Impairment of the Mitochondrial Oxidative Phosphorylation System and Oxidative Stress in Liver of Crucian Carp (*Carassius auratus* L.) Exposed to Microcystins

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ABSTRACT: Hepatotoxic microcystins (MCs) are produced by cyanobacteria in diverse water bodies and the pathophysiology includes induction of reactive oxygen species and adenosine triphosphate (ATP) depletion in cells. In this study, we evaluated MCs induced changes in the oxidative phosphorylation (OXPHOS) system in mitochondria of crucian carp liver. Fish were subdivided into two groups that were intraperitoneally injected with two doses of MCs (50 and 200 MC-LReq μg/kg bw) and were sacrificed at 1, 3, 12, 24, and 48 h postinjection. The activities of five enzyme complexes of electron transport chain and mRNA expression of mitochondrial-encoded genes (*cox1, cox2, cox3,* and *atp6*) were significantly reduced in a time-dependent pattern after injection. There were also changes in mitochondrial ultrastructure, decreases in ATPase activities and reduction in antioxidant level after MCs exposure. Disorder in the OXPHOS system and decreased activities of antioxidative enzymes might contribute to bioenergy deficiency and consequent hepatocyte damage induced by MCs. © 2011 Wiley Periodicals, Inc. Environ Toxicol 29: 30–39, 2014.

Keywords: microcystins; mitochondrion; impairment; OXPHOS system; crucian carp

INTRODUCTION

In recent decades, cyanobacterial blooms in water have generated great public concerns worldwide due to their ability to produce toxic substances known as cyanotoxins. Among these toxins, microcystins (MCs) are considered to be the most common and potent (Howard and Boyer, 2007). Many studies have confirmed the toxicity of MCs to different organisms including humans (Jochimsen et al., 1998; Chen et al., 2009; He et al., 2010; Rogers et al., 2011). It is well known that MCs inhibit protein phosphatases 1 and 2A, leading to increase in protein phosphorylation (Gulledgea et al., 2002).

Aquatic organisms are exposed to MCs by different routes. They can come into direct contact with toxins after the senescence and lysis of cyanobacterial cells (Amado and Monserrat, 2010). Evidence abound that the adverse effects of MCs on mammals and fish are closely related to oxidative stress processes and apoptosis (Brzuzan et al.,

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2009; Puerto et al., 2009; Zhao et al., 2009). Mitochondrial impairment is considered a significant contributor to the formation of reactive oxygen species (ROS) and the regulation of apoptotic pathway (Indo et al., 2007). As a primary site of cellular energy generation and oxygen consumption, the mitochondrion presents itself as a likely target for various toxins or threats, which may explain cellular toxicity of toxins (Binukumar et al., 2010).

Oxidative damage accumulates more in mitochondria because electrons continually leak from the electron transport chain (ETC) (Ott et al., 2007). This oxidative damage may modify mitochondrial proteins, DNA and lipids, which may lead to mitochondrial bioenergetic failure, and consequently to necrosis or apoptosis (Chuang, 2010). The mitochondrial oxidative phosphorylation (OXPHOS) machinery, residing in the inner membrane, is composed of five multi-subunit complexes. Damage to any part of the electron transport machinery could cause mitochondrial impairment and cellular dysfunction (Reinecke et al., 2009; Tripathy and Mitra, 2010).

Previous studies strongly suggest that mitochondria play a critical role in MC-induced damages (Ding and Ong, 2003; Wei et al., 2008). However, MC-induced mitochondrial impairment and subsequent contribution to hepatocyte damages are still largely unknown. There have been limited *in vivo* studies on the toxic effects of MCs on the OXPHOS system, especially in aquatic organisms. Ding and Ong (2003) pointed out that the disruption of ETC may be associated with MC-induced apoptosis. Our previous study showed that MCs exposure impaired mitochondria in mammals (Zhao et al., 2008). To broaden the understanding of mitochondrial changes induced by MCs, we investigated the changes in the mitochondrial OXPHOS system in hepatocytes of fish exposed to MCs, by detecting both the activities of mitochondrial core enzymes and mRNA expression of some mitochondrial-encoded genes. This information may be helpful in understanding the general cellular and molecular mechanisms of MC-induced hepatotoxicity in animals.

