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Mitochondrial energy metabolism in hepatopancreas of freshwater crab (*Sinopotamon henanense*) after cadmium exposure

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Abstract Energetic homoeostasis is a fundamental requirement in the defense against cadmium (Cd) stress. Using the freshwater crab *Sinopotamon henanense* as an example, we explored the effects of Cd on submicroscopic structures in hepatopancreatic cells through transmission electron microscopy (TEM) and energy-related parameters such as adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratios, reduced/oxidized nicotinamide adenine dinucleotide ($NADH/NAD^+$) ratios, and the mitochondrial membrane potential ($\Delta\psi m$). The impact of Cd on carbohydrate and protein metabolism, and metallothionein (MT) were also investigated. An experimental 10-day exposure increased NADH, NAD^+ and provided a higher $NADH/NAD^+$ ratios and $\Delta\psi m$ in hepatopancreatic cells. Corresponding significantly increased levels of ATP and ATP/ADP ratio in hepatopancreas supported high energy production. The up-regulation of MT content in exposed crabs suggests that protein synthesis for detoxification could partially be a major ATP consumer. With increasing exposure time, however, energy production was in decline. Excessive energy consumption was explained by substrate mobilization and mitochondrial impairment. Less carbohydrate and enhanced protein catabolism was observed. Ultrastructurally, there were changes in mitochondria with swelling, membrane disruption, shortening of cristae or the rupture and disappearance of entire mitochondria. The rough endoplasmic reticulum (rER) displayed expansion and membrane rupture, suggesting the destruction of protein-synthesizing structures in hepatopancreatic cells. Our findings suggest that energy-related parameters could be used as biomarkers in the monitoring of metal pollution, and quantitative risk assessments of pollutant exposures.

Keywords Cadmium, Mitochondria, Energy equivalents, Mitochondrial membrane potential, ATP/ADP, $NADH/NAD^+$

Introduction

Over the last few years, cadmium (Cd) contamination of the aquatic environment has increased, mainly due to human industrial activities such as burning of coal in power stations and metal smelting (Jozef et al., 2009; Pirrone et al., 2010; Ciliberti et al. 2011)[1-3]. Cd pollution incidents are frequently found in aquatic systems (Bai et al., 2012; Feng et al., 2010; Lin et al., 2002)[4-6]. Some examples from China are provided by Shaoguan city of Guangdong North River in 2005, and the Longjiang Cd pollution event in Hechi, Guangxi, 2012 (Zhou et al., 2012)[7]. The Cd level could reach 12.05 ± 1.47 mg/l in some valleys near Cd-rich mines (Yuan et al., 2010)[8]. Cd pollution will have a negative impact on the fitness and survival of aquatic organisms as indicated by a decrease in biodiversity in polluted areas. A decline and extinction of some species has been reported (Han et al., 2008)[9]. Cd exposure could stimulate the formation of reactive oxygen species (ROS) such as hydroxyl radicals and singlet oxygen. ROS could cause oxidative stress by reacting with macromolecules, leading to membrane lipid peroxidation (Pan and Zhang, 2006)[10]. The cellular mechanisms of toxicity, such as Cd-induced cell necrosis (Gennari et al., 2003) and apoptosis (Liu et al., 2011)[11, 12], have been intensively studied. In addition, Cd is also reported to affect lipid, and protein metabolism in fish (De and Blust, 2001; Pierron et al., 2007)[13, 14], and birds (Lucia et al., 2010)[15].

The acute or chronic intoxication of organisms and a variety of adverse effects from Cd is becoming a global environmental problem that provides damage for aquatic organisms and aquatic ecosystems (Sousa et al. 2008; Moreirinha et al. 2011)[16, 17]. Therefore, it is necessary to develop reasonable biological markers from representative species to indicate environmental pollution in aquatic systems. Biomarkers are useful as indicators of biological states, and as indicators of biological processes, pathogenic processes, responses to stressors, and the monitoring of environmental quality (McCarthy and Shugart, 1990)[18]. In the monitoring of aquatic systems several different organisms were used as model organisms for environmental pollution studies (Nogueira et al., 2010; Oliveira et al., 2011)[19, 20]. Among them, crustaceans are considered as suitable indicators because of their sediment-associated and less mobile life styles. Some crustacean species, such as crayfish *Astacus leptodactylus* (Malev et al., 2010)[21] and the marine crab *Carcinus maenas* (Ben-Khedher et al., 2013)[22], were chosen for the study of aquatic contamination. In the present study, the freshwater crab *Sinopotamon henanense* became the subject of our molecular approaches. This sentinel crab species is widely distributed in the Yangtze

River drainage, Huaihe River drainage and Yellow River Valley of China. It lives at the interface of sediments and the water column where Cd is deposited (Zhao et al., 2012)[23]. The hepatopancreas of *S. henanense* is involved in a variety of metabolic and secretory functions, such as lipid storage and synthesis, food digestion. It also has the ability to synthesize antioxidants, and plays a major role in the detoxification of toxic compounds. Therefore, the hepatopancreas of crabs could be used as a model organ to explore novel molecular mechanisms involved in the reaction to xenobiotics.

