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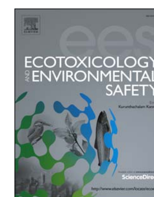

Oxidative damage, ultrastructural alterations and gene expressions of hemocytes in the freshwater crab *Sinopotamon henanense* exposed to cadmium

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ABSTRACT

Toxicity of Cd was tested with the hemocytes of the freshwater crab, *Sinopotamon henanense*, which were exposed to concentrations of 0, 0.725, 1.450, and 2.900 mg L^{-1} Cd for 7, 14 and 21 d. We investigated the effects of Cd on the total antioxidant capacity (TAC), and oxidative damage of biomarkers, such as malondialdehyde (MDA), protein carbonyl derivates (PCO), and DNA–protein crosslink (DPC). Transmission electron microscopy (TEM) was applied to assess ultrastructural changes of hemocytes. The mRNA expression levels of prophenoloxidase (*proPO*), lysozyme (*LSZ*), metallothionein (*MT*), and the activity of phenoloxidase (PO) were also determined. Our results showed that TAC was inhibited by Cd, resulting in an increase of MDA contents, PCO contents, and DPC levels in hemocytes, respectively. Ultrastructural observations revealed that chromatin condensation, nucleus deformation, mitochondrial dilation, rough endoplasmatic reticulum (rER) degranulation and secondary or tertiary lysosomes were observed in hemocytes of crabs exposed to Cd. Meanwhile, the expression levels of *proPO* were down-regulated, while the activity of PO was up-regulated in hemocytes. The expression levels of *LSZ* and *MT* were up-regulated to some extent. Our findings suggest these parameters could be used as biomarkers in the monitoring of heavy metal pollution and quantitative risk assessments of pollutant exposure.

1. Introduction

Due to anthropogenic activities from industry, agriculture and combustion, cadmium (Cd) became ubiquitous in the environment, being present in air, soil, and water. Mainly due to industrial activities, Cd contamination of the aquatic environment has increased in recent years (Pacyna et al., 2009; Yang et al., 2015), resulting in Cd pollution in aquatic systems. Thus, adverse effects of Cd are becoming a complicated environmental issue, with damage to various aquatic organisms and ecosystems. Cd could inhibit activities of antioxidant enzymes and displace Zn and Se in metalloenzymes as well as suppressing their activities (Ma et al., 2013). This in turn enhances the generation of reactive oxygen species (ROS), resulting cellular structure damage or dysfunction (Datta et al., 2000; Liu et al., 2011) or even death. Oxidative stress is recognized as an important molecular mechanism of Cd toxicity (Matović et al., 2015). Consequently, ROS induces oxidative stress by reacting with macromolecules and causing damage such as the peroxidation of lipids, destruction of protein structures, and mutations of DNA (Valko et al., 2006; Pierron et al., 2007; Wu et al., 2014).

ROS can not only harm cellular functions, but also mediates the signaling pathways of physiological activities (Pacitti et al., 2014). As a unique defense system of invertebrates, the prophenoloxidase (proPO) activating system (proPO-AS) plays a vital role in melanization as a response to environmental stress (Yang et al., 2014). The inactive proPO zymogen, which is present in granular and semi-granular hemocytes (Söderhäll and Smith, 1983), is converted to active phenoloxidase (PO), and PO oxidizes phenols to quinines, which are further polymerized to melanin (Cerenius et al., 2008; Yang et al., 2014). Thus, hemocytes have been considered as the principal source of proPO in crustacea (Cerenius and Söderhäll, 2004; Terwilliger and Ryan, 2006; Ai et al., 2009; Matozzo and Marin, 2010). Besides, lysozyme (LSZ), as a classic enzyme of lysosomes located in granular hemocytes, is released during the process of lysosomal membranes damaged by Cd exposure (Zhou et al., 2016). Lysozyme is also used as a sensitive and reliable biomarker to evaluating the toxic effects of environmental contaminants (Mondon

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et al., 2000). In addition, metallothionein (MT), a low-molecular-weight, cystein-rich and metal-binding protein, is mainly implicated in metal ion detoxification processes (Kumari et al., 1998). Importantly, it has been demonstrated that *MT* mRNA expression is predominantly regulated by metals at the transcription level (Li et al., 2011b), while the induction of *MT* is proposed to be a key role in decreasing Cd toxicity such as oxidative damage (Amiard et al., 2006; Li et al., 2015).