MATERIALS AND METHODS

Toxin

The cyanobacterial material used in this experiment was collected from surface blooms of Lake Dianchi, Yunnan in China. MCs were extracted from freeze-dried crude algae three times with 5% acetic acid and extracts were suspended in distilled water for the toxic experiment. MCs were quantitated using a reverse-phase high-performance liquid chromatography (LC-20A, Shimadzu Corporation, Kyoto, Japan). MCs concentrations were determined by comparing the peak areas of the test samples with those of the standards available (MC-LR and MC-RR, Wako Pure Chemical Industries, Japan). The toxin-containing solution was finally diluted with distilled water to 167.7 μg/mL MC-RR and 47.0 μg/mL MC-LR.

Animals and Their Care

Healthy crucian carp weighing 240.0 $\pm$ 20.4 g obtained from a local fish hatchery in Wuhan City, China, were acclimated in a 150 L (95 cm L $\times$ 55 cm W $\times$ 40 cm H) aquarium containing dechlorinated tap water for 14 days. During the acclimation period, dissolved oxygen value in the water was maintained at between 6.2 and 7.3 mg/L by continuous aeration. The fish were fed with commercial crucian carp food at a rate of 2.0% of body weight per day. Feeding was terminated 2 days before initiation of the experiment, and no food was supplied to fish throughout the experiment.

Exposure and Sampling

After acclimation, specimens of crucian carp were divided into three groups, one control and two treated ones that received intraperitoneal (i.p.) injections of MCs. Six acclimated fish without administration were expressed as 0 h and were sampled 2 h before injection. Fish in the low-dose group ($N = 30$) and high-dose group ($N = 30$) were injected i.p. with crude MCs extractions (mainly MC-RR and -LR) at 50 and 200 MCLReq μg/kg bw (body weight), respectively; and the control fish ($N = 30$) were injected i.p. with equal volume of distilled water. Fish in each dose group and the control were subdivided equally into three aquariums. Five sampling points were set during a period of 48 h in the experiment (1, 3, 12, 24, and 48 h post treatment). At each sampling point, six individual fish from each group were sacrificed. Our previous study (Zhang et al., 2007) showed that the dose of MCs lethal to 50% of the crucian carp population is well above 200 MCLReq μg/kg bw.

Isolation of Mitochondria

Mitochondria were isolated from fish liver by standard differential centrifugation according to the method of Frezza et al. (2007). Livers were homogenized in 0.1 g/mL ice-cold homogenization medium (215 mM mannitol, 0.75 M sucrose, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM ethylene diamine tetra-acetic acid (EDTA), 0.1% bovine serum albumin (BSA), pH 7.4) by homogenizer. Homogenate was centrifuged at 1000 $\times$ g for 10 min at 4°C. Pellet was resuspended in isolation buffer and centrifuged again at 10 000 $\times$ g for 10 min at 4°C. The brown mitochondrial pellet was washed twice and resuspended in 0.5 mL of respiratory buffer (70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.1 mM EDTA,

TABLE I. All the primers used in the real-time PCR

Target Gene	Primer Sequence (5′-3′) Forward	Reverse	Size (bp)
cox1	CCCACGCAGGAGCATCAGT	AGAAGGAGGACGGCGGTT	178
cox2	CCAGTCCGTGTCTTAGTATCCG	CGTGAGGCGATGAAAGCAGT	119
cox3	TACCACTTTACATCCGAAC	GAGCCTCATCAATAGATAGAC	110
atp6	AGGAGGACATAAATGAGCC	AATCAGTGGGATGGGTGT	226

pH 7.4). Mitochondrial protein was determined by the method of Lowry et al. (1951) using BSA as standard.

Transmission Electron Microscopic Observation

The dissected liver sections were prefixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C. Postfixation was in cold 1% aqueous osmium tetroxide for 1 h. After rinsing with phosphate buffer again, the specimens were dehydrated in a graded ethanol series and then embedded in Epon/812. Ultra-thin sections were sliced with glass knives on a LKB-V ultramicrotome (Nova, Sweden), stained with uranyl acetate and lead citrate and examined under a HITACHI, H-600 electron microscope.

Analysis of mRNA by Real-Time PCR

Total RNA Extraction and cDNA Synthesis

All fish were killed and their livers were quickly removed, minced, and stored frozen in liquid nitrogen. Total RNA was isolated from 50 to 70 mg sections of liver using Trizol reagent (Invitrogen) and quantified by determination at OD260. The purified total RNA (2 μg) was then reverse transcribed using first strand cDNA synthesis kit (TOYOBO, Japan). The resultant cDNA was then diluted 20-fold and kept at −20°C for later use.