Early laboratory studies focussed on Cd effects on the antioxidant system comprising enzymes, such as the cellular activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), malonyldialdehyde (MAD), and metallothionein (MT). These antioxidant enzymes could also be used as routine biomarkers (Ma et al., 2007; Li et al., 2008; Wang et al., 2008)[24-26]. Recently, more studies have focused on another group of biomarkers derived from energy parameters provided by the mitochondrial energy metabolism (Padmini and Usha, 2011; Sokolova et al., 2012)[27, 28]. They are considered to be more sensitive to contaminant exposure compared to routinely used antioxidant and oxidant stress parameters in biomonitoring programs. For the energy metabolism of eucaryotes, mitochondrial respiration and ATP synthesis represent two most important pathways. Actually, mitochondrial respiration is the oxidation of mitochondrial NADH. Protons coupled with NAD^+ are pumped across the mitochondrial inner membrane, generating an electrochemical gradient of protons (Δp) by the electron transport chain which leads to a membrane potential $\Delta\psi m$. Via mitochondrial ATP synthase, these protons are returning to the mitochondrial matrix across the membrane where they also generate ATP. Levels of NADH and $\Delta\psi m$ are, therefore, critical for ATP synthesis. Moreover, the main substrate for the respiratory chain is supplied by fatty acids, glucose, and amino acids through interconnected pathways. The products of ATP synthesis are used by a large number of cellular pathways. The most important process may be protein synthesis, which also provides enzymes for the defence of xenobiotics such as antioxidant enzymes, and metallothioneins (Brown, 1992; Rolfe and Brown, 1997)[29, 30].

In the present study, the ratios of ATP/ADP, $NADH/NAD^+$, and $\Delta\psi m$ were measured, and mitochondrial submicroscopic structures of hepatopancreatic cells were studied by TEM. The main objective of the present study was to examine the effects of Cd on energy metabolism. Another purpose was to explore the possible effects of Cd on carbohydrates, protein metabolism, and intracellular antioxidants.

Material and methods

Ethics statement

The place where crabs were caught is privately owned. Our study was carried out with the permission from the landowner. We also confirm that the current study did not involve endangered or protected species. The work described in this article was performed in accordance with National and Institutional guide-lines for the protection of animal welfare.

Treatments and sample preparation

Freshwater crabs, *S. henanense*, were purchased from Taiyuan Wulongkou fish market. These crabs were known to be collected from the mostly unpolluted Qinghe River near Qingyan, Henan province in China. Before the experiment, mature female crabs of comparable weight were acclimated for 3 weeks in glass aquaria (45 cm × 30 cm × 30 cm) that were filled with dechlorinated tap water that was filtered through charcoal (dissolved oxygen 8.0-8.3 mg/l, pH 7.5 ± 0.13, and temperature 20 ± 2 °C). The aquaria were shielded using a black plastic to reduce optical disturbance. After acclimation, crabs were randomly divided into a control group and three treatment groups. Crabs in each group were also placed in five different tanks with 20 individuals each.

Crabs were exposed to Cd in the water using a static renewal method. They were exposed to 1.45, 2.9, and 5.8 mg/l $CdCl_2$ (Sailboats Chemical Reagent Co., Ltd., Tianjin, China), corresponding to 1/160, 1/80, 1/40 of the 96h LC_{50} according to Wang et al. (2008) for 10, 15 and 20 days. These concentrations were similar to the Cd levels in a few serious pollution incidents. Water was renewed to clean tanks every two days with treatment batches of the same Cd concentration. During the acclimation and exposure period, crabs were fed a commercial feed (Hanye, Beijing, China), containing 30 % protein and 15 % fat every evening (5 % animal wet weight/day). Tissues were sampled after 10, 15 and 20 days. After heparin (400 units per kg body weight) was injected into the abdomen, a haemolymph sample of 0.2 ml was taken 5 min later, centrifuged to collect serum, and frozen temporarily at -20 °C for the subsequent measure of glucose. After dissection of the cephalothorax, the hepatopancreas was immediately sampled, and was also divided into several parts for the experiments.

171 Cd concentration assay in water

172 Because the treatment medium was changed every 48 h, the water samples from each group of
173 only 0 and 48 h of Cd exposure were collected, and Cd concentration in water samples was
174 determined. Cd concentration in water was measured by electrothermal atomic absorption
175 spectrophotometry (SHIMADZU AA-6300, Kyoto, Japan). The standard Cd solution (Shanxi
176 Environment Protection Department, China) was used as a control of metal concentration. Cd
177 concentration was expressed as mg/l. The same experiment was repeated three times.

178 Extraction and assay of NADH and NAD^+

179 The procedure for the extraction of NADH/NAD^+ was based on the modification described by
180 Zhao et al. (1987)[31]. Fresh hepatopancreas, 0.1 g, was homogenized in 1 ml of 0.1 mol/l HC1 for
181 NAD^+ or in 1 ml of 0.1 mol/l NaOH for NADH determination. The homogenates were then heated
182 in a boiling water bath for 5 min, cooled in an ice bath, and centrifuged at 10,000g for 10 min at
183 4 °C. Supernatants were neutralized by 0.1 mol/l NaOH or HC1, respectively, and then centrifuged
184 at 10,000g for 10 min at 4 °C. Finally, supernatants were kept on ice for NADH/NAD^+ assays. The
185 procedure of NADH/NAD^+ assays was performed according to the method described by
186 Matsumura and Miyachi (1983)[32]. Because of the light sensitivity of phenazine ethosulfate (PES)
187 and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), measurements were
188 carried out at low light. Equal volumes of 0.1 mol/l Tris-NaOH buffer, 40 mmol/l EDTA, 4.2
189 mmol/l MTT, 16.6 mmol/l PES, and 5 mol/l ethanol were mixed just before the assay, and 500 µ1
190 of this mixture was transferred to microtubes. Nicotinamide adenine dinucleotides or biological
191 samples were added to the mixture and the volume was brought to 900 µ1 with 0.1 mol/l NaCl.
192 Tubes containing the assay media were incubated for 5 min in a 37 °C water bath. Enzyme cycling
193 was initiated by adding either 100 µ1 of 100 U/ml alcohol dehydrogenase solution (ADH, Sigma
194 Chemical Co., St. Louis, MO, U.S.A.) for NAD^+ and NADH determination. With each biological
195 sample, a blank measurement was also made by adding 0.1 mol/l Tris-NaOH buffer instead of
196 enzyme. After 40 min of cycling time, the reactions were stopped by adding 500 µ1 of the 6 mol/l
197 NaCl stock solution. The microtubes with NaCl were centrifuged for 5 min at 4°C and 10,000g.
198 The supernatant was carefully siphoned off and the pellet was solubilized in 1 ml 96% ethanol.
199 Absorbance at 570 nm and spectra from 400 to 800 nm were determined using a Spectra Max M5
200 microplate reader (Molecular Devices Corp., San Francisco, CA, U.S.A.).