The freshwater crab *Sinopotamon henanense*, as an important representative of decapod crustaceans, is commonly found in the freshwaters of China. This crab was selected as an experimental animal in our research since it lives close to the sediment/water column interface of aquatic environments whereby it tends to accumulate Cd and other toxicants (Yan et al., 2008; Sun et al., 2016). It was also confirmed that Cd can accumulate in main organs such as the hepatopancreas, gills, and hemocytes of this crab (Li et al., 2015; Sun et al., 2016; Zhou et al., 2016). Previous studies have demonstrated that Cd accumulation levels could reach 12.46 ± 1.88 mg kg^{-1} in surface sediment of the Qin River, the second longest river in Shanxi Province in China, which was also the habitat of *S.henanense* (Han et al., 2008). Moreover, in some valleys near Cd-rich mines, the Cd level could reach up to 12.05 ± 1.47 mg L^{-1} in the nearby waters (Yuan et al., 2010), which posed a high ecological risk. Considering the serious deterioration of the ecological environment, we chose a series of Cd concentrations of 0.725, 1.450 and 2.900 mg L^{-1}, according to the 96 h LC_{50} for Cd to *S. yangtsekiense* (Wang et al., 2008). These concentrations may be higher than general environmental Cd levels, but were similar to or even lower than the Cd levels in a few serious pollution incidents.

The aim of this study is to investigate the total antioxidant capacity (TAC), the extend of oxidative damage to lipids (malondialdehyde, MDA), proteins (protein carbonyl derivates, PCO) and DNA (DNA–protein crosslinks, DPC) in hemocytes of *S.henanense*. Transmission electron microscopy (TEM) was also applied to assess ultrastructural changes induced by Cd exposure. The mRNA expressions of *proPO*, *LSZ*, *MT*, and the activity of PO in hemocytes of the crab were determined as well. Our results could provide the cellular and molecular evidences for the possible toxic mechanisms of Cd.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade. Cadmium chloride·$2H_2O$ ($CdCl_2·2H_2O$) was purchased from Tianjin Fengchuan Chemical Reagent Co., Ltd. 2,4-dinitrophenylhydrazine (DNPH) was bought from Tianjin Kermal Chemical Reagent Development Center. Total Antioxidant Capacity (TAC) Assay Kit and MDA test kits were obtained from the Nanjing Jiancheng Bioengineering Institute, China.

2.2. Animals and treatments

Freshwater crabs, *S. henanense*, were purchased from a local dealer in Taiyuan, at the Wu Longkou Dong - an aquatic product wholesale market in Shanxi Province of China. Prior to experiments, crabs were acclimated in aquaria (50 cm × 30 cm × 25 cm) that were filled with dechlorinated, carbon-filtered tap water (pH 7.5, dissolved oxygen 8.0–8.3 mg L^{-1}) for two weeks. The temperature was maintained at 20 ± 2 °C. Crabs were fed a commercial diet (Jin Kangda feed Co., Ltd, Jiang Su, China) twice a week during the acclimation. The water was renewed every two days.

After acclimatization, healthy adult crabs with a homogeneous weight (20.0 ± 0.5 g) were selected and randomly divided into 12 groups of 6 specimens for each treatment. The crabs were exposed to a gradient of Cd concentrations (0.725, 1.450 and 2.900 mg L^{-1}) and one control group for 7, 14 and 21 d, respectively. During the treatment period, crabs were fed twice a week. The treatment medium was changed once every two days in order to maintain the given experimental Cd concentration. All other conditions were kept like those used for acclimation.

