metal–thiolate bonds is infirm, and this has been validated by previous studies.[16,17] In their experiment, Pb *in vitro* was found to not easily form Pb_7-MTs by replacing Zn in the Zn_7–MTs which naturally exist in vertebrate animals.

Zinc (Zn) and copper (Cu) in the tissues of *C. carpio* of all experimental groups and control group mainly come from fry and feed. The original Zn and Cu may exist as the binding forms of Zn/Cu-MTs in fish tissues like other vertebrates. It

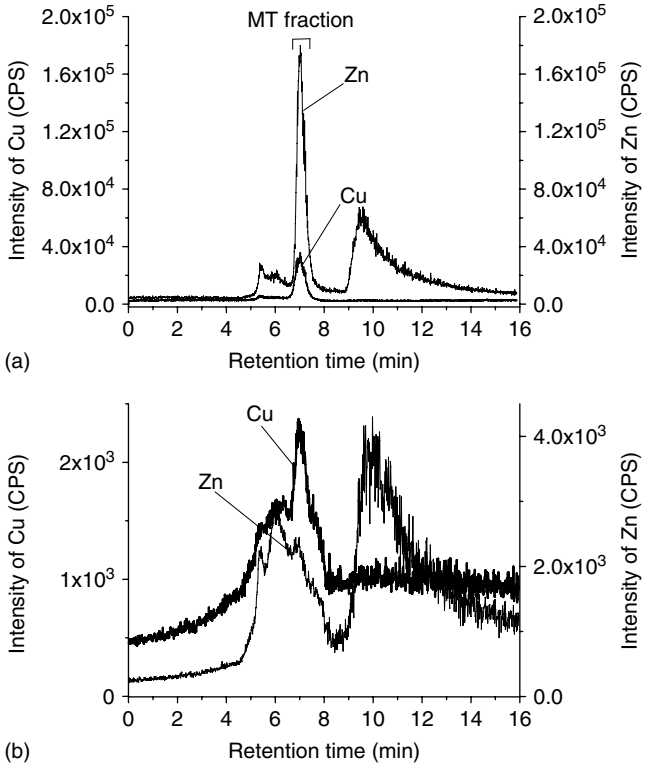

Figure 3. Chromatographic profiles of essential metal-binding MT fractions in the gills of *C. carpio.* (a) Experimental group III; (b) control group.