Assessment of mitochondrial membrane potential (Δψm) using JC-1

The method of mitochondrial isolation was a modification of the method described by Kun et al. (1979)[33]. Fresh hepatopancreas was chilled on ice and washed in a separation medium containing 0.4% bovine serum albumin (BSA) (0.22 M mannitol, 0.07 M sucrose, 0.02 M 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), 2 mM Tris-HCl, pH 7.2, 1 mM ethylenediaminetetraacetic acid (EDTA)) in a prechilled glass petri dish. Tissues were suspended with separation medium (1:20, w/v) containing 0.4% BSA and then homogenized with a prechilled glass homogenizer. The homogenate was centrifuged at 3,000 g for 1.5 min at 4 °C, and the supernatant was collected and centrifuged at 17,500 g for 2.5 min at 4 °C. The resulting pellet was washed in separation medium and then centrifuged at 17,500 g for 4.5 min. The pellet was resuspended in suspension medium (0.22 M mannitol, 0.07 M sucrose, 0.01 M Tris-HCl, pH 7.2, 1 mM EDTA and was centrifuged at 17,500 g for 4.5 min. The pellet was finally resuspended in suspension medium at a ratio of 1.0 m1 suspension medium per 1.0 g of starting material. JC-1 is an important fluorescent carbocyanine dye for the assessment of the mitochondrial membrane potential (Δψm). Depending on the mitochondrial membrane potential, it accumulates in the mitochondrial membrane in two forms (monomers or dimers). Negative potential of the inner mitochondrial membrane facilitates the formation of JC-1 aggregates, which results in a shift of JC-1 aggregate fluorescence. The measurement of the JC-1 aggregate fluorescence is a convenient and reliable method for the estimation of the mitochondrial membrane potential. According to the described requirements of the mitochondrial membrane potential assay kit with JC-1 (Beyotime Institute of Biotechnology, Jiangsu, China), 20 μl purified mitochondria sample were added to 180 μl JC-1 (1 μg/ml), and were then placed in black 96-well flat-bottom microtiter plates. The JC-1 aggregate fluorescence values were measured by spectrophotometer. The excitation and emission wavelength of the spectrophotometer was 490 nm and 590 nm, respectively. The membrane potential of the sample was expressed in the value of fluorescence emissions.

Determination of ATP/ADP ratios

According to the modified method of Anderson and Murphy (1976)[34], the ratio of ATP to ADP was determined by high performance liquid chromatography (HPLC). Hepatopancreas (0.1g) were homogenized with 1.5 ml ice-cold 0.6 mol/l perchloric acid in an ice bath for 20 min. The homogenate was centrifuged by a refrigerated centrifuge (Eppendorf 5415R, Hamburg, Germany)

for 30 min at 16,000 g at 0 °C. The clear supernatant was removed and neutralized to pH 7 using 6 mol/l KOH. After storage for 2 hours at 4 °C, the neutralized supernatant was centrifuged for 10 min at 1,468 g at 0 °C to precipitate potassium perchlorate. Potassium perchlorate was removed by paper filtration, and the clear supernatant was stored at - 25 °C. Before the measurement by HPLC, the supernatant was filtered through a 0.5 µm filter and diluted to 0.5 ml. Calibration curves were constructed using ATP and ADP standards (Sigma Chemical Co., Poole, Dorset, UK). The presence of ATP and ADP in the samples was analyzed using a HPLC system (Shimadzu, Kyoto, Japan) including a SCL-10Avp controller, a LC-10Avp pump, and a SPD-10Avp UV-visible spectrophotometric detector with the peaks being detected and analyzed at 254 nm. Chromatographic separation was achieved on a Diamonsil 5U C18 column (250 mm × 4.6 mm, Dikma technologies Inc., USA). The mobile phase consisted of 0.5% methanol of 0.05 M ammonium dihydrogen phosphate pH 6.0 (adjusted using 1 mol/l KOH). The flow rate of the mobile phase was 1.0 ml/min, and the injection volume was 10 µl. The retention times for ATP and ADP were 9.3 and 11.5 min, respectively. ATP and ADP in the samples were identified by comparison with the retention time of standards, while their concentrations were determined using an external standard method. The results were expressed in terms of micrograms of ATP or ADP nmol/mg protein from which the ratio of ATP to ADP was calculated. The protein concentration in the tissue was determined using the method of Bradford (1976)[35] where bovine serum albumin was used as a standard.

Transmission electron microscopy

Following the method of Liu et al. (2013)[36], fresh hepatopancreas were immediately fixed in 2% (v/v) glutaraldehyde for 2h and postfixed in 1% (v/v) osmic acid. After dehydration in a graded series of acetone, the samples were rinsed in propylene oxide and infiltrated with epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and electron micrographs were prepared with a transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan).

