Quantification of metallothionein on the liver and kidney of rats by subchronic lead and cadmium in combination

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Abstract

The combined subchronic effects of exposure to lead acetate and cadmium chloride on oxidative stress and metallothionein (MT) gene expression were detected in the liver and kidney of rats to investigate the hazards of environmentally relevant, low-dose exposure to these compounds. Pb and Cd co-induced oxidative stress in liver and kidney tissues. This result was indicated by a significant ($P < 0.01$) increase in the maleic dialdehyde level and decreased levels of reduced glutathione, superoxide dismutase, catalase, and glutathione peroxidase. MT mRNA and protein significantly increased ($P < 0.01$) in the liver and kidney of rats. Furthermore, the expression levels of MT-1 mRNA and MT-2 mRNA differed between the liver and kidney. The findings indicate that Pb combined with Cd induced oxidative damage in the liver and kidney of rats, and MT may be a biochemical environmental indicator.

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1. Introduction

The Agency for Toxic Substances and Disease Registry classifies lead (Pb) and cadmium (Cd) as nonessential environmental pollutants and toxic compounds, which enter the environment because of natural and human-related activities (Bajpai and Upreti, 2012; Mol, 2011). The two elements are often released into the environment because of their wide industrial uses and untreated sewage from chemical plants. These elements then become concentrated in the environment. Thus, particular interest has been devoted to the safety of public food supplies for human health (Bajpai and Upreti, 2012; Iwegbue, 2011). Given that the liver and kidneys are the major targets of Pb and Cd, these elements are highly dangerous to hepatic and renal function. Furthermore, the synergism between the toxic effects of Pb and Cd has stimulated interest in the study of toxic substances in the biological system (Wang et al., 2010).

Pb and Cd indirectly contribute to oxidative stress (Winiarska Mieczan, 2013). Although specific differences in the toxicities of Pb and Cd may be related to differences in solubility, absorbability, transport, chemical activity, and the complexes formed within the body, evidence suggests...
that one of the basic mechanisms involved in metal-induced toxicity is via the increased level of lipid peroxidation (LP) (Stohs and Bagchi, 1995). Moreover, Pb/Cd inhibits the activities of the antioxidant defense system, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as reduction of glutathione (GSH), which will increase the susceptibility of cells to free radical-induced toxicity (Bolin et al., 2006; Kara et al., 2005). Oxidative stress has been suggested to contribute to Pb/Cd-associated tissue injury in the liver, kidney, brain, and other organs (Pandya et al., 2012; Patra et al., 2001; Winiarska Mieczan, 2013).

Susceptibility to Cd toxicity increases in animals that are unable to synthesize metallothionein (MT). MTs have a molecular weight of approximately 6–7 kDa (apparent molecular weight of 13 kDa), and are characterized by their ability to bind metals in metallothionein clusters (Cd and Hg) (Lacorn et al., 2009). When the Cd content in the liver and kidneys exceeds the binding capability of MT, the non-MT-bound Cd ions cause hepato- and nephrotoxicity (Goyer et al., 1989; Liu et al., 2009). However, minimal information is available on the mechanism of MT induction by Pb compounds. Although MT may function in Pb detoxification, the administration of Pb does not affect MT levels or distribution in the liver and kidney alone (Salińska et al., 2012; Yu et al., 2009). Furthermore, MT functions as an antioxidant agent to prevent tissues from oxidative damage (Klaassen et al., 1999).

In the majority of previous animal studies, only one type of metal has been used in high concentration. However, in the environment, a population receives simultaneous multiple exposures (Teuschler and Hertzberg, 1995). Thus, further experiments are needed on combinations of toxic metals. Historically, numerous models have been explored to predict the potential combined effects of chemicals for an individual species or population group. These models, especially concentration addition and independent action, have been applied to numerous mixtures (Spurgeon et al., 2010).