Quantitative Real-Time PCR

The primers were designed based on the gene sequence of carp present on the NCBI website (http://www.ncbi.nlm.nih.gov). All the primers used in the real-time PCR are listed in Table I. Specification of each pair of primers was tested by randomly sequencing three clones, and further confirmed by the melting curve analysis using quantitative real-time PCR (Q-PCR). The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid. Only primers with similar amplification efficiency were used in this experiment. One commonly used housekeeping gene GAPDH was used in analyzing samples. The cycling parameters for PCR were as follows: 4 min at 94°C, 40 cycles of 20 s at 94°C, 20 s at 60°C or 62°C, and 25 s at 72°C. Real-time PCR reactions were performed in triplicate for each cDNA sample, and melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Data were analyzed with Option Monitor software 2.03 Version (MJ Research, Cambridge, MA).

Enzyme Assays

The measurement of the enzyme activity was performed as described by Jarreta et al. (2000) with slight modifications. NADH dehydrogenase (complex I) activity was measured in 20 mM potassium phosphate (pH 7.4), 0.1 mM NADH, and 1 mM potassium ferricyanide. Ferricyanide reduction was followed at 420 nm. Succinate dehydrogenase (SDH, complex II) activity was determined according to the kit protocol (Nanjing Jiancheng Bioengineering Institute, China). Cytochrome c reductase (complex III) activity was measured in a solution containing 1 mM EDTA, 250 mM sucrose, 2 mM KCN, and 50 μM oxidized cytochrome c. After the addition of 80 μM reduced DB (DBH_2), reduction of cytochrome c was measured at 550 nm. Cytochrome c oxidase (COX, complex IV) activity was assayed in 25 mM sucrose, 120 mM KCl, 0.025% *n*-dodecyl-β-D-maltoside, and 30 μM reduced cytochrome c. The oxidation of cytochrome c was measured at 550 nm. Adenosine triphosphate (ATP) synthase (complex V) was determined by using a glucose/hexokinase trap system, according to the method of Feniouk et al. (2007). Phosphorylation was determined in terms of disappearance of inorganic phosphorus.

Lipid Peroxidation and Antioxidant Activities Analyses

Lipid peroxidation (LPO) level in mitochondria was determined using the malondialdehyde (MDA) assay kit (Nanjing Jiancheng Bioengineering Institute, China). And LPO was calculated as nanomoles of MDA per milligram of protein. Mitochondrial superoxide dismutase (SOD) activity was measured using the xanthine oxidase-cytochrome c method as described by McCord and Fridovich (1969) with some modifications. SOD activity was determined spectrophotometrically at 505 nm. Glutathione peroxidase

(GPx) activity was estimated by the method of Flohe and Gunzler (1984). The decrease in absorbance was monitored at 340 nm.

Measurement of ATPase Activities

Na^+-K^+-ATPase activity determination was performed according to the kit protocol (Nanjing Jiancheng Bioengineering Institute, China). Ca^{2+}-Mg^{2+}-ATPase activity was determined by measuring the initial rate of release of Pi from ATP. The assay was measured according to the method of Castilho et al. (2001). The reaction mixture consisted of 4 mM disodium ATP, 4 mM magnesium chloride, 35 mM potassium chloride, 0.3 M sucrose, 1 mM EGTA, 200 μM sodium vanadate, 4 μg/mL oligomycin, 10 mM Mops and Tris (to adjust pH to 7.4). The assay was initiated by the addition of melanosomes in a total volume of 0.4 mL. Phosphate released is proportional to time up to 60 min of incubation. ATP free samples were used as controls for Pi content.

Statistical Analysis

The data were tested for statistical differences by one-way ANOVA followed by Duncan's multiple comparison test using STATISTICA software package (Version 6.0, Statsoft, Tulsa, Oklahoma) to compare data of control fish and the ones exposed to MCs. Statistical differences were determined at the $P < 0.05$ and $P < 0.01$ levels for all analysis.

RESULTS

Ultrastructural Change of Mitochondria

Mitochondrial damage was severe at 3 and 12 h with obvious vacuolization and loss of cristae and matrix, while at 24 and 48 h the vacuolization disappeared and the cristae became visible in both low and high MCs groups (Fig. 1). In the high dose group, mitochondria lost their regular morphology and were highly swollen at 3 h [Fig. 1(F)] while at 48 h, mitochondria showed compact and concentrated forms [Fig. 1(I)] after MCs exposure.

mRNA Expression of Mitochondrial-Encoded Genes (*cox1, cox2, cox3, and atp6*)

The expression pattern of mRNAs for mitochondrial-encoded genes (*cox1, cox2, cox3*, and *atp6*) of the ETC complexes IV and V is shown in Figure 2. The expression of four genes slightly changed in the low dose group compared with the control at 1 h. All the genes significantly decreased their mRNA expression as the exposure time prolonged.