Biochemical analysis

The glycogen concentration in hepatopancreas was measured using the method of Seifter et al. (1950)[37]. Tissue digestion was performed by the addition of a concentrated sodium hydroxide solution (3 µl/ 1 mg wet tissue) and heating for 20 min at 100 °C. After cooling, the glycogen

hydrolysis was colored with 0.2 % anthrone sulfuric acid in a boiling water bath. The colored mixture was determined spectrophotometrically by absorption at 620 nm using glucose as standard. The glycogen level was expressed as mg/g tissue. The alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) activities of homogenates were measured by commercially available kits (Jiancheng Nanjing Institute of Bio-engineering, Nanjing, China). Alanine and aspartate were used as the substrates of ALT and AST, respectively. For the measurement of each enzyme activity, relevant substrate, ketoglutarate and assayed sample were incubated at 37 °C for 30 min. The reaction was stopped with 2, 4-dinitrophenyl hydrazine. The substance was colored with sodium hydroxide and spectrophotometrically assayed at 505 nm. ALT and AST enzyme activities were expressed as U/mg protein.

MT Measurement by enzyme linked immunosorbent assay (ELISA)

Hepatopancreas were homogenated in precooled homogenization buffer (1:9, w/v) including 0.5 mol/l NaCl, 0.26 mol/l EDTA, 0.1mmol/l phenylmethylsulfonyl fluoride (PMSF), and 0.02 mol/l Tris-HCl pH 7.6. The homogenate was centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was collected and stored at -20 °C for MT measurement. Polystyrene microtiter plate (Corning, NY, USA) was coated with different concentrations of MT standard ranging from 3.90 and 500.00 ng/ml or samples diluted by coating buffer (50 mM sodium carbonate, pH 9.6). After 12 hours at 4 °C, the coated liquid was discarded, and the plate was washed 5 x with PBST (10 mM PBS in 0.05% Tween-20, pH 7.2). The nonspecific binding sites were blocked by incubating the plate with PBST containing 1% bovine serum albumin (BSA) for 2 h at 37 °C. After 5 x washings with PBST, anti-MT polyclonal antiserum raised in mouse and diluted to 1:160000 with 1% BSA was added, and the plate was incubated for 2 h at 37 °C. Plates were then washed 5 x with PBST, and incubated for 1 h with goat antimouse IgG-HRP (Sigma, St. Louis, MO, USA) diluted to 1:2000 with the 1% BSA. 3, 3', 5, 5' -tetramethylbenzidine (TMB) chromogenic reagent (Solarbio, Beijing, China) was added to the plate after washing 5 x with PBST. After incubation for 30 min, 50 µl of 2 M H_2SO_4 was added to plots to stop the reaction. The absorbance was read at 450 nm on a spectrophotometer.

Data analysis

Statistical computations were performed with SPSS 17.0 (SPSS Inc., Chicago, USA). Data

distributions and the homogeneity of variance were tested using the Kolmogorov–Smirnov and Levene tests. Significant differences between treated and control groups were calculated by one-way ANOVA analysis with a Dunnett's post-hoc test. If the prerequisites for parametric tests could not be satisfied, differences were tested using non-parametric ANOVA analyses with Kruskal-Wallis test. All the data representing mean values of five independent sets of experiments are given as means ± standard deviation (S.D). Probability values less than 0.05 were considered as significant.

Results

Cd concentration assay in water

Cd concentrations in the water from each treatment group after 0 and 48h of subchronic Cd exposure were presented in Table 1. At 0 h, no significant changes in the Cd concentration from each treatment groups were observed compared to the nominal exposure concentration. However, the Cd concentrations in water of all treatment groups declined significantly (p<0.05) after 48h of Cd exposure compared with the nominal exposure concentration. The experiment was repeated three times with similar results.

Tab. 1. Cd concentration analysis in water of freshwater crab (*Sinopotamon henanense*) exposed to subchronic Cd.

Nominal exposure concentration (mg/l)	Measured exposure concentration in water (mg/l)					
	1-0h	1-48h	2-0h	2-48h	3-0h	3-48h
Control	0	0	0	0	0	0
1.45	1.39 ± 0.04	1.02 ± 0.02*	1.42 ± 0.01	0.98 ± 0.03*	1.44 ± 0.05	0.91 ± 0.02*
2.9	2.83 ± 0.03	2.01 ± 0.05*	2.78 ± 0.07	1.95 ± 0.08*	2.84 ± 0.05	2.12 ± 0.10*
5.8	5.72 ± 0.08	4.56 ± 0.11*	5.83 ± 0.10	4.63 ± 0.09*	5.78 ± 0.13	4.58 ± 0.07*

Data are expressed as mean ± standard deviation (n = 5). Statistical significance is denoted by * p < 0.05 compared to the respective nominal exposure concentrations.

Impact of Cd stress on NADH/NAD$^+$ and Δψm

The NADH, NAD$^+$, and NADH/NAD$^+$ ratios were measured as an important H^+ carrier during mitochondrial energy metabolism. As shown in Fig. 1A-1C, all three indexes were higher than those of the controls after 10-day Cd exposure. With increasing exposure time, the levels decreased, while no significant difference was observed at 15-day exposure. The levels in 20-day exposed crabs showed a significant decrease compared to that of the control, 10- and 15-day. Mitochondrial membrane potential was determined by evaluating the changes of JC-1 aggregate fluorescence. As shown in Fig. 1D, the value of JC-1 aggregate fluorescence in hepatopancreas showed a close trend with the NADH/NAD$^+$ ratio. Compared to the control at 10-day exposure, a significantly increased value suggested an increase of Δψm. With time, Δψm in exposed crabs decreased, and a significantly lower value of JC-1 aggregate fluorescence was observed in that of 20-day exposure.