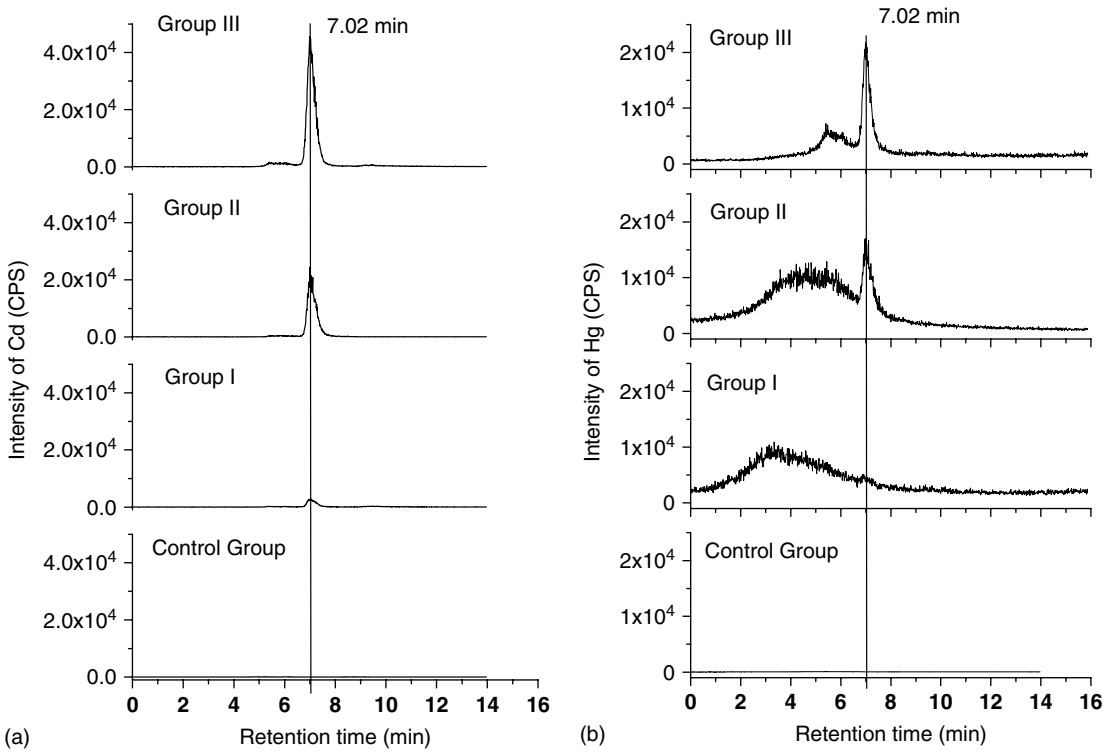

Figure 4. Formation of Cd and Hg binding MTs in the of gills of *C. carpio* belonging to control and experimental groups: (a) Cd-MTs; (b) Hg-MTs.

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has been validated that the role of MTs in detoxification against Hg and Cd is to form Hg/Cd binding MTs by substituting Zn and/or Cu from Zn/Cu-binding MTs in tissues. Therefore, the Zn/Cu-binding MTs in tissues are very important for the detoxification against toxic metals. Figure 3 shows that Zn, Cu-binding MTs in the gills of experimental group III are significantly synthesized compared with that of the control. For example, the amounts of Zn-binding MTs and Cu-binding MTs were 20 and 11 times higher than those of the control group. Additionally, the increase of Zn, Cu-binding MTs is also observed in other tissues of treated groups. A similar result was observed in the mammalian animals such as rats in our previous report.[6] It may be assumed that MTs *in vivo* play an important role in the homeostatic regulation of essential metals such as Zn and Cu because Zn/Cu-binding MTs are generally considered as the storage sites for toxic metal ions such as Cd^{2+} and Hg^{2+}. Thus, MTs play a protective role against the toxicity of non-essential metals.[18,19]

In the present experiment, the quantity of Cd/Hg binding metallothionein fractions increases with the duration of exposure (Fig. 4). For example, the amounts of Cd-binding MTs formed in group II and group III were 7.9 and 14.6 times more than that of group I. Similarly, the amounts of Hg-binding MTs in group II and group III were 3.6 and 4.7 times more than that of group I. The results suggest that gill may significantly accumulate Cd and Hg, and synthesize many Cd/Hg-binding MTs, which play a role of detoxification against the toxic metals. Therefore, MTs in the gills may be used as a marker to reflect the toxic metal pollution.

Studies on MTs in fish exposed to waterborne toxic metals have been mainly restricted to single metal exposure.[9,20–22] To our knowledge, there have been few reports on MT induction in fish exposed to a mixture of metals, such as Cd, Hg and Pb. De Smet *et al*.[8] observed that Cd, Zn–MT concentrations increased in the kidney of *C. carpio* exposed to 20 μM of cadmium for 4 days, while increases were found in the gills for 29 days. Also, no significant changes of Cd, Zn-MT levels in liver occurred. Similar results observed by Cosson *et al*.[23,24] were that there was an increase (14%) of MTs in gills but no significant increase in the liver of *C. carpio* exposed to 1 μg ml^{-1} Cd for 1 week. Similar results are also observed in our experiment, with the concentration order gill > kidney > liver for Cd-binding MTs in experimental group III. However, the concentration pattern of Hg-binding MTs in the same experimental group is different from Cd-binding MTs. For example, the order of quantity of Hg-binding MTs in exposed group III was kidney > liver > gill. The results imply that MT synthesis in fish organs varies with the different intracellular toxic metals. It should be noted that MTs synthesis in fish organs is influenced by other factors, such as the mixture of exposure metal ions and the relative concentrations of metals.[8]

The induced Cd/Hg-binding MTs in the liver and kidney of *C. carpio* are also analyzed and discussed. The results in Fig. 5 indicate that the amounts of Cd/Hg-binding MTs in the liver and kidney of all the experimental groups are significantly