Respiratory Chain Enzymes

Activities of all enzymes decreased at 1, 3, 12, and 24 h, and then returned to the control levels at 48 h after MCs exposure. Activity of mitochondrial NADH dehydrogenase in the low dose group was significantly decreased by 31.7%, 31.9%, 24.8%, and 32.6% at 1, 3, 12, and 24 h, respectively, and decreased by 30.8%, 25.2%, and 34.7% at 1, 3, and 12 h in the high dose group, respectively [Fig. 3(a)]. In the low dose group, SDH activity was significantly decreased by 63.4% and 78.9% at 1 and 3 h, respectively, while in the high dose group, it was decreased by 72%, 55.1%, 28.9%, 39.3%, and 41.9% at 1, 3, 12, 24, and 48 h, respectively [Fig. 3(b)]. In the low dose group, activity of cytochrome c reductase was decreased by 40.9% and 38.7% at 3 and 12 h, respectively, and by 45.4% at 12 h in the high dose group [Fig. 3(c)]. Cytochrome c oxidase activity was reduced by 75.6% and 76.7% at 12 and 24 h in the low dose group, and by 49.0% and 59.2% at 1 and 3 h in the high dose group, respectively [Fig. 3(d)]. Activity of ATP synthase had 53.3% and 45.8% reduction at 12 and 24 h in the low dose group and 47.2% and 55.6% reduction at 24 and 48 h in the high dose group, respectively [Fig. 3(e)].

Antioxidant Activities and MDA Level

Mitochondrial SOD activity was significantly decreased by 50.7%, 52.3%, 47.6%, 55.1%, and 30.7% at 1, 3, 12, 24, and 48 h in the low dose group and by 32.5%, 46.3%, 63.3%, 57.0%, and 91.3% in the high dose group, respectively [Fig. 4(a)]. GPx activity also showed obvious reduction in both dose groups [Fig. 4(b)]. In the high dose group, MDA level in mitochondria was increased by 47.2% and 79.2% at 12 and 24 h, respectively, but no significant change was observed in the low dose group [Fig. 4(c)].

ATPase Activities of Mitochondria

As shown in Figure 5, obvious reductions occurred in the activities of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase in mitochondria in the two dose groups. Na^+-K^+-ATPase activity was reduced by 60.4%, 69.7%, 68.0%, and 53.5% at 3, 12, 24, and 48 h in the low dose group and by 36.8%, 58.5%, 60.8%, 39.2%, and 42.8% at 1, 3, 12, 24, and 48 h in the high dose group, respectively [Fig. 5(a)]. Ca^{2+}-Mg^{2+}-ATPase activity was decreased by 60.4%, 47.7%, 69.6%, and 53.4% at 3, 12, 24, and 48 h in the low dose group and by 24.0%, 52.0%, 54.3%, 42.6%, and 35.9% at 1, 3, 12, 24, and 48 h in the high dose group, respectively [Fig. 5(b)].

DISCUSSION

Our study clearly indicated that MCs exposure significantly impaired the activities of mitochondrial complexes I, II, III,