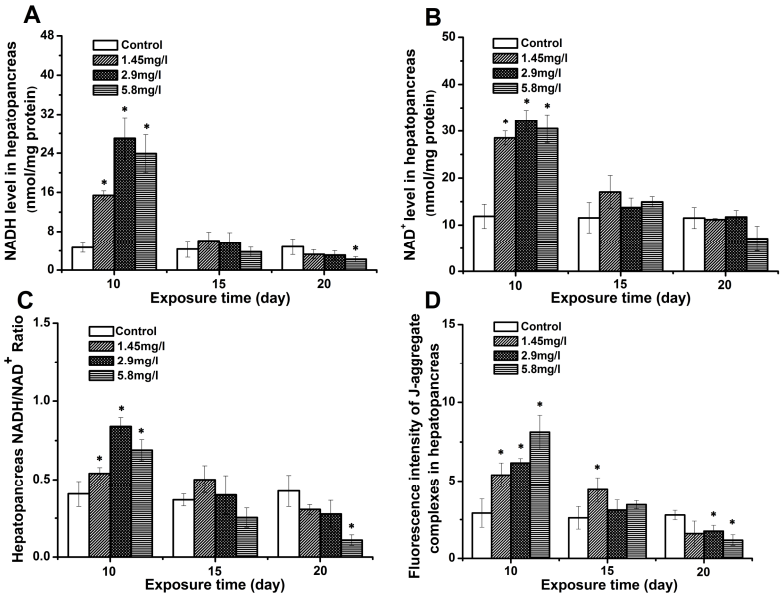


Fig. 1. The level of NADH (A), NAD (B), NADH/NAD ratio (C), and fluorescence intensity of J-aggregate complexes (D) in hepatopancreas of control and Cd-exposed crabs after 10, 15 and 20 days of experimentation. Data are expressed as mean ± standard deviation (n = 5). Comparison between the control and treatment groups is notified by * $p < 0.05$.

Bioenergetic status in response to Cd stress

To evaluate the impact of Cd stress on the crab energy status, the levels of ATP, ADP and the ratio of ATP to ADP in hepatopancreas were measured by HPLC (Fig. 2). After 10-day exposure,

the levels of ATP and ADP of hepatopancreas (Fig. 2A; Fig. 2B) in exposed crabs increased significantly, and the ATP/ADP ratio of hepatopancreas (Fig. 2C) was higher than that of the control. As the exposure time elapsed, the ATP and ADP levels were decreasing compared to a 10-day exposure. However, the ADP level at 15-day exposure was still higher than that of the control, leading to a decreased ATP/ADP ratio. A significant decrease was also observed lowest levels at 20-day exposure.

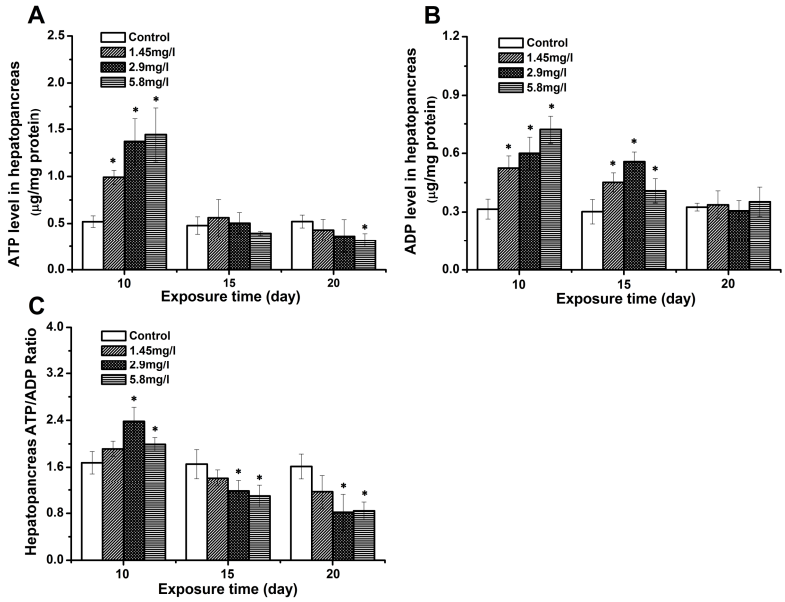


Fig. 2. The levels of ATP (A), ADP (B) and ATP/ADP ratio (C) in the hepatopancreas after 10, 15 and 20 days for control and Cd-exposed crabs. Data are expressed as mean ± standard deviation (n = 5). Statistical significance is denoted by * $p < 0.05$ compared to the respective control.

Ultrastructural observations

Fig.3 and Fig.4 showed the effects of Cd on submicroscopic structures of hepatopancreatic cells in the control and 5.8 mg/l Cd exposure group. In mitochondria of normal hepatopancreatic cells discernible cristae and moderate electron dense matrices were observed (Fig. 3A). After 10-day Cd exposure, numerous mitochondria and mitochondrial cristae were found to be well-preserved in the cytoplasm of hepatopancreatic cells at the 5.8 mg/l Cd concentration (Fig. 3B), and no apparent changes appeared. However, mitochondrial injury in hepatopancreatic cells appeared gradually with increasing Cd exposure time. Volume expansion and swollen matrices were observed in some mitochondria of 15-day Cd exposed crab (Fig. 3C). Following exposure for 20-day, volume expansion and swollen matrices in more mitochondria could lead to a disturbance in

mitochondrial morphology, such as mitochondrial membrane disintegration and the disappearance of cristae (Fig. 3D). As shown in Fig. 4A, the normal rER present as single short cisternae is distributed throughout the cell, and is generally associated with mitochondria. In 10- and 15-day Cd exposed crabs, normal rER and large numbers of ribosomes on the surface of the rER could be found (Fig. 4B; Fig. 4C). However, dilation and vesiculation of rER cisternae were frequently observed after 20-day Cd exposure. Membrane degranulation and breaks of rER became visible, and some ribosomes were detached from the surface of the rER (Fig. 4D).