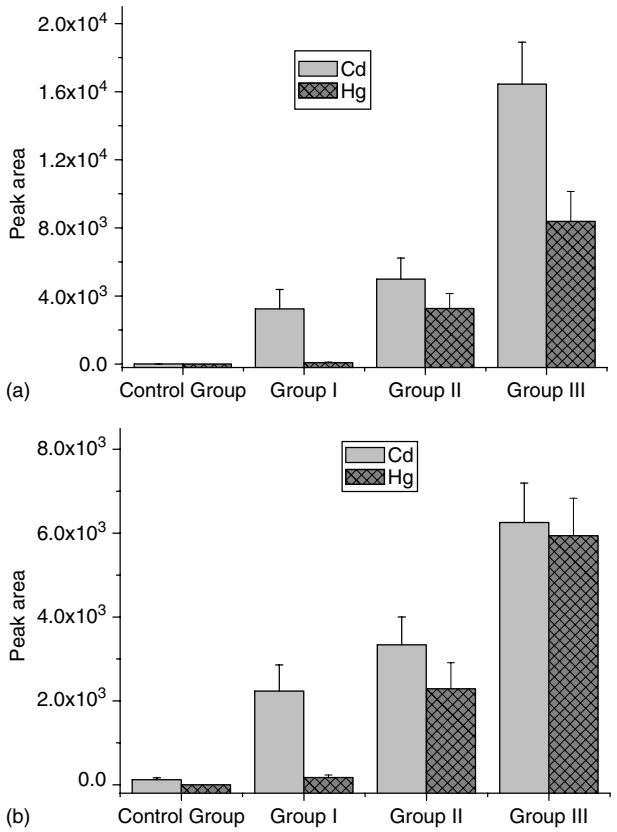

Figure 5. Peak areas of Cd- and Hg-binding MTs in different tissues of *C. carpio*: (a) kidney; (b) liver. Significant differences ($p < 0.05$) of Hg and Cd-binding MTs among the different groups in kidney and liver, respectively.

higher than those of the control group ($p < 0.05$), and the quantity of Cd/Hg-binding MTs induced in the two organs increases with the exposure time. In addition, the amounts of Cd/Hg-binding MTs in the kidney are higher than those in the liver for the same exposure period. For example, the amounts of Cd-binding MTs and Hg-binding MTs in the kidney of experimental group III are 2.24 and 2.28 times higher than those in the liver, respectively. The results show that Cd and Hg are easily transferred and accumulated in the kidney during the metabolizing process, and the induced Hg/Cd-binding MTs are one of the important ways for detoxification against toxic metals.

Another interesting result observed is that heavy metals including Cd, Hg and Pb are found to bind with the high molecular weight proteins (HMWP) in the gills of the exposed fish at 5.43 min retention time [Fig. 1(a)]. The metal-binding HMWP is also observed in the kidney and liver of the exposed fish. Similar results were previously reported by Kito *et al*.,[25] that cadmium in the gills and kidneys of carp exposed to 5 mg l^{-1} Cd for 31 days was able to bind with HMWP during the earlier periods of exposure and then with MTs in the later stages. The metal-binding HMWP is thermally stable because the sample is heated at 85 °C for 10 min in the preparing

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procedure, and this may be attributed to the product of metallothionein's polymerization due to the oxidation of MTs, according to our previous report.[26]

CONCLUSION

It can be concluded that fish exposed to a mixture of toxic metals such as Hg, Cd and Pb accumulate these heavy metals in tissues like gill, kidney, liver and muscle (except for Cd and Pb in muscle). The metal concentrations in the tissues depend on different kinds of toxic metals and different exposure times. Metal-binding MTs can be significantly induced in the gill, kidney and liver of the exposed groups in comparison with the control group. Despite the higher intracellular Hg contents and the stronger binding affinity of Hg–SH, the amount of Cd-binding MTs is much higher than that of Hg-binding MTs in the tissues of exposed fish. The results indicate that MTs synthesis in fish tissues is clearly metal-specific. MTs in gill may be used as a marker to reflect the pollution caused by Hg and Cd.

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