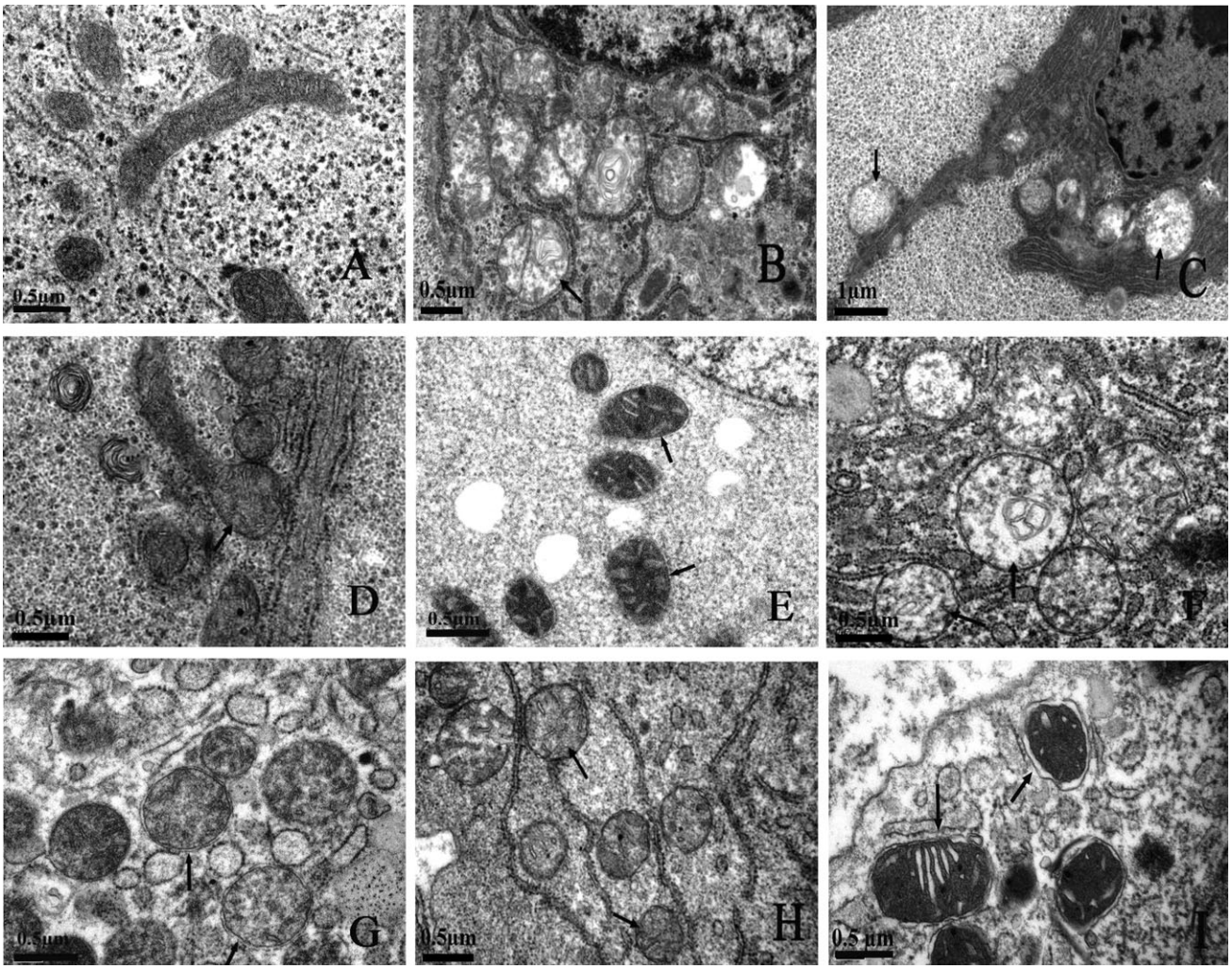

Fig. 1. Ultrastructural change of mitochondria in liver after MCs exposure. (A) mitochondria of control fish. (B), (C), (D), and (E) showing mitochondrial changes at 3, 12, 24, and 48h, respectively, in the low dose group and (F), (G), (H), and (I) showing changes at 3, 12, 24, and 48h, respectively, in the high dose group. (B) and (C) showing obvious vacuolization and loss of cristae and matrix in mitochondrion. (F) and (G) showing obvious vacuolization and swelling. (D), (E), (H), and (I) showing slightly damaged mitochondria with condensed cristae and matrix.

IV, and V and the expression of mitochondrial-encoded genes of the ETC complexes IV and V in crucian carp liver. This is the first study combining both transcriptional expression and enzyme activities of mitochondrial complexes to assess MC-induced changes in the OXPHOS system. Defects in any of the enzyme complexes responsible for oxidative metabolism may lead to mitochondrial cytopathy (Mehndiratta et al., 2000). *Cox1, cox2, cox3*, and *atp6* encode the subunits of complexes IV and V, respectively. The expression of these genes and enzyme activities of mitochondrial ETC complexes were significantly decreased after MCs exposure. These may synergistically alter the biochemical functions of the OXPHOS system in stressed cells.

ETC contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of superoxide production (Jezek and Hlavatá, 2005). And there is growing evidence that most of the superoxide anions are generated at complex I and III of the respiratory chain, especially when mitochondrial respiration is suppressed (Ott et al., 2007). Therefore, the decrease in complexes activities induced by MCs may result in excessive production of ROS. Oxidative stress arises from a significant increase in concentrations of ROS to the levels that are toxic to biomolecules, including DNA, proteins, and lipids (Schrader and Fahimi, 2006). Excessive harmful ROS are decomposed by protective antioxidant system in healthy cells (Kakkar and Singh, 2007). In this study, the activities