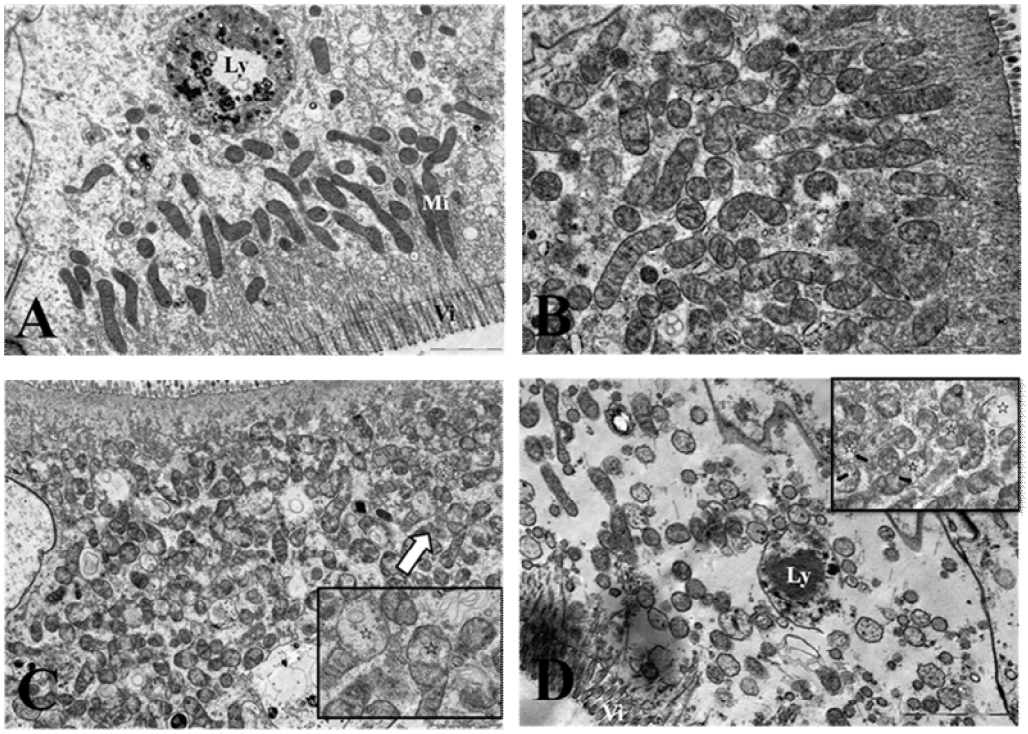


Fig. 3. Effects of Cd on the ultrastructure of the hepatopancreatic mitochondria of *S. henanense*. (A) Normal mitochondria. Bar = 2 μm. (B) Mitochondria in the Cd 5.8 mg/l of the 10-day exposure group. Bar = 2 μm. (C) Minor mitochondrial injury in the Cd 5.8 mg/l of the 15-day exposure group. Bar = 2 μm; Mitochondria on the upper left corner shows an edematous mitochondrial matrix. Bar = 1 μm. (D) Mitochondria in the Cd 5.8 mg/l of the 20-day exposure group. Bar = 2 μm. Mitochondria on the upper right corner show a serious edematous mitochondrial matrix and a fragmented outer membrane. Bar = 1 μm. (Vi: microvillus; Mi: mitochondria; Ly: lysosome; ☆: edematous mitochondrial matrix; ⬆: mitochondrial outer membrane damage)