2.3. Sample preparation

After Cd exposure, the crabs from each group were anaesthetised on ice for 10 min, and the hemolymph (at least 1000 µl/crab) was immediately drawn from the last pereiopod using a 1 mL sterile syringe containing a corresponding volume of ice-cold anticoagulant buffer (2.05*g* glucose, 0.8g citrate and 0.42g NaCl per 100 mL of double-distilled water, pH = 7.2). The hemolymph was centrifuged at 800g for 10 min at 4 °C to obtain hemocytes (10^6/mL). 200 µl of the hemocytes were for the TEM assay, 300 µl were for the RNA extraction, then 300 µl of each sample with 10^6 hemocytes/mL were homogenized using a Fluko Superfine Homogenizer at 1000 rpm for about 30 s, centrifuged at 12,000g at 4 °C, and the supernatants were aliquoted for TAC, MDA, PCO, DPC coefficient, PO activity, and protein content assays.

2.4. Total antioxidant capacity (TAC) determination

TAC was assayed by the TAC Assay Kit according to the instructions of the manufacturer. Three hundred microliters of each sample with 10^6 hemocytes/mL were homogenized using a Fluko Superfine Homogenizer at 1000 rpm for about 30 s. The samples were centrifuged at 12,000*g* at 4 °C. Then, 20 µl of catalase working solution and 170 µl 2,′-Azinobis-(3-ethylbenzthiazo-line-6-sulfonate) (ABTS) working solution were added to 10 µl each sample and incubated at room temperature for 10 min. The TAC was measured at 414 nm using a microplate reader (Spectramax M5, Molecular Devices, San Francisco, CA, USA). Trolox was used as a standard. Results were expressed as concentration of corresponding Trolox/ mg protein in each sample.

2.5. MDA, PCO and DPC assay

The content of malondialdehyde (MDA), as a convenient index for determining the extent of lipid peroxidation, was measured by the thiobarbituric reactive species (TBARS) assay, which measured the production of MDA that reacts with thiobarbituric acid, according to the method described by Livingstone et al. (1990).

Measurements of PCO and DPC were performed as described by Li et al. (2011a). PCO content was determined with 2,4-dinitrophenylhydrazine (DNPH), which reacted with protein carbonyl derivates to form 2,4-dinitrophenylhydrazone. The OD values were measured at 370 nm (Spectramax M5, Molecular Devices, San Francisco, CA, USA) and results were expressed as nmols of carbonyl groups/mg protein using a molar extinction coefficient of 22,000 M/cm for aliphatic hydrazones.

The amount of DPC was assayed with KCl–SDS, which was used to precipitate the crosslink to separate free DNA with protein-bound DNA. Hoechst 33258 was added to combine with DNA. The fluorescence values were measured at a wavelength (excitation: 350 nm; emission: 460 nm) (Spectramax M5, Molecular Devices, San Francisco, CA, USA). The ratio of fluorescence was calculated as a percentage of protein-bound DNA to total DNA.

2.6. Ultrastructural observations of hemocytes

Crabs exposed to Cd (0, 0.725 and 2.900 mg L^{-1}) for 21 d were assayed for ultrastructural changes of hemocytes. Hemocytes were fixed with an equal volume of 2.5% (v/v) glutaraldehyde for 2 h, then centrifuged at 2000 rpm for 10 min at 4 °C. The pellets were postfixed with 1% (v/v) osmic acid, dehydrated in a graded series of ethanol and acetone from 30% to 100% (v/v), and embedded in Epon 812. The samples were cut with an ultramicrotome (Leica, UC-6, Germany), stained with uranyl acetate and lead citrate, and the hemocyte specimens were observed for each group by TEM (JEM-1011, JEOL, Tokyo, Japan).

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