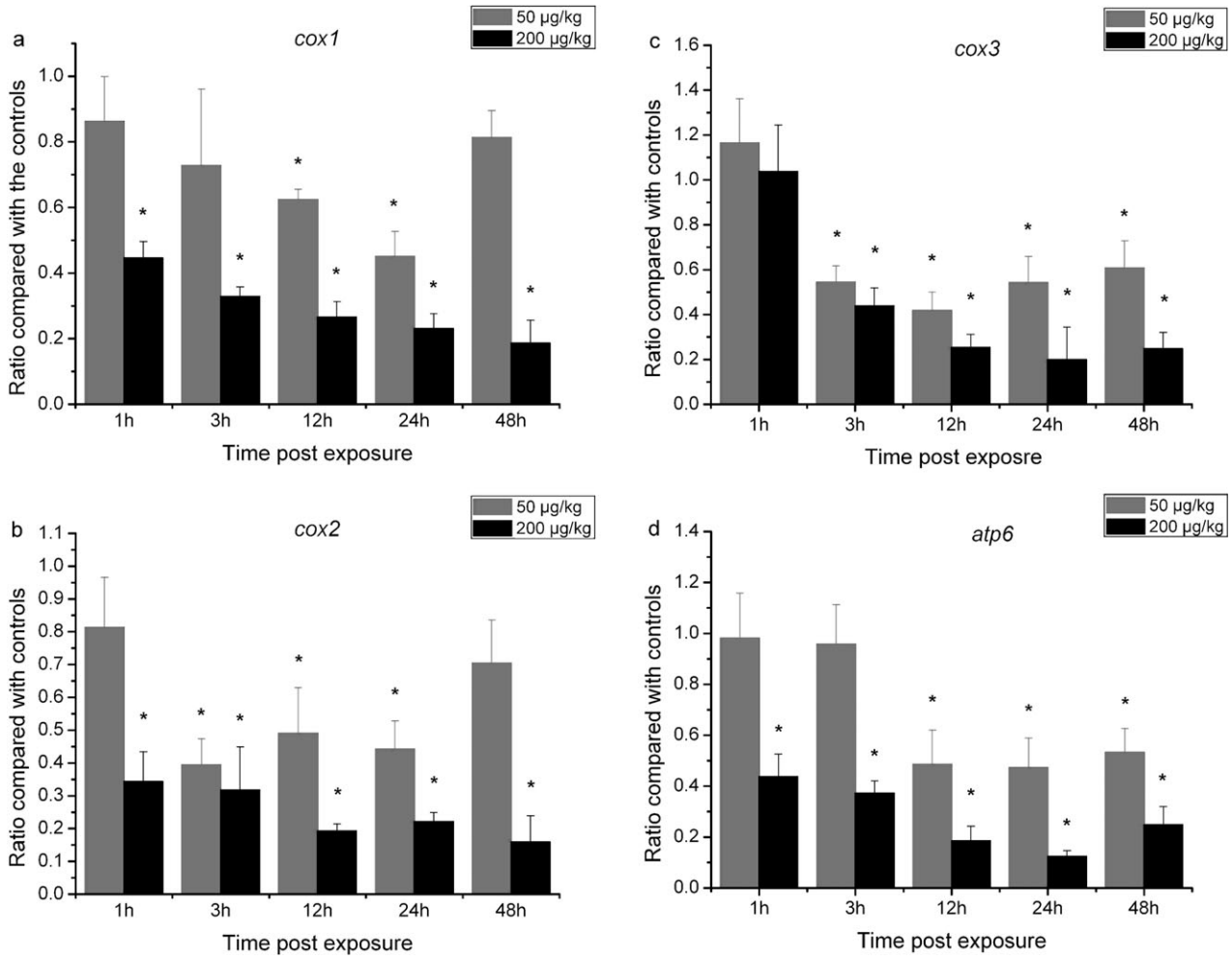

Fig. 2. The temporal changes of mitochondrial encoded gene transcript after MCs exposure compared with the controls. All expression values were normalized to the value of GAPDH gene. (a), (b), (c), and (d) showing *cox1*, *cox2*, *cox3*, and *atp6*, respectively. Significance is indicated as: $^{*}P < 0.05$.

of antioxidant enzymes in mitochondria were significantly decreased after MCs exposure. Therefore, the impairment of OXPHOS system and the decrease in antioxidant may be mechanisms leading to oxidative stress and pathological changes. An important consequence of the effect of oxidative stress to cells is apoptosis which is a continuing process in the normal ontogenesis and life of organisms. Thus, disorder in the OXPHOS system may play a significant role in the widely reported MCs-induced apoptosis of hepatocytes (Huang et al., 2010; Li et al., 2011). However, the exact mechanisms of the OXPHOS system defects in regulating apoptosis after MCs exposure still needs further researches.