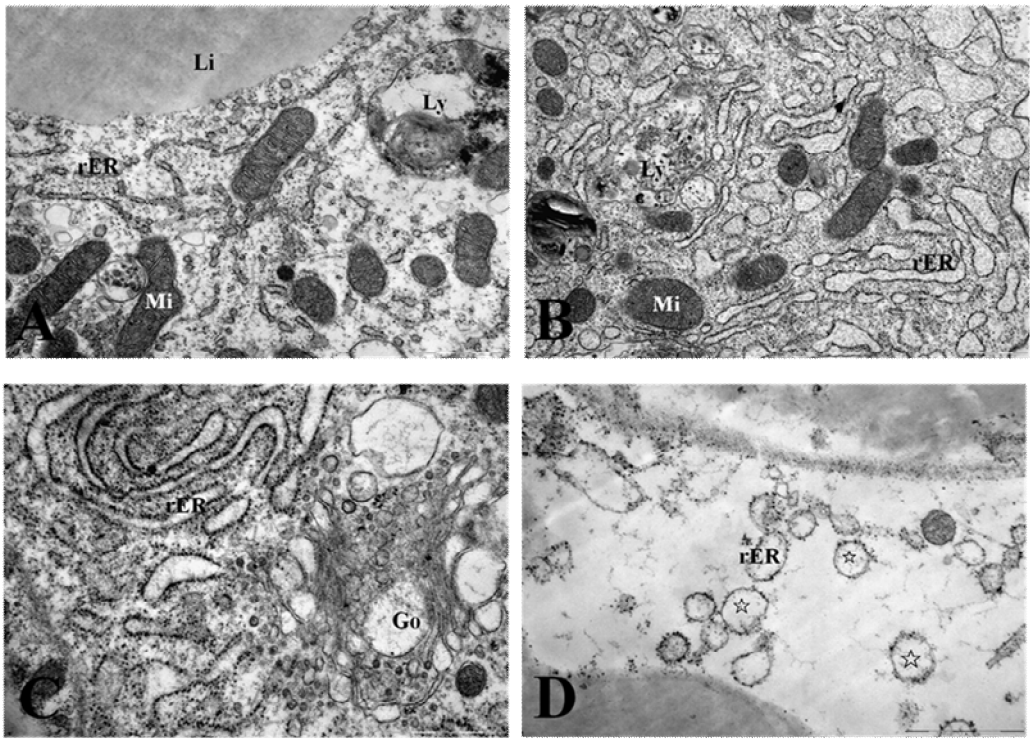
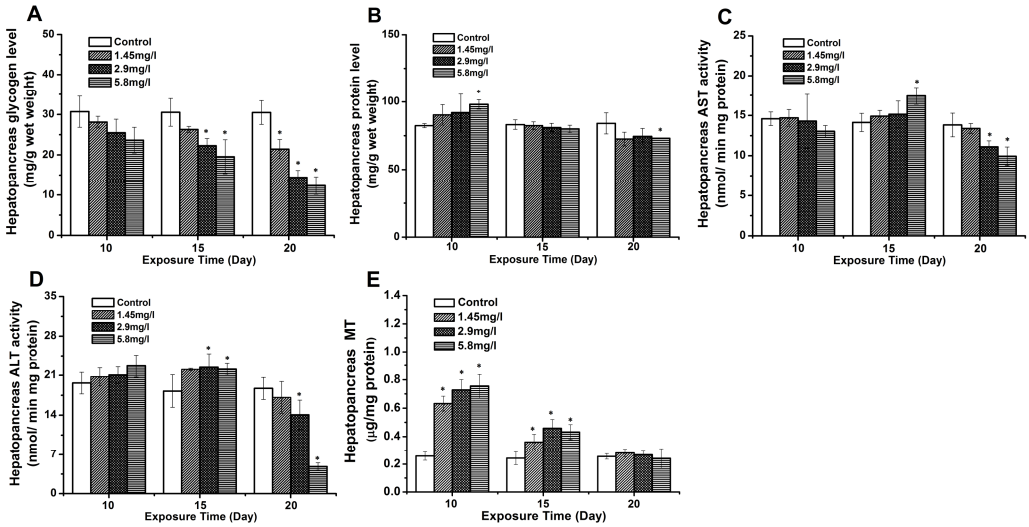


Fig. 4. Effects of Cd on the rough endoplasmic reticulum and ribosome of *S. henanense*. (A) Rough endoplasmic reticulum, ribosome and mitochondria in normal group. Bar = 1 um. (B) Rough endoplasmic reticulum and ribosome in the Cd 5.8 mg/l of 10-day exposure group. Bar = 1 um. (C) Slightly damaged Golgi complex in the Cd 5.8 mg/l of 15-day exposure group. Bar = 1 um. (D) Damaged rER in the Cd 5.8 mg/l of 20-day exposure group. Detached ribosomes from rER were observed. Bar = 2 um. (rER: rough endoplasmic reticulum; Mi: mitochondria; Li: lipid; Ly: lysosome; Go: Golgi complex; ☆: rER with few ribosomes)

Effect of Cd on carbohydrate, protein metabolism and MT synthesis of *S. henanense*

Significant decreases in hepatopancreatic glycogen was observed at 15- and 20-day, and showed a time- and dose-dependent pattern (Fig. 5A). Protein levels in hepatopancreas in the 5.8 mg/l Cd concentration group at 10-day exposure were higher than those of the control, and also showed decreasing levels with time (Fig. 5B). Correspondingly, increasing activities of the ALT and AST were observed at 10- and 15-day exposure. However, both enzymes activities were significantly down-regulated at 20-day compared to the control (Fig. 5C, Fig. 5D). Fig. 5E showed changes of hepatopancreatic MT levels in Cd exposed crabs. The MT levels in hepatopancreas of 10-day exposed crabs were higher than the control, and showed a dose-dependent pattern. A significant up-regulation of MT levels was observed at 15-day, while these levels were lower than those at 10-

384 day. Though no significant difference was observed at 20-day exposure to that of the control, the
385 down-regulation of MT levels at 20-day was still lower compared to that of 10- and 15-day.



386

387 **Fig. 5.** Effect of Cd on carbohydrate, protein metabolism, and of *S. henanense*. Hepatopancreatic
388 glycogen (A), protein level (B), AST activity (C), ALT activity (D) and MT levels (E) after 10, 15,
389 and 20 days of experimentation. Data are expressed as mean ± standard deviation (n = 5).
390 Comparison between the control and treatment groups is notified by * $p < 0.05$.

391 **Discussion and Conclusion**

392 The reduction of Cd concentration in water from treatment groups after subchronic Cd exposure
393 indicated that Cd from the ambient water could be transferred to the body of the crabs. These
394 observations were supported by our earlier study of Yang et al. (2013)[38] and Ma et al. (2007)[24],
395 which demonstrated the high Cd accumulation in tissues of exposed crabs. Moreover,
396 hepatopancreas were found to be the major targets for Cd distribution. In the present experiments,
397 we found a variation of the bioenergetic status as a response to Cd stress, suggesting the
398 interference of metabolic functions and energy balance in organisms. After 10-day exposure,
399 higher levels of ATP, ADP, and the ratios of ATP/ADP in hepatopancreas of exposed crabs
400 suggested an enhanced energy metabolism. However, with increasing exposure time, decreased
401 energy production was observed. A failure of the cellular energy metabolism could be one of the
402 factors of early cell damage upon xenobiotic exposure (Sims and Muyderman, 2010)[39]. A severe
403 depletion of ATP might result in the dysfunction, destabilization of several regulatory pathways
404 mediated by enzymes and the impairment of lipids (Lucia et al., 2010; Pike et al., 2011)[15, 40]. A
405 longer exposure time or an increase in reactive species induced by pollutants and various other