In our previous research, Pb(NO$_3$)$_2$ and CdCl$_2$·2.5H$_2$O were used in an equitoxic mixture ratio design. The fixed mixture ratio was based on the single toxicant LD$_{50}$-value (median lethal dose value) for an oral acute study (Lu et al., 2012). The result showed that Pb and Cd with the mixture toxicant LD$_{50}$-value of 2696.54 mg/kg also have an additive effect for rat models. In this study, we chose low-dose exposure via the subchronic experiment because ingestion is the most important route of human and animal exposure that naturally occurs in the environment, and pollutants emanate mainly from industrial sources. The experimental model of rats treated with relatively low Pb and Cd levels were based on the mixture toxicant LD$_{50}$-value of our oral acute study for a 90 d period as a model of Pb/Cd-induced subchronic toxicological evaluation.

The objective of this study was to examine the effects of simultaneous exposure to Pb and Cd on the antioxidant defense system, and determine their functions in the discrepancies of MT mRNA and protein synthesis in liver and kidney of rats at exposure levels without anticipated overt clinical indicators of toxicity.

2. Materials and methods

2.1. Reagents and antibodies

Lead acetate [Pb(NO$_3$)$_2$] and cadmium chloride (CdCl$_2$·2.5H$_2$O), which were of analytical grade (AR > 99.0%), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pb and Cd calibration standard solutions for inductively coupled plasma spectrometry (ICP, GSB04-1766-2004) were provided by the National Research Center for Certified Reference Materials (Beijing, China). Nitric acid and hydrogen dioxide (guaranteed reagent [GR] grade) were obtained from Xilong Chemical Co., Ltd. (Chengdu, China). CAT (A007-1), SOD (A001-1), GSH-Px (A005), MDA (A003-1), and GSH (A006) reagent kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Anti-MT antibody (UC1MT) and EXPOSE Mouse Specific HRP/DAB Detection IHC kit for immunohistochemistry (IHC) were purchased from Abcam Ltd. (Hong Kong, China). Other reagents, which were of the highest quality available, were obtained from commercial sources.

2.2. Study design

This study was performed in accordance with the following references: (A) Food and Drug Administration Redbook (FDAN, 2000): Chapter IV.C.4.a Subchronic Toxicity Studies with Rodents; (B) Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals, Section 4 Health Effects (Part 408) (OECD, 1998): Repeated Dose 90 d Oral Toxicity Study in Rodents; and (C) Chinese Center for Disease Control and Prevention (CDC, 2008): chemical-test method of repeated-dose oral toxicity study in rodents. The study was conducted in accordance with the OECD principles of Good Laboratory Practice.

2.3. Experimental animals

Four-week-old male and female specific pathogen-free Sprague–Dawley rats weighing 100 ± 5 g were purchased from the Chengdu Dossy Experimental Animals Co., Ltd. (License No.SCXK (Sichuan) 2008-24, China), and kept in the animal house at the Sichuan Agriculture University (Ya’an, China). Rats were housed separately in the laboratory animal house at 20–25 °C, 50–60% humidity, and a 12 h light/dark cycle with the lights off at 7 PM. These conditions were based on the Guidelines of the International Committee on Laboratory Animals. Finally, rats were fed with a standard diet from Nuvital Nutrients (Colombo/PR, Brazil), allowed access to distilled water ad libitum, and had been acclimated to laboratory conditions for 7 d.

2.4. Treatments

Three groups of 20 rats, each group containing 10 females and 10 males, consumed a daily dose of 29.96 (Group II, 29.25 ± 0.71), 89.88 (Group III, 87.74 ± 2.14), and 269.65 (Group IV, 263.23 ± 6.42) mg/kg body weight (b.w.) with a mixture of Pb(NO$_3$)$_2$ and CdCl$_2$·2.5H$_2$O for at least 90 consecutive days. In each case, the product volume administered by gavage was
10 mL/kg b.w. A vehicle-control group (Group I) formed by 20 rats consumed distilled water as drinking water during a 90 d period.

After the exposure period (90 d), rats were anesthetized with diethyl ether. Whole blood samples were collected from the femoral vein and placed into ion-free tubes treated with 10% nitric acid for Pb and Cd analyses. Rats that fasted were sacrificed by cervical decapitation under ether anesthesia, and their livers and kidneys were rapidly removed and stored on ice. Some tissues were minced and homogenized (10%, w/v) in appropriate buffer (PBS, pH 7.4), and then centrifuged (3000 rpm for 10 min, 4 °C). The resulting clear supernatant was used for various enzymatic and non-enzymatic biochemical assays. Then, plastic tweezers were used immediately to dissect and separate the liver and renal cortex for quantitative real-time polymerase chain reaction (qRT–PCR). In each group, six rats (three males and three females) were anesthetized and perfused through the left ventricle with 300 mL of 0.9% saline preheated at 37 °C, followed by 500 mL of ice-cold, freshly made 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for IHC.

2.5. Analysis of Pb and Cd contents

The collected whole blood samples were digested with 10% HNO₃ (1 mL of blood: 2.5 mL of HNO₃, GR grade, ≥65%, Xilong, Chengdu, China) for 3 d in a clean room hood. The supernatants were then used to detect Pb and Cd. Meanwhile, the liver and renal cortex tissue samples (approximately 400 mg wet weight) were dried to constant weight (dry weight) at 80 °C for 48 h. Then, the dried samples were weighed and placed in 10 mL conical flasks with polypropylene lids containing 3 mL of HNO₃ at room temperature until the solution became clear. Then, 1 mL of 30% H₂O₂ was added to the samples. After the effervescence ceased, the samples were heated at 80 °C until HNO₃ evaporated. The samples were then cooled to room temperature. The final volume was increased to 10 mL with the addition of 2% HNO₃. The blank reagents, which did not contain blood, were subjected to the same procedure. The blood and tissue samples were then analyzed for Pb and Cd contents using ICP-optical emission spectrometry (ICP-OES, SPECTRO ARCOS, SPECTRO Analytical Instruments GmbH, Kleve, Germany). The methods used and suitable conditions are outlined in the previous article (Langkammer et al., 2010). The suitable wavelengths for Pb and Cd were 220.353 and 228.802 nm, respectively.

2.6. Antioxidant enzyme activities and LP parameters

Protein determinations were conducted using the dye-binding method of Bradford in the liver supernatant and kidney homogenate (Bradford, 1976). Bovine serum albumin was used to construct the standard curve. Then, renal and hepatic CAT, SOD, and GSH-Px activities, as well as GSH and MDA contents, were assayed using commercial reagent kits (Nanjing, China).

2.7. IHC

All liver and renal cortex specimens were fixed by immersion for at least 1 d in 10% buffered formaldehyde phosphate, and subsequently dehydrated and embedded in paraffin wax to cut sections at a thickness of 4 μm. For immunohistochemical staining, a HRP/DAB detection IHC kit (ab80436 Abcam, China) was used according to the manufacturer’s protocol. The sections were then deparaffinized, and the formalin-fixed paraffin-embedded tissue sections were rehydrated. Hydrogen peroxide block was added to cover the sections, which were incubated for 10 min. After antigen retrieval (100 × Citrate Buffer, ab64236 Abcam, China) for 20 min in a domestic pressure cooker and blocking non-specific binding sites with protein block, the sections were immunoreacted with anti-MT antibody (MT; 1:100 dilution of monoclonal mouse IgG1; ab12228, Abcam, China) overnight at 4 °C. For negative controls, the sections were immersed in PBS instead of the specific antibody. Then, mouse-specific HRP was applied to conjugate and incubate for 15 min at room temperature. DAB was applied to the tissue sections counterstained with hematoxylin.

For immunohistochemical quantification, two slices were selected from one integration receptor sample. Five 400× microscopic views of cortex and medulla per slide were selected randomly and photographed using a Nikon ECLIPSE 80i microscope equipped with a Nikon DS-Ri1 camera (Nikon Corporation, Tokyo, Japan). The immunopositive reactions in the photographs were analyzed by Software Image Pro-Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA), according to a previously introduced method (JiangFeng et al., 2011; Wang et al., 2009). All photographs were taken and measured in the same parameter setting to ensure that the data were comparable. The area of positive staining was measured in pixels using Image-Pro Software, which detected brown staining in the liver and renal cortex. The average optical density (AOD) was defined as the percentage of positive area in the total area of livers and renal cortices.

2.8. qRT-PCR

Total RNA was extracted from the livers and kidneys of rats using RNAiso Plus (D9108A, TaKaRa, Dalian, China), according to the manufacturer’s instructions. RNA concentration and purity were assessed using OD260 and the OD260/OD280 ratio, respectively, and the ratio exceeded 1.9 for all preparations. Total RNA (1 μg) was reversely transcribed in a 25 μL reaction volume using TranScript™ First Strand cDNA Synthesis Super-Mix Kits (TranGen Biotech, Beijing, China), according to the manufacturer’s instructions. Primer information for MT-1 gene, MT-2 gene, and intergenic receptor were listed in Table 1. qRT–PCR analysis was conducted in triplicate for each sample with Bio-Rad CFX96 Real-Time PCR System and Bio-Rad CFX96 Manager Software (Bio-Rad, Hercules, CA, USA) using SYBR® Premix Ex Taq™ II (RR820A, TaKaRa, Dalian, China). Analysis was performed in a 25 μL reaction volume according to the manufacturer’s instructions. The thermal protocol was as follows: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. A melting curve of the PCR products (60–90 °C) was also obtained to ensure the absence of artifacts. The qRT–PCR reaction was performed in triplicate for each sample, and a mean value was used to calculate mRNA levels. Six biological replicates were measured for each group. Amplification of the GAPDH gene RNA fragment was used for normalization of
expression data. The delta–delta Ct method was used to calculate relative fold-change values between samples (Schmittgen and Livak, 2008).

2.9 Statistical analysis

All data were expressed as the means ± standard error of the means, and analyzed by one-way ANOVA with Duncan’s multiple-range tests after using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The significant values at either $P < 0.05$ (*) or $P < 0.01$ (**) were represented as asterisks.

3. Results

3.1 Effect of Pb/Cd treatment on the performance of rats

Neither treatment-related mortality nor obvious clinical signs, including hair loss, scabbing, soft or mucoid feces, relatively decreased defecation or feces, wet yellow material in the urogenital area, or vocalization upon handling, were observed in any of the treated groups throughout the experimental period. Thus, these patients were all inclined to be normal. Animals from all treatment groups did not exhibit serious diseases at the end of the study period. Heavy metal treatment had no effect on weight gain or on water and food consumption in rats.

3.2 Distribution of Pb and Cd in the blood, liver, and renal cortex

Tissue Pb and Cd concentrations were determined by ICP–OES, and the results are shown in Fig. 1A and B. Pb and Cd ingestion resulted in a significant elevation of Pb and Cd in the blood, as well as the accumulation of Pb and Cd in the liver and renal cortex in the exposed rats (Groups II to IV). Pb and Cd levels in the tissue gradually increased with increasing doses of Pb and Cd ingestion. Pb and Cd contents in the blood, liver, and renal cortex of Groups II to IV significantly increased ($P < 0.001$, $P < 0.01$) compared with those of the control group (Group I). By comparing Pb and Cd levels in the blood with those in the liver and renal cortex, we found that Pb was mainly distributed in the blood, but Cd accumulated particularly in the liver and kidney.

3.3 Antioxidant enzyme activities and LP assessments

Changes in the hepatic and renal antioxidant enzyme activities and LP parameters are shown in Tables 2 and 3. Compared with the control group, Pb and Cd co-exposure administration (Groups II to IV) caused a significant ($P < 0.01$) increase in LP as measured by the MDA levels, as well as a significant ($P < 0.01$) decrease in the non-enzymatic antioxidant GSH. Pb and Cd co-exposure administration (Groups II to IV) also caused significant ($P < 0.01$) decreases in the antioxidant enzymes SOD, CAT, and GSH-Px in the 90 d period.

3.4 Immunohistochemical analyses

MT immunostaining was observed in the hepatocytes and renal proximal tubular cells (Figs. 2 and 3). The control groups showed a small area of scattered focal positive staining (Figs. 2a and 3a). The intensity and amount of immunostaining of low-dose groups (Figs. 2b and 3b) showed a fewer increase than those of the control group. Moreover, MT immunostaining was primarily localized within hepatocytes and proximal convoluted tubules, and was weak or absent from distal convoluted tubules. By contrast, MT staining of the intermediate- (Figs. 2c and 3c) and high-dose groups (Figs. 2d and 3d) was spread throughout the liver and renal cortex. No detectable MT immunostaining was present in glomeruli for all treatments. The AOD of MT was significantly higher in the exposed rats (Groups II to IV) than that in the control group ($P < 0.01$, Table 4).

3.5 MT-1 and MT-2 gene expression

Results of MT-1 and MT-2 gene expression analysis in the rat livers and renal cortices exposed to Pb and Cd are shown in Fig. 4. The MT-1 and MT-2 expression levels were significantly upregulated for both liver and kidney in the exposed rats. After 90 d of exposure, the MT-1 relative expression levels of the exposed rats significantly increased in the livers (Groups II to IV) by 3.38-, 6.95-, and 10.47-fold, respectively, whereas MT-2 increased by 2.37-, 5.45-, and 7.81-fold, respectively. Compared with the controls, the relative expression levels in the kidneys (Groups II to IV) also increased by 3.18-, 5.66-, and 8.88-fold for MT-1, respectively, and 2.25-, 3.65-, and 6.76-fold for MT-2, respectively.

4. Discussion

This study is the first attempt to report the equitoxic mixture ratio of Pb and Cd as the experimental animal model method for long-term toxicity experiments. After introducing Pb and Cd co-exposure in rats, no significant changes in body weight and intoxication were observed. Thus, the general metabolic condition of the animals was within normal range. Moreover, this animal model may help in detecting the early events of chronic Pb/Cd intoxication with relatively low and environmentally realistic concentrations.

Pb was mainly distributed in the blood compared with the levels of Pb and Cd in the liver and renal cortex, but Cd accumulated in the liver and kidney. These results indicate the characteristics of the two heavy metals. Cd was considered the principal MT inductor. With repeated administration, Cd
accumulated primarily in the liver, whereas MT sequestered the majority of intracellular Cd (Campana et al., 2003). Cd-MT was then slowly released from the liver, and exogenous Cd-MT was rapidly degraded in the renal cells by lysosomal enzymes to liberate the Cd ions that cause nephrotoxicity (Shaikh et al., 1999). However, Pb was a relatively hard metal with a lower sulfhydryl-binding affinity, and was a weak inducer of MT (Campana et al., 2003). With repeated administration, Pb mainly attached to the red cell membrane, damaged the hematopoietic system, and caused body anemia.

In addition to the key enzymatic activities, we investigated the function of oxidative damage in Pb- and Cd-induced changes in steroidogenesis in the liver and kidney. We observed that co-exposure to Pb and Cd at low levels for 90

Table 2 – Effect of lead and cadmium on hepatic enzymatic and non-enzymatic biochemical assays in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>(G-I) 0</th>
<th>(G-II) 29.96</th>
<th>(G-III) 89.88</th>
<th>(G-IV) 269.65</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>nmol mg⁻¹ prot</td>
<td>2.13 ± 0.38</td>
<td>2.80 ± 0.38</td>
<td>3.76 ± 0.23</td>
<td>4.31 ± 0.43</td>
</tr>
<tr>
<td>SOD</td>
<td>U mg⁻¹ prot</td>
<td>95.67 ± 6.67</td>
<td>87.42 ± 3.32</td>
<td>80.54 ± 1.88</td>
<td>69.25 ± 2.03</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>U mg⁻¹ prot</td>
<td>223.68 ± 23.12</td>
<td>182.30 ± 6.34</td>
<td>161.81 ± 6.47</td>
<td>128.59 ± 5.66</td>
</tr>
<tr>
<td>CAT</td>
<td>U mg⁻¹ prot</td>
<td>17.45 ± 1.56</td>
<td>13.35 ± 1.09</td>
<td>12.45 ± 1.15</td>
<td>8.45 ± 1.43</td>
</tr>
<tr>
<td>GSH</td>
<td>mg g⁻¹ prot</td>
<td>59.00 ± 5.56</td>
<td>29.77 ± 2.33</td>
<td>23.55 ± 3.44</td>
<td>17.87 ± 1.87</td>
</tr>
</tbody>
</table>

MDA maleic dialdehyde, SOD superoxide dismutase, GSH-Px glutathione peroxidase, CAT catalase, GSH glutathione. The values are presented as means ± standard deviation (n = 6). Compared with control group, *P < 0.05, **P < 0.01.
Table 3 – Effect of lead and cadmium on renal enzymatic and non-enzymatic biochemical assays in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>(G-I) 0</th>
<th>(G-II) 29.96</th>
<th>(G-III) 89.88</th>
<th>(G-IV) 269.65</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>nmol mg⁻¹ prot</td>
<td>1.59 ± 0.33</td>
<td>2.12 ± 0.45**</td>
<td>2.56 ± 0.67**</td>
<td>3.12 ± 1.00**</td>
</tr>
<tr>
<td>SOD</td>
<td>U mg⁻¹ prot</td>
<td>127.46 ± 4.87</td>
<td>98.77 ± 4.87**</td>
<td>77.54 ± 3.45**</td>
<td>59.49 ± 3.67**</td>
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<tr>
<td>GSH-Px</td>
<td>U mg⁻¹ prot</td>
<td>211.59 ± 3.88</td>
<td>55.99 ± 2.45**</td>
<td>130.12 ± 4.44**</td>
<td>105.33 ± 6.44**</td>
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<tr>
<td>CAT</td>
<td>U mg⁻¹ prot</td>
<td>7.87 ± 1.56</td>
<td>5.55 ± 1.09**</td>
<td>4.66 ± 0.89**</td>
<td>3.68 ± 0.99**</td>
</tr>
<tr>
<td>GSH</td>
<td>mg g⁻¹ prot</td>
<td>53.44 ± 2.77</td>
<td>33.12 ± 3.11**</td>
<td>28.66 ± 4.76**</td>
<td>22.68 ± 1.87**</td>
</tr>
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</table>

MDA maleic dialdehyde, SOD superoxide dismutase, GSH-Px glutathione peroxidase, CAT catalase, GSH glutathione.

The values are presented as means ± standard deviation (n = 6). Compared with control group, *P < 0.05, **P < 0.01.

Fig. 2 – Immunohistochemistry with specific antibody against MT in liver tissues. There are a few positive cells (brown stain) in the control group (a). The positive cells are slightly increased in the low dose group (b) and markedly increased in the intermediate and high dose groups (c, d). Negative immunostaining with PBS replaces MT in the control group (e). The arrows demonstrate positive immunoreation. Immunohistochemical labeling of HRP–streptavidin detection and HE counterstain, final magnification 400×, bars = 50 μm.
Fig. 3 – Immunohistochemistry with specific antibody against MT in renal cortex tissues. There are a few positive cells (brown stain) in the control group (a). The positive cells are slightly increased in the low dose group (b) and markedly increased in the intermediate and high dose groups (c, d). Negative immunostaining with PBS replaces MT in the control group (e). The arrows demonstrate positive immunoreaction. Immunohistochemical labeling of HRP–streptavidin detection and HE counterstain, final magnification 400×, bars = 50 μm.

Table 4 – The average optical density (AOD) of MT positive cells in the liver and renal cortex.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Doses (mg/kg/day)</th>
<th>Liver</th>
<th>Renal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0</td>
<td>0.52 ± 0.31</td>
<td>0.57 ± 0.39</td>
</tr>
<tr>
<td>Group II</td>
<td>29.96</td>
<td>3.62 ± 0.65**</td>
<td>2.61 ± 0.59**</td>
</tr>
<tr>
<td>Group III</td>
<td>89.88</td>
<td>6.81 ± 0.68**</td>
<td>7.01 ± 0.81**</td>
</tr>
<tr>
<td>Group IV</td>
<td>269.65</td>
<td>7.14 ± 0.92**</td>
<td>7.92 ± 1.83**</td>
</tr>
</tbody>
</table>

Group I: control group; Group II: low dose group; Group III: intermediate dose group; Group IV: high dose group. Values represent means ± S.E.M.s (n = 10, %).

** P < 0.01 represents a significant difference to controls.
d resulted in a significant increase in LP, as indicated by a marked increase in MDA and a significant decrease in GSH in the liver and kidney compared with the controls. LP may be a direct consequence of membrane damage because Pb and Cd interact with cell membranes (Casalino et al., 1997; El-Nekeety et al., 2009). GSH, which is one of the most important intracellular non-enzymatic antioxidants, is the largest component of an endogenous cellular “redox buffer” (Liu et al., 2012). The decrease in GSH may be due to the decrease in GSH levels by the binding ability between Pb and Cd and the SH-group, which interferes with the antioxidant activity (Pandya et al., 2012). In this study, the activities of antioxidant enzymes, such as SOD, CAT, and GSH-Px, decreased. These antioxidant enzymes are the primary enzymatic defense against toxic oxygen reducttion metabolites, and each enzyme has an integral function in free radical modulation. Thus, the accumulated free radical could consume SOD, CAT, and GSH-Px in the kidney and liver. Moreover, if the balance between reactive oxygen species (ROS) production and antioxidant defense was disrupted, the enzyme may be exhausted and its concentration may be depleted (Liu et al., 2010). The enhanced LP in this study indicates failure of the antioxidant defense mechanism, which was consistent with our results on the antioxidant activity of enzymes.

The human body has evolved a protective mechanism against toxic metals through MT. MT, which is an important complexing protein, reduced the bioavailability of highly toxic free Cd\textsuperscript{2+}. Pb also bound MT, but did not appear to displace Cd or zinc. MT is not induced by Pb, but MT sequesters Pb in the cell (Pandya et al., 2012). Among the four common MTs, MT-1 and MT-2 were expressed in most tissues; MT-3 was predominantly present in the brain; and MT-4 was restricted to the squamous epithelium. The expression of MT-1 and MT-2 was easily induced by heavy metals (Sabolić et al., 2010).

Both MT mRNA and protein levels in livers and kidneys of the exposed rats (Groups II to IV) were higher than those in the controls. This result was related to the constant transportation and detoxification of the liver, from where Cd-MT was slowly released. In the kidney, Cd-MT was ultimately degraded in lysosomes of proximal tubule cells. Released Cd could then induce MT resynthesis as a stimulus in proximal tubule cells (Zhang et al., 2012). Although the gene expression of MT-1 was higher than that of MT-2 in the tissues of co-exposed rats, MT-1 and MT-2 were similarly regulated in rodents, and the proteins were functionally equivalent (Searle et al., 1984). MTs were constitutively expressed in the co-exposed rat hepatocytes and renal proximal tubular cells at levels higher than those in Cd exposure in vivo (Nolan and Shaikh, 1986; Wang et al., 2010). Exposure to low-dose Pb combined with Cd resulted in additive or synergistic effects on MT expression in the liver and kidney of rats in a 90 d period. Thus, Cd induced MT mRNA without increasing MT protein in the kidney. The stability of MT protein in the kidney exhibited an equilibrium state of MT synthesis and MT degradation. Our results show that Pb and Cd co-exposure treatment could also significantly induce MT protein synthesis. In particular, MT could directly function as an antioxidant. In addition, cysteine in the structure of MT could also be used for GSH synthesis, and ROS could increase MT mRNA transcriptional response (Kara et al., 2005; Wang et al., 2004). These changes may be one of the possible mechanisms of inhibition of steroidogenic enzyme activity, testosterone levels, and sperm count/sperm motility after Pb and Cd co-exposure.

In summary, the combined exposure to Pb and Cd disrupted the antioxidant defense mechanisms. The effects produced by the combined treatment of metals were not only antagonistic, but increased the MT mRNA and protein levels. MTs may be biochemical environmental indicators for Pb/Cd.
levels in parenchymal tissues of mammals based on increased environmental levels.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Acknowledgement**

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