Animal cells derive more than 90% of the required energy from OXPHOS. Cellular metabolism depends on the continuous supply of ATP, cellular activities can, therefore, be adversely affected when the mitochondrial OXPHOS system is damaged (Binukumar et al., 2010). Defects in the OXPHOS system may result in failure to supply sufficient ATP to meet cellular needs. Na^{+}-K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase are ATP-dependent ion-pumps located at the mitochondrial membrane and are responsible for ion homeostasis and maintaining normal resting membrane potentials. ATP deficiency may directly influence these ATP-dependent reactions inside the mitochondrion (Smeitink et al., 2006). In the present study, decreases in Na^{+}-K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase activities in mitochondria were detected after MCs exposure, and this may disrupt mitochondrial ion homeostasis and enhance cellular dysfunction. Accumulating evidence endorses a close relationship between ionic homeostasis and apoptosis, especially the regulation of apoptosis by K^{+} and Ca^{2+} homeostasis (Yu, 2003; Ouyang et al., 2011). Ca^{2+} homeostasis is involved in mitochondrial pathway apoptosis. Increase in mitochondrial Ca^{2+} induces opening of permeability transition pore and causes apoptosis. Zhang et al. (2008) reported increased fish lymphocyte apoptosis

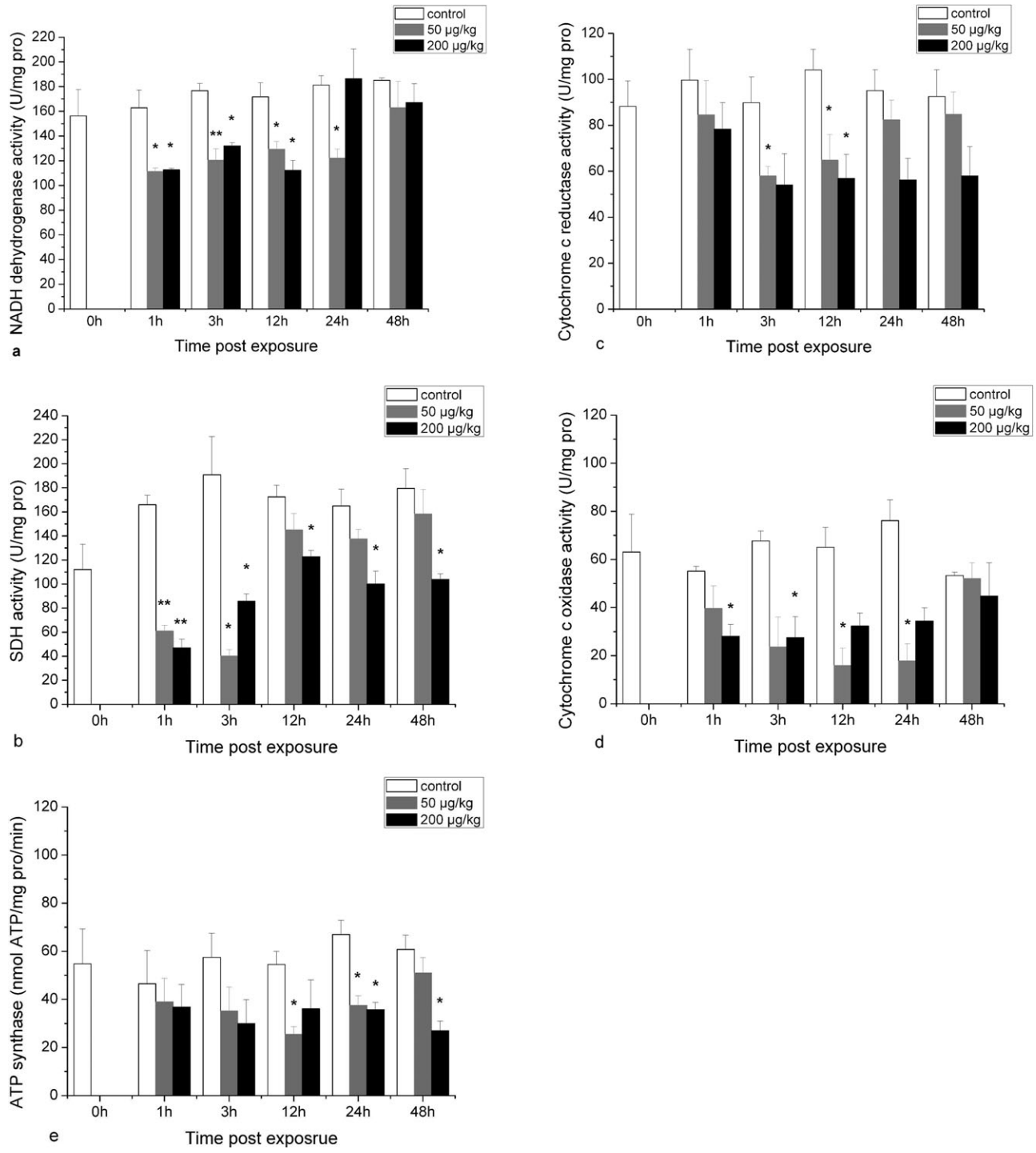

Fig. 3. Respiratory chain enzymes NADH dehydrogenase, SDH, Cytochrome c reductase, Cytochrome c oxidase, and ATP synthase activity of mitochondria in liver of control fish and fish exposed to MCs. The values are expressed as mean ± SE. The significance levels observed are $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with control group values.

with massive Ca^{2+} influx, ROS elevation, MMP disruption, and ATP depletion in mitochondria as a result of MC-RR toxicity. Therefore, dysfunction of the transport enzyme and potential ATP deficiency caused by the defective OXPHOS system may play important roles in hepatocyte damages induced by MCs.

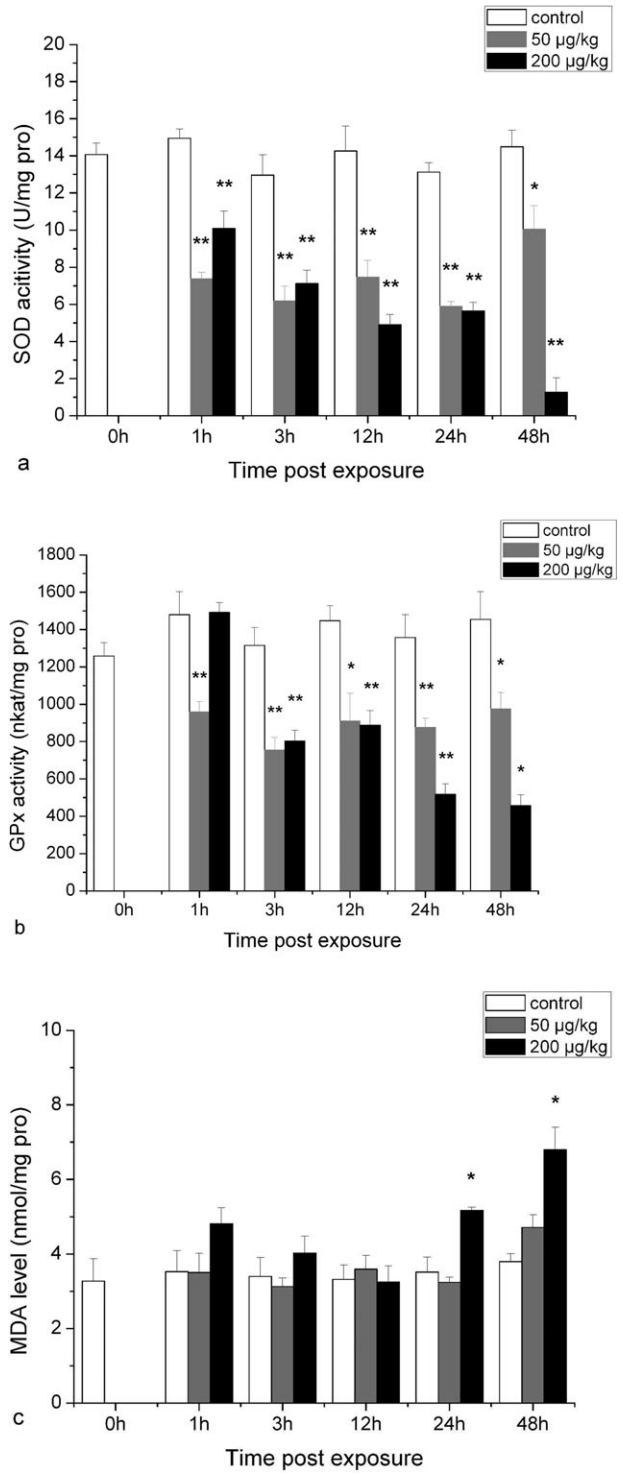

Fig. 4. SOD, GPx, and LPO values of mitochondria in liver of control fish and fish exposed to MCs. The values are expressed as mean ± SE. The significance levels observed are $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with control group values.

In this study, MCs exposure also induced mitochondrial ultrastructural change with obvious vacuolization and highly swollen forms in the two dose groups; however,