406 sources caused a transient drop in cellular ATP (Kabakov et al., 2002)[41]. The latter report
407 confirmed observed results of decreased ATP and increased ADP and hence altered ATP/ADP
408 ratios in response to 15- and 20-day Cd stress in the hepatopancreas of exposed crabs. By the
409 synthesis of ATP, mitochondria are the major supplier of energy. By TEM studies, a specific
410 cellular ultrastructural finding and difference between exposed and control crabs was revealed.
411 With the time of Cd exposure, serious mitochondrial injury in hepatopancreatic cells appeared
412 gradually, such as volume expansion, and swollen matrices of mitochondrial membrane
413 disintegration. A reduction and disappearance of the cristae together with mitochondrial membrane
414 disintegration could contribute to less ATP production. Mitochondrial impairment could lead to a
415 significant disturbance in mitochondrial morphology. In accordance to our results, Martin and
416 Forkert (2004)[42] and Padmini and Usha (2011)[27] also found that the dysfunction of hepatic
417 mitochondria resulted from the impairment of mitochondria caused by environmental
418 contaminants.

419 $\Delta\psi m$, reflecting the pumping of hydrogen ions across the inner membrane during the process
420 of electron transport and oxidative phosphorylation, is the driving force behind mitochondrial ATP
421 production and also a key indicator of cellular viability. Hence, a mitochondrial dysfunction
422 associated with the impairment of mitochondrial membranes and a disturbance in membrane
423 potential causes abatement in ATP production. Accordingly, changes of hepatopancreatic
424 mitochondria in Cd exposed crabs could affect the level of $\Delta\psi m$ and the $NADH/NAD^+$ ratio.
425 Moreover, the level of $\Delta\psi m$ and $NADH/NAD^+$ ratio in the present study provided a positive
426 correlation with ATP levels in both control and exposed crabs, reflecting a disturbance in ATP
427 production during alterations in $\Delta\psi m$ and NADH. This view has also been supported in a study on
428 human leukemic cell lines (Bradbury et al. 2000)[43].

429 The main substrate for the respiratory chain was supplied by glucose and amino acids by two
430 interconnected pathways. It was necessary, therefore, to study the relationship between substance
431 metabolism and mitochondrial energy metabolism. For most organisms, carbohydrates provided
432 the immediate source of energy (Batatinha et al., 2013)[44]. Under Cd stress conditions,
433 carbohydrate reserves are mobilized to meet the energy demands (Pretto et al., 2014)[45]. In
434 accordance to this, Cd-exposed crabs typically contain fewer carbohydrates in hepatopancreas
435 compared to the control. This indicates increased energy demands triggered by Cd. Elevated
436 cellular energy demands for the basal maintenance often leads to changes in energy allocation in

order to cover maintenance needs in the biochemical defence of Cd stress.

Cd could also result in the upregulation of cellular protective mechanisms, such as antioxidant defense systems and cellular repair pathways (Cherkasov et al., 2006; Ivanina and Sokolova, 2008)[46, 47]. Cd-induced expression of various proteins of functional importance, such as the SOD, GSH-Px, CAT, and MT, could be observed in tissues of the freshwater crab *S. henanense* (Li et al, 2008; Wang et al., 2008)[25, 26]. Among them, MT is one of the important metal-binding proteins and also plays a key role in the defense against Cd-stress. In the present study we found an up-regulation in the expression of MT in the hepatopancreas following exposure to Cd stress. Hand and Hardewig (1996)[48] and Cherkasov et al. (2006)[46] showed that protein synthesis is a major ATP sink in the total ATP consumption of organisms, especially under stress. Thus, the above mentioned elevated basal metabolism, specifically the up-regulation of protein synthesis involved in the defense against Cd-stress during 10-day Cd exposure, could be an important energy consumption mechanism during exposure to pollutants (Larade and Storey, 2007)[49]. However, the protein in hepatopancreas of 15-day exposure showed decreasing levels compared to that of 10-day exposure. Accordingly, the activity of ALT and AST, two main aminotransferases, were up-regulated after 15-day Cd exposure. The transamination being an important pathway for the amino acid metabolism may be suggestive of an increasing utilization of amino acids during Cd exposure. Prolonged 20-day Cd exposure resulted in the destruction of protein-synthesizing structures, such as membrane degranulation of rER and a loss of ribosomes detached from the surface of the rER, which may further affect the protein synthesis and the ability to metabolize amino acids within the hepatopancreas.

In the present study, a significant disturbance in hepatopancreatic morphological structures was observed in the hepatopancreas of exposed crabs compared to the control. The analysis of three stress parameters namely ATP/ADP ratio, $NADH/NAD^+$ ratio and $\Delta\psi m$ at the same experimental conditions revealed a significant alteration in the bioenergetic status, and enhanced induction of MT in exposed crabs in response to Cd stress. Hence, evaluation of these stress parameters as biological markers could provide quantitative risk assessments of potentially deleterious environmental exposures in comparison with routinely used biomarkers in biomonitoring programs.

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Environmental impact statement

The intoxication of organisms and a variety of adverse effects from Cd is becoming a global environmental problem that provides damage for aquatic organisms and aquatic ecosystems. It is necessary to develop reasonable biological markers from representative species to indicate environmental pollution in aquatic systems. Therefore, the main objective of the present study was to examine the effects of Cd on energy metabolism, carbohydrates, protein metabolism, and intracellular antioxidants. Another purpose was to provide quantitative risk assessments of potentially deleterious environmental exposures in comparison with routinely used biomarkers in biomonitoring programs by evaluation of these stress parameters.