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Cadmium induced oxidative damage and apoptosis in the hepatopancreas of *Meretrix meretrix*

Liping Xia[1] · Sihan Chen[1] · Hans-Uwe Dahms[2] · Xueping Ying[1] · Xue Peng[1]

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Abstract Even trace amounts of cadmium (Cd), a non-essential metal, are known to be toxic to aquatic organisms. Here we investigated the relationship between cadmium ion (Cd^{2+}) exposure and oxidative damage and apoptosis in the hepatopancreas of the clam *Meretrix meretrix*. Clams were exposed to different concentrations of Cd^{2+} (0, 1.5, 3, 6 and 12 mg L^{-1}) for 5 days. We monitored both antioxidant enzyme activity, including that of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), and levels of malondialdehyde (MDA), glutathione (GSH) and glutathione disulfide (GSSG). Apoptosis of hepatopancreatic cells was detected by DNA laddering and AO/EB double fluorescent staining. The results show that the rate of apoptotis, MDA levels, and caspase-3 activity, increased with Cd^{2+} concentration, whereas GPx activity and the ratio of GSH/GSSG, decreased. SOD and CAT enzyme activity first increased, then decreased, with increasing Cd^{2+} concentration; peak activity of these enzymes was recorded in the 3 mg L^{-1} Cd^{2+}-treatment group. These results show that Cd-induced oxidative damage can both induce, and aggravate, apoptosis in the hepatopancreatic cells of clams, even at Cd^{2+} concentrations far below the semi-lethal dose for adult clams. The observed changes in caspase-3 activity enhanced significantly at lower Cd^{2+} concentrations, indicating that caspase-3 is a suitable biomarker for heavy metal pollution, especially cadmium pollution, in marine organisms.

Keywords Cadmium · *Meretrix meretrix* · Hepatopancreas · Apoptosis · Antioxidant enzymes

Abbreviations

AO/EB	Acridine orange/ethidium bromide
CAT	Catalase
Cd	Cadmium
GPx	Glutathione peroxidases
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione transferase
LC50	Medial lethal concentration
MDA	Malondialdehyde
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Heavy metal pollution in aquatic ecosystems probably has long-lasting effects, even in marine ecosystems. Cadmium (Cd) is a widespread heavy metal in the marine environment that poses a serious risk for human health when it enters and becomes bio-magnified in food webs (Wagner et al. 1998; Waalkes 2000; Wang et al. 2008). Cd is a non-essential metal that can cause a series of toxicological changes in organisms (Geret et al. 2002; Ketata et al. 2007; Ji et al. 2012). As peroxide inducers, cadmium and other

✉ Xueping Ying
xpying2008@wzu.edu.cn

[1] College of Life and Environmental Sciences, Wenzhou University, Wenzhou 325035, Zhejiang, People's Republic of China

[2] Department of Biomedical Science and Environmental Kaohsiung, Medical University Biology, No. 100, Shin-Chuan 1st Road, Kaohsiung 80708, Taiwan, ROC

heavy metal contaminants can stimulate the production of excess free radicals.

Oxidative stress occurs when the generation of reactive oxygen species (ROS) exceeds an organism's capacity to neutralize these molecules (Wang et al. 2011; Cao et al. 2012). Excess free radicals have a range of deleterious effects, including causing lipid peroxidation and blocking the mitochondrial electron transport chain (Bolduc et al. 2004; Shih et al. 2004). These effects can cause DNA damage and other abnormalities leading to cellular damage and apoptosis (Nzengue et al. 2008; Wang et al. 2011; Ali et al. 2012; Ji et al. 2012; Zhang et al. 2012). MDA has recently been suggested as a potential biomarker of oxidative stress (Otitoloju and Olagoke 2011) in which case quantification of MDA could allow oxidative stress to be detected before lethal, or pathological, effects occur (Wang et al. 2013; Zhang et al. 2014). Caspase-3, an effector caspase, has been shown to play a critical role in apoptosis (Shih et al. 2004; Liu et al. 2013).

Organisms have evolved effective anti-ROS defense systems, mainly comprised of low molecular weight compounds, such as vitamin A, C, E, glutathione (GSH), uric acid, and major antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione transferase (GST) and others (Chandran et al. 2005; Ivanina et al. 2008; Loro et al. 2012; Zhang et al. 2014). These anti-ROS systems work by scavenging free radicals, thereby reducing oxidative stress and inhibiting apoptosis (Angkeow et al. 2002; Wang et al. 2013). The potential of using elements of the antioxidant defense system as biochemical biomarkers for environmental monitoring made it the focus of intensive research (Otitoloju and Olagoke 2011).

Bivalve mollusks inhabiting intertidal zone sediments are widely distributed globally. Because these invertebrates are suspension feeders with poor locomotor ability, they are particularly vulnerable to heavy metal contamination. For this reason, bivalves (mussels, oysters, clams, etc.) have been widely used in the biological monitoring of marine heavy metal pollution (Byrne and O'Halloran 2001; Hamad et al. 2011; Meng et al. 2013).

Meretrix meretrix (Bivalvia, Veneridae) is an economically important shellfish that lives in the sediments of the neritic intertidal zone, including estuaries, along the coast of China. Several studies have shown that heavy metals, such as zinc (Zn^{2+}) and cadmium (Cd^{2+}), have serious toxic effects on the Veneridae, including retarding the development of both gonads and larvae (Wang et al. 2009a, b; Zhang et al. 2011). For example, exposure to these heavy metals is followed by a dramatic decrease in GSH levels and GPx activity in the gonads, membrane lipid peroxidation (Zhang et al. 2011), and a significant increase in antioxidant enzyme activity in *M. meretrix* larvae (Wang et al. 2010). The effects of Cd^{2+} exposure on the antioxidant system of adult *M. meretrix* and hepatopancreatic cells are, however, currently unknown.

The present study makes use of several biochemical parameters, including DNA fragmentation, antioxidants (SOD, CAT, and GPx), the key enzymes of apoptosis (caspase-3), and the level of GSH, GSSG and MDA in the *M. meretrix* exposed to different concentrations of Cd^{2+}. We particularly focused on the relationships between Cd^{2+} exposure, antioxidant defenses, oxidative damages and apoptosis in the hepatopancreatic cells of *M. Meretrix*. The main objective of this study was to explore the toxic effect of Cd^{2+} on the clam, and to improve our understanding of the antioxidant defense mechanisms of clam against Cd^{2+} stress. A secondary goal was to provide baseline information of biomarkers' selection for the assessment of Cd^{2+} pollution in aquatic ecosystems.

Materials and methods

Reagents

Analytical grade cadmium chloride ($CdCl_2 \cdot 2.5\ H_2O$) was purchased from Tianlian Fine-Chemical Co., ltd. (Shanghai, China). A DNA extraction assay kit was obtained from Tian Gen Biotech Co., Ltd (Beijing, China). Caspase-3, GSSG/GSH assay kits were acquired from Beyotime Institute of Biotechnology (Shanghai, China). MDA, SOD, GPx, and CAT assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). AO/EB double fluorescent stain was purchased from Nanjing Keygen Biotech Co., Ltd (Nanjing, China).

Animals and treatments

Meretrix meretrix clams which was 2 years old were purchased from the Linkun aquafarm, Wenzhou, Zhejiang, China, between June and July 2013. The Cd concentration levels of total fraction in seawater and deposit sediment of the clam farm were 0.54 μg L^{-1} and 0.89 mg kg^{-1}, respectively. Clams were acclimated in 15 % artificial sea water without food for 2 days in auto-temperature control aquarium at a temperature of 22 ± 1 °C before beginning experiments. The sea water was renewed, and the aquarium thoroughly cleaned, daily. Any dead clams were immediately removed. After acclimation, 300 healthy clams (see Table 1 for size and weight) were selected for a semi-lethal Cd^{2+} concentration (LC_{50}) test. The LC_{50} toxicity experiment was conducted using a modified method outlined by Wang (Wang et al. 2009a, b). The 48 h LC_{50} and 96 h LC_{50} Cd^{2+} levels were 40.78 and 15.01 mg L^{-1}, respectively.

Table 1 The size and weight of *M. meretrix* clams used in experiments to determine LC50 and acute toxicity of cadmium

Class	Shell length (mm)	Shell width (mm)	Shell height (mm)	Mass (g)
LC_{50}	47.39 ± 0.33	24.16 ± 0.17	38.43 ± 0.30	28.90 ± 0.52
	(43.07–53.78)	(22.10–27.27)	(34.49–43.89)	(24.00–36.60)
Acute toxicity	47.24 ± 0.32	23.93 ± 0.24	38.28 ± 0.23	27.79 ± 0.25
	(36.37–50.14)	(21.09–33.29)	(33.16–41.23)	(25.12–31.90)

Clams were then randomly assigned to either a control group, or one of four Cd^{2+}-treatment groups, each comprised of 30 clams. The control and treatment groups each had three replicates so the experiment involved a total of 450 clams, accommodated in 15 aquariums. The four treatment groups were exposed to 1.5, 3, 6 or 12 mg L^{-1} concentrations of cadmium solution, corresponding to 1/10, 1/5, 1/2.5, 1/1.25 of the previously determined 96 h LC_{50} (15.01 mg L^{-1}). To maintain both good water quality and heavy metal absorption, the different concentrations of cadmium solution were renewed daily throughout the experiment. All other experimental conditions were kept the same as during acclimation. No mortality occurred in any treatment group. All clams were collected and dissected after 5 days of cadmium exposure, after which the hepatopancreas of each animal was quickly removed and immediately frozen in liquid nitrogen before being stored at −80 °C in a refrigerator until required for analysis. Basic parameters of the clams used in the LC_{50} test and acute toxicity experiments are listed in Table 1.

Cadmium content determination

Concentrations of Cd were measured following the Chinese National Standard for determination of cadmium in food (GB1738.6-2007) with some modifications. After exposure, hepatopancreas tissues were excised and weighted (approximate 2 g wet weight), cut into small pieces, and excess water on surface of tissues was removed with absorbent paper. Then tissues were digested in HNO_3 (analytical grade) and $HClO_4$ (analytical grade) over a hot plate at about 120–150 °C, under a reflux cap. Cd concentrations of each sample were measured with an atomic absorption spectrophotometer (Shimadzu AA-6300, Japan). Standard Cd solution was used for the analysis of metal concentrations. The carrier gas was acetylene. Cd content was expressed as mg kg^{-1} wet weight tissue.

Detection of apoptosis with fluorescent microscopy

Following the methods described by Ribble et al. (2005) and Kasibhatla et al. (2006), acridine orange/ethidium bromide (AO/EB) staining of hepatopancreatic cells was carried out to estimate the degree of cell damage induced by Cd^{2+}. 60 mg of hepatopacreatic tissue was prepared as a cell suspension in precooling 1 % PBS (pH = 7.2). A 25 μL suspension of each sample was mixed with 1 μl aqueous AO/EB solution (100 μg ml^{-1} of AO in PBS; 100 μg ml^{-1} of EB in PBS) and incubated for 5 min before being examined under fluorescent microscopy (Nikon Ti-s, Japan).

Because AO penetrates intact cell membranes and stains nuclear DNA with bright green fluorescence, whereas EB penetrates damaged cell membranes and stains nuclear DNA with orange fluorescence, fluorescent microscopy allows to distinguish different types of cells. Viable cells had bright green nuclei, whereas early-stage apoptotic cells were yellow with nuclear chromatin condensation, and late-stage apoptotic cells and dead cells had orange or red nuclei with intact structures. A minimum of 100 cells were counted at least 3 times from each treatment group. Data from the AO/EB test were expressed as the percentage of apoptotic cells.

Detection of apoptosis by DNA laddering

50 mg of hepatopancreatic tissue from the treatment and control groups was added to separate vessels containing 500 μL physiological saline, thoroughly ground into a suspension on ice and centrifuged at 11,200 g (4 °C) for 1 min. After centrifugation, the supernatant was removed and the sediment used for DNA fragment extraction. DNA was extracted with a DNA extraction kit (Beyotime, C008) according to the manufacturer's instructions. A DNA sample mixture (8 μL DNA extract, 3 μL Bromophenol blue) was added to each lane of a 1 % agarose gel (containing 0.1 mg ml^{-1} ethidium bromide) in 0.5 × TBE buffer and subject to electrophoresis at 58 V for 1 h. The resultant bands were photographed under ultraviolet light with Gel Doc 2000 (Tannon, Shanghai, China).

Caspase-3 activity assay

Caspase-3 activity was measured with a corresponding caspase-3 Activity Assay Kit (Beyotime, Shanghai, China). Nine hepatopancreatic tissue samples from each treatment group were randomly collected, weighted and homogenized on ice (5 % w/v) in lysate with a SCIENTZ DY89-II type motor-driven homogenizer (Zhejiang, China). After homogenization, samples were incubated for 5 min on ice,

then centrifuged at 18,000 g for 20 min at 4 °C. The supernatant was collected for caspase-3 activity measurements according to the kit manufacturer's instructions. One unit of enzymatic activity was taken to be the amount of enzyme required to cleave 1.0 nmol of the colorimetric substrate to produce 1.0 nmol *p*NA per hour at 37 °C under saturated substrate concentrations. Optical density values were read at 405 nm on a multifunction microplate detector (Biotek Epoch, Vermont, USA).

Antioxidant enzyme activity assay

Hepatopancreas samples were macerated on ice to form a 10 % (w/v) homogenate in 0.9 % physiological saline using a SCIENTZ DY89-II type motor-driven homogenizer. Supernatants were collected after centrifugation at 3500 rpm for 15 min at 4 °C for SOD, GPx and CAT activity measurement. All enzyme activity was assayed according to the kit manufacturer's instructions (Nanjing Jiancheng, China) and defined as units of activity per mg of protein (U mg^{-1}).

MDA, GSH and GSSG assay

Tissue MDA content, a biomarker of lipid peroxidation, was determined with a colorimetric MDA assay kit (Nanjing Jiancheng, China) according to the manufacturer's instructions. GSH and GSSG in the hepatopancreas were measured using GSH and GSSG Assay Kits (Beyotime, China) based on the method described by Tietze (1969), according to the kit manufacturer's instructions. GSH and GSSG levels were expressed in mg per g protein (mg g^{-1}), and MDA levels as nmol per mg protein (nmol mg^{-1}).

Total protein assay

The total protein content of the hepatopancreas was measured according to the method described by Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

The data were analyzed using the SPSS Statistical Package (Version 16.0, Chicago, USA for Windows). The significance of differences among experimental groups was determined using a one-way ANOVA followed by Tukey's Multiple Comparison Test. $P < 0.05$ was taken to be statistically significant, and $P < 0.01$ was considered highly significant. The Least Significant Difference test (LSD) was used to perform multiple comparisons among different treatment groups. All data are expressed as mean ± standard error (SE).

Results

Cd bio-accumulation in hepatopancreas of *M. meretrix*

The original pre-exposure Cd concentration levels of the hepatopancreas of *M. meretrix* was 0.29 ± 0.01 mg kg^{-1} wet tissue. After Cd exposure for 5 days, Cd levels of the hepatopancreas increased sharply, and reached maximum levels in the 12 mg L^{-1} exposure groups (Table 2). There was significantly different among control group and all Cd^{2+}-treatment groups ($P < 0.05$). And Cd concentrations in the hepatopancreas of *M. Meretrix* showed a higher bio-accumulation and concentration-dependent manner.

Morphological observation of apoptosis with fluorescent microscope

Three types of cells could be distinguished under fluorescent microscopy after AO/EB staining; live cells (green), early-stage apoptotic cells (yellow), late-stage apoptotic (orange), and dead cells (red) (Fig. 1a–e). The control group had almost all normal cells (green) indicating no apoptosis (Fig. 1a). As the Cd^{2+} concentration increased, both the number of apoptotic cells and the number of late-stage apoptotic cells, significantly increased (Fig. 1b–e). At the highest concentration (12 mg L^{-1}) (Fig. 1e), almost all cells were in the late stage of apoptosis (orange), and some were already dead.

We calculated the rate of apoptosis in each group according to the following formula: apoptosis ratio = (early-stage apoptotic cells + late-stage apoptotic cells)/total cells × 100 %. The result showed that the apoptosis ratio increased with the Cd^{2+} concentration, and that the rate in each Cd^{2+}-treatment group was significantly different to that in the control group ($P < 0.05$) (Table 3). The apoptosis ratio in the 12 mg L^{-1} Cd^{2+}-treatment group reached 90.75 %, whereas that in the control group was just 3.89%. This indicated that the apoptosis ratio was positively correlated with the Cd^{2+} concentration.

DNA fragmentation analysis by agarose gel electrophoresis

Agarose gel electrophoresis was used to detect DNA degradation in hepatopancreatic cells (Fig. 2, lanes M–E). With the exception of two bright DNA bands in the low-molecular area near the loading slot, no obvious DNA laddering was detected in the control group (Fig. 2; lane E). In contrast, DNA ladders were apparent in all Cd^{2+}-treatment groups (Fig. 2, lanes A–D). The distances between each ladder were nearly 200 bps, a metric which is

Table 2 The Cd levels of hepatopancreas in *M. Meretrix* after exposure to different concentrations of Cd^{2+}

Group (mg L^{-1})	0	1.5	3	6	12
Cd level (mg kg^{-1} wet tissue)	0.30 ± 0.01^{e}	4.12 ± 0.10^{d}	7.03 ± 0.11^{c}	18.63 ± 0.19^{b}	37.90 ± 0.73^{a}

Data are means ± standard errors, values in the same column with different superscripts are significantly different ($P < 0.05$)

indicative of apoptosis. The degree of apoptosis increased with Cd^{2+} concentration, and maximal DNA fragmentation occurred in the 12 mg L^{-1} Cd^{2+}-treatment group, which suggests that Cd^{2+} induced apoptosis in a dose-dependent fashion.

GSH and GSSG content

Figure 3 shows the trend in GSH and GSSG levels in hepatopancreatic cells under different Cd^{2+} concentrations. GSH levels in the 3 and 6 mg L^{-1} Cd^{2+}-treatment groups were significantly lower than in the control group ($P < 0.05$), and the GSH level in the 12 mg L^{-1} Cd^{2+}-treatment group was extremely low (0.31 ± 0.01 mg g^{-1}) compared to the control ($P < 0.01$). In contrast, with the exception of the 12 mg L^{-1} Cd^{2+}-treatment group which was significantly different to all other groups ($P < 0.05$), no significant differences in GSSG levels were found among the groups.

The ratio of GSH/GSSG declined with Cd^{2+} concentration (Fig. 3). The lowest ratio was recorded in the 12 mg L^{-1} Cd^{2+}-treatment group, which was significantly different to all other groups ($P < 0.01$). There was no significant difference in GSH/GSSG ratio between the 1.5 mg L^{-1} Cd^{2+}-treatment group and the control group (Fig. 3).

MDA content

MDA levels increased with Cd^{2+} concentration reaching the highest recorded level of 2.94 ± 0.20 nmol mg^{-1} protein in the 12 mg L^{-1} Cd^{2+}-treatment group; significantly higher than in the control group and all other Cd^{2+}-treatment groups. There was no significant difference in MDA levels of the control, the 1.5 mg L^{-1} treatment group, or the 3 mg L^{-1} Cd^{2+}-treatment group (Fig. 4).

Caspase-3 enzyme activity

Caspase-3 activity increased sharply with Cd^{2+} concentration (Fig. 5) and was significantly higher in all Cd^{2+}-treatment groups than in the control ($P < 0.01$).

Antioxidant enzyme activity

Cellular defenses were taken against oxyradicals including enzymes, particularly superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx). GPx activity was lowest in the 12 mg L^{-1} Cd^{2+}-treatment group (15.20 ± 1.0 U mg^{-1} protein) ($P < 0.01$), and was significantly lower in the 3 and 6 mg L^{-1} Cd^{2+}-treatment groups compared to the control group (Fig. 6). GPx activity in the 1.5 mg L^{-1} Cd^{2+}-treatment group did not differ significantly from that in the control group (Fig. 6).

The relationship between SOD activity and Cd^{2+} concentration was not linear. SOD activity in the 3 mg L^{-1} Cd^{2+}-treatment group (36.51 ± 1.27 U mg^{-1} protein) was significantly higher than in all other treatment groups ($P < 0.05$) except the 6 mg L^{-1} Cd^{2+}-treatment group (Fig. 6). SOD activity in the 12 mg L^{-1}-treatment group (28.08 ± 3.10 U mg^{-1} protein) was, however, lower than in all other treatment groups (Fig. 6; $P < 0.05$).

The relationship between CAT activity and Cd^{2+} concentration was similar to that between SOD and Cd^{2+} concentration (Fig. 6). CAT activity in the 3 mg L^{-1} Cd^{2+}-treatment group (2.95 ± 0.13 U mg^{-1} protein) was significantly higher than that in the control group and the 12 mg L^{-1} Cd^{2+}-treatment group (Fig. 6; $P < 0.05$). There was no significant difference in the CAT activity of the 1.5, 3, and 6 mg L^{-1} Cd^{2+}-treatment groups, and CAT activity in the 12 mg L^{-1} Cd^{2+}-treatment group was similar to that in the control group (1.75 ± 0.21 U mg^{-1} protein) ($P > 0.05$) and significantly lower than in all other treatment groups (Fig. 6; $P < 0.05$).

Discussion

Previous research has shown that the distribution and accumulation of heavy metal pollutants in aquatic systems can differ between different aquatic organisms and between different organs in the same organism (Silvestre et al. 2005; Rainbow and Black 2007; Zhao et al. 2012). Waterborne heavy metals are taken in via the gills and intestines by aquatic animals. Then they are transported to other parts of the body, commonly by the circulatory system if this is present. Digestive gland (hepatopancreas) is one of the most vulnerable and sensitive organs to heavy metal pollutants because of the function of metal homoeostasis, excretion and detoxification (Orbea et al. 2000; Yan et al. 2007; Liu et al. 2008).

Therefore, the bio-accumulation of Cd in hepatopancreas of *M. meretrix* was investigated in this study. The Cd concentration level of total fraction in seawater of the clam

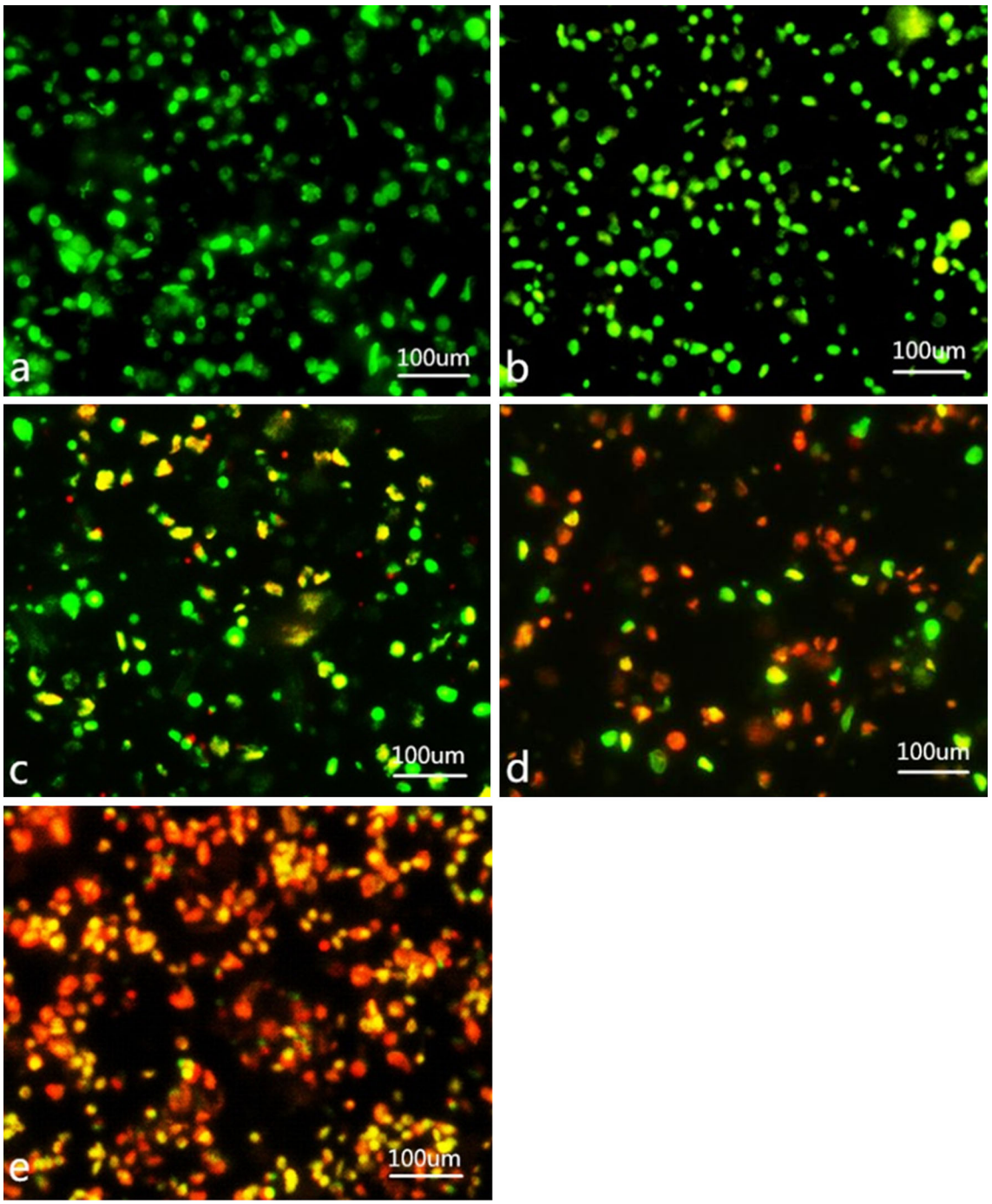

Fig. 1 Results of AO/EB double fluorescent staining of *M. meretrix* hepatopancreatic cells exposed to different Cd^{2+} concentrations; **a** control group (0 mg L^{-1} Cd^{2+}); **b** 1.5 mg L^{-1} Cd^{2+}; **c** 3.0 mg L^{-1} Cd^{2+}; **d** 6.0 mg L^{-1} Cd^{2+}; **e** 12 mg L^{-1} Cd^{2+}. *Green* indicates healthy cells whereas *yellow*, *red* or *orange* indicate different stages of apoptosis (Color figure online)

farm and the hepatopancreas of *M. Meretrix* were 0.54 μg L^{-1} and 0.29 mg kg^{-1}, respectively. When two years' old *M. meretrix* was exposed to different concentrations of Cd for 5 days, the concentration of Cd in hepatopancreas increased significantly (Table 2). The bioaccumulation of Cd was in a concentration-dependent manner. And the maximum values of accumulation in 12 mg L^{-1} treatment groups reached 37.90 ± 0.73 mg kg^{-1}, which was 130.69 fold than pre-exposure group. This observation supported the result of Li (Li et al. 2015), which demonstrated that exposure to Cd led to a significant accumulation of Cd in the hepatopancreas of *Sinopotamon*

Table 3 The apoptosis rate in hepatopancreatic cells in *M. meretrix* induced by exposure to different concentrations of Cd^{2+}

Group (mg L^{-1})	NC	EAC	LAC	Total cell number	Apoptosis rate (%)
0	214.00 ± 0.58	8.67 ± 0.88	0	222.67 ± 1.45	3.89 ± 0.37^{e}
1.5	173.00 ± 3.46	24.00 ± 1.73	14.67 ± 4.91	211.67 ± 10.11	18.05 ± 2.29^{d}
3	118.00 ± 5.20	23.67 ± 0.88	50.00 ± 4.62	191.67 ± 1.45	38.47 ± 2.25^{c}
6	70.67 ± 6.64	55.00 ± 1.16	38.00 ± 0.58	163.67 ± 5.49	57.00 ± 2.70^{b}
12	20.00 ± 4.62	117.67 ± 3.18	76.33 ± 3.76	214.00 ± 5.20	90.75 ± 1.94^{a}

NC normal cells, *EAC* early apoptotic cells, *LAC* late apoptotic cells

Data are means ± standard errors, values in the same column with different superscripts are significantly different ($P < 0.05$)

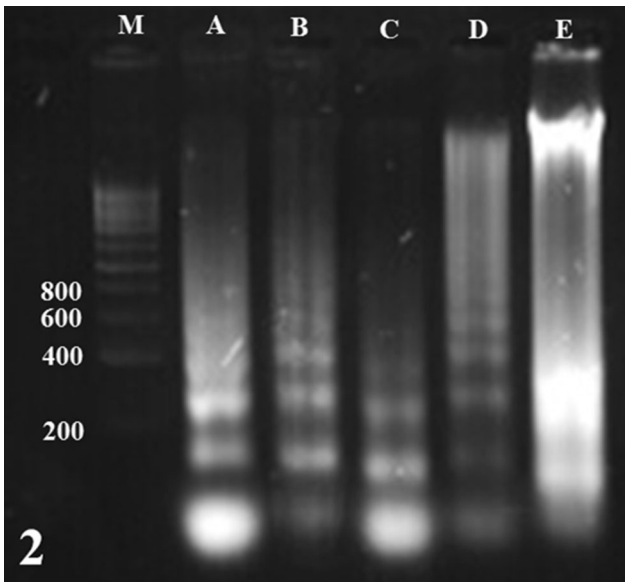

Fig. 2 Laddering of *M. meretrix* hepatopancreatic cell DNA induced by exposure to different Cd^{2+} concentrations. *Lane labels* are: *M* marker, *A* 12 mg L^{-1} Cd^{2+}, *B* 6 mg L^{-1} Cd^{2+}, *C* 3 mg L^{-1} Cd^{2+}, *D* 1.5 mg L^{-1} Cd^{2+}, *E* control group

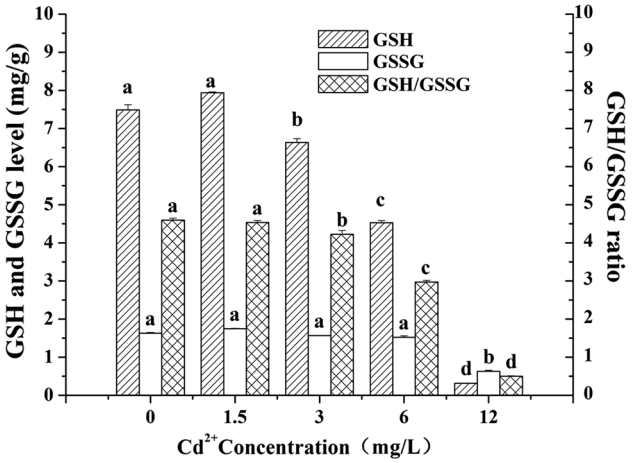

Fig. 3 GSH and GSSG levels, and GSH/GSSG ratio, in the hepatopancreas of *M. meretrix* induced by exposure to different concentrations of Cd^{2+}. *Note* Significant differences between groups are indicated by *different letters* above. *Bars* ($P < 0.05$)

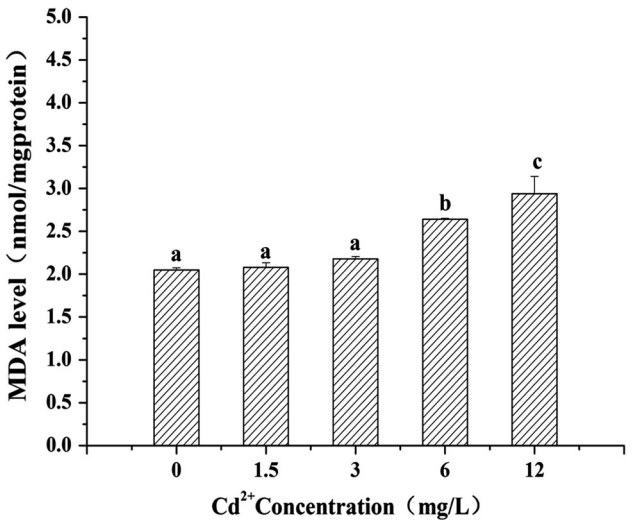

Fig. 4 MDA levels in the hepatopancreas of *M. meretrix* induced by exposure to different concentrations of Cd^{2+}

henanense in a time and concentration-dependent manner. The accumulation of Cd^{2+} in hepatopancreas of *M. meretrix* would induce apoptosis and oxidative damage. The number of apoptotic and dead cells was significantly increased with increasing Cd^{2+} concentration (Table 3; Figs. 1, 2), which is in accordance with the previous study of the effects of Cd^{2+} on the freshwater crab *S. henanense* (Liu et al. 2013). Apoptosis was associated with increased DNA fragmentation, which also increased with Cd^{2+} concentration (Xiang et al. 2001). That Cd^{2+} caused DNA damage is evident from the fact that clams exposed to higher Cd^{2+} concentrations had significantly more DNA fragmentation than those exposed to lower concentrations (Fig. 2, lanes A–C). Taken together, the data presented in Figs. 1, 2 and Table 3 demonstrate that Cd^{2+} induced apoptosis of hepatopancreatic cells in *M.*

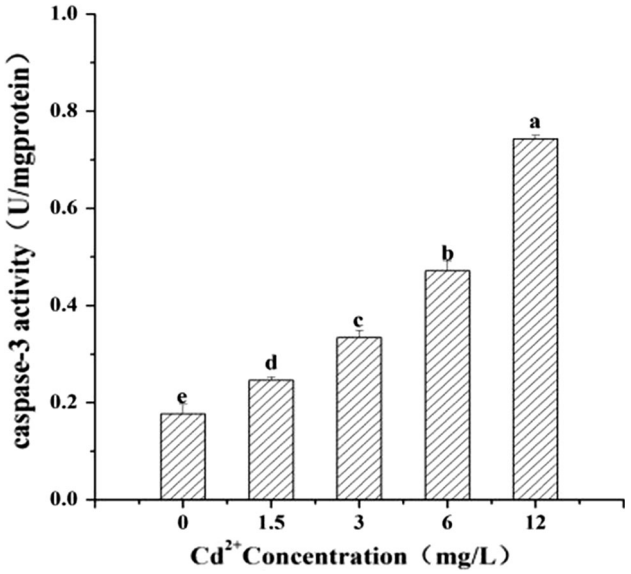

Fig. 5 Caspase-3 activity in the hepatopancreas of *M. meretrix* induced by exposure to different Cd^{2+}concentrations

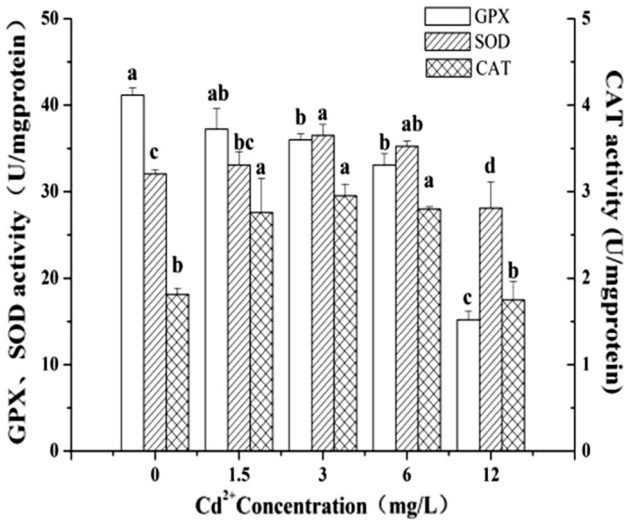

Fig. 6 Effects of exposure to different concentrations of Cd^{2+} on *GPx*, *SOD*, and *CAT* activity in the hepatopancreas of *M. meretrix*

meretrix in a dose-dependent fashion. This is consistent with the findings of other studies. For example, Wätjen et al. (2002) found that Cd^{2+} induced apoptosis of C6 glioma cells in a both time and concentration-dependent way.

Caspase-3 activity also increased with increasing Cd^{2+} concentration (Fig. 5). Caspases are a family of cysteine-dependent aspartate directed proteases that play a critical role in the initiation and execution of apoptosis (Hagara and Malki 2014). Our results showed that exposure to Cd^{2+} increased the activity of caspase-3 in the hepatopancreas of *M. Meretrix*. The activation of the caspase family of proteases is consistent with the hypothesis that cadmium exposure causes DNA damage leading to apoptosis (Shih et al. 2004; Liu et al. 2013). However, in contrast, Liu et al. (2013) found that caspase-3 activity in the hepatopancreas of *S. henanense* was lower in animals exposed to higher levels of Cd^{2+} than in those exposed to more moderate levels. They postulated that Cd-induced apoptosis occurred via a mitochondrial caspase-dependent pathway in low-dosage treatment groups.

The role of antioxidants as a possible defense against heavy metal toxicity has been a focus of toxicological research for the last decade (Orbea et al. 2000; Yan et al. 2007; Wang et al. 2011; Zhang et al. 2012). When heavy metals induce excessive ROS production, the resultant oxidative stress damages macromolecules, including DNA, proteins, and membranes. Many organisms have evolved an antioxidant defense system to protect themselves from these harmful effects of oxidative stress (Geret et al. 2002; Chandran et al. 2005; Ali et al. 2012). Endogenous antioxidants such as GSH, SOD, CAT and GPx, are key components of such systems (Geret et al. 2002; Otitoloju and Olagoke 2011; Loro et al. 2012).

Glutathione (GSH) is an important non-enzymatic antioxidant that is the first line of defense against ROS (Waalkes 2000; Liu et al. 2008; Cao et al. 2012). GSH can inhibit free radical formation through its ability to stabilize components in its oxidative state (Yan et al. 2007; Ivanina et al. 2008; Loro et al. 2012). Under oxidizing conditions, two molecules of GSH are linked by a disulfide bridge to form a molecule of oxidized glutathione (GSSG). Thus, changes in total GSH content, or in the ratio of GSH/GSSG, reflect changes in the normal redox state of cells.

Our results showed that although both GSH and the GSH/GSSG ratio decreased with Cd^{2+} concentration, there was no obvious change in GSSG levels, except in the 12 mg L^{-1} Cd^{2+}-treatment group (Fig. 3). A decrease in GSH in the digestive gland appears to be a common response of mollusks to heavy metal exposure that can partly be explained by the GSH molecule's high affinity for metal (Chandran et al. 2005; Ali et al. 2012).

We observed that a decline in GSH levels, and the GSH/GSSG ratio, was accompanied by an increase in MDA levels (Figs. 3, 4). This negative correlation between GSH and MDA is consistent with the results of previous studies (Chandran et al. 2005; Otitoloju and Olagoke 2011; Ali et al. 2012). We used MDA as an indicator of lipid peroxidation because of its sensitivity to oxidative injury in cells (Geret et al. 2003; Wang et al. 2013; Zhang et al. 2014). Differences in MDA levels between treatment groups clearly indicate that Cd^{2+} induced lipid peroxidation in hepatopancreatic cells, and that MDA levels increased significantly with Cd^{2+} concentration (Fig. 4). This suggested that Cd^{2+} exposure damaged hepatopancreatic cells by elevating ROS levels. A negative

relationship between MDA and GSH levels is consistent with this hypothesis.

Antioxidant enzymes are recognized as important biomarkers of heavy metal contamination (Ivanina et al. 2008; Zhang et al. 2009; Loro et al. 2012). SOD, CAT, and GPx play important roles in scavenging free radicals (Orbea et al. 2000; Valavanidis et al. 2006). With GSH as a catalyst, GPx protects tissues from oxidative damage by converting free hydrogen peroxide into water and disulfide glutathione (GSSG) (Geret et al. 2003). Cd^{2+}-induced GPx activity was, however, down-regulated in the hepatopancreas of *M. meretrix* (Fig. 6). This finding is consistent with that of Chandran et al. (2005) who found that cadmium suppressed GPx activity in the kidney and digestive gland of *Achatina fulica*. Similarly, Wu et al. (2013) found that GPx activity in the hepatopancreas, intestine and stomach of *S. henanense* were lower in a high (29.0 mg L^{-1}) Cd^{2+}-treatment group than that in the control group ($P < 0.01$). Collectively, these results indicate that Cd^{2+} has a significant inhibitory effect on GPx. GPx activity is dependent on selenium levels. By binding to Cd, GPx's active site (Se-Cys) can reduce the toxic effects of Cd on an organism. In addition to changes at the active site, this causes GPx to become deactivated (Iszard et al. 1995).

SOD and CAT are also part of the cellular enzymatic antioxidant defense systems that act in combination to counteract oxidative stress (Waalkes 2000; Zhang et al. 2009; Feng et al. 2013; Zheng et al. 2013). SOD catalyzes the breakdown of superoxide radicals to hydrogen peroxide. CAT prevents oxidative stress by degrading hydrogen peroxide (Wang et al. 2011). There is evidence that different concentrations of Cd^{2+} produce different trends in the activity of these two enzymes in different organisms (Geret et al. 2002; Yan et al. 2007; Wang et al. 2011; Feng et al. 2013). We found the same trend in both CAT and SOD levels in response to Cd^{2+} concentration. There was a negative relationship between the activity of these enzymes and higher Cd^{2+} concentration, and the peak activities of CAT and SOD were observed in the 3 mg L^{-1} Cd^{2+}-treatment group (Fig. 6). This is consistent with the results observed by Yan et al. (2007) in the hepatopancreas of *S. yangtsekiense*, and those of Wu et al. (2013) who found a negative correlation between Cd^{2+} concentration and SOD and CAT activity in the hepatopancreas, intestine, and stomach of *S. henanens* with peak activities in the 7.25 mg L^{-1} Cd^{2+}-treatment group.

These results can be explained by an initial increase in SOD activity in response to the formation of superoxide radicals induced by Cd^{2+} exposure. Superoxide is considered as a central component of the signal transduction system that triggers the genes responsible for antioxidant enzyme synthesis, including SOD and CAT (Cong et al. 2012). Low exposure to heavy metals may activate antioxidant protective mechanisms but higher exposure may suppress SOD and CAT activity through the binding of heavy metal ions to the active center of these enzymes, ultimately leading to cytotoxicity (Zhang 2010; Wang et al. 2013).

Exposure to 0.5 and 1 mg L^{-1} of $CdCl_2$ for 2 days was followed by a decrease in both SOD and CAT activity in the digestive gland and kidney of *Achatina fulica* (Chandran et al. 2005). Similarly, Company et al. (2006) also found that SOD and CAT activity in the gills of the mussel *Bathymodiolus azoricus* decreased significantly ($P < 0.05$) after exposure to Cd^{2+} (100 μg L^{-1}) for 24–48 h. After 6 days, a significant increase in SOD activity occurred in the gills but there was no significant difference in CAT activity between the control and Cd^{2+}-exposed treatment groups. These results indicate that, irrespective of whether antioxidant enzyme activity is up- or down-regulated, certain dosages and durations of heavy metal exposure can have adverse effects on the activity of some antioxidant enzymes in mollusks (Chandran et al. 2005; Zhang et al. 2009; Cong et al. 2012).

Conclusions

Cadmium induced significant apoptosis and oxidative stress in the hepatopancreas of the clam *M. meretrix*, even at concentrations far below the LC_{50}. The higher the Cd^{2+} will be the more cell damage will be caused. Cd^{2+} inhibited GSH and GPx activity but increased MDA levels and caspase-3 activity. Low Cd^{2+} concentrations stimulated CAT and SOD activity, but higher Cd^{2+} concentrations inhibited the activity of these enzymes.

On the basis of these results, we expect that exposure to low levels of cadmium activates the antioxidant system but higher levels inactivate it resulting in cell damage and apoptosis. Caspase-3 activity in *M. meretrix* was enhanced at lower Cd^{2+} concentrations, suggesting Caspase-3 as a suitable biomarker for the detection of low levels of Cd^{2+} in aquatic systems.

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Compliance with ethical standards

Conflict of interest Liping Xia declares that he has no conflict of interest. Sihan Chen declares that she has no conflict of interest. Hans-Uwe Dahms declares that he has no conflict of interest. Xueping Ying declares that she has no conflict of interest. Xue Peng declares that she has no conflict of interest.

Research involving human and animal rights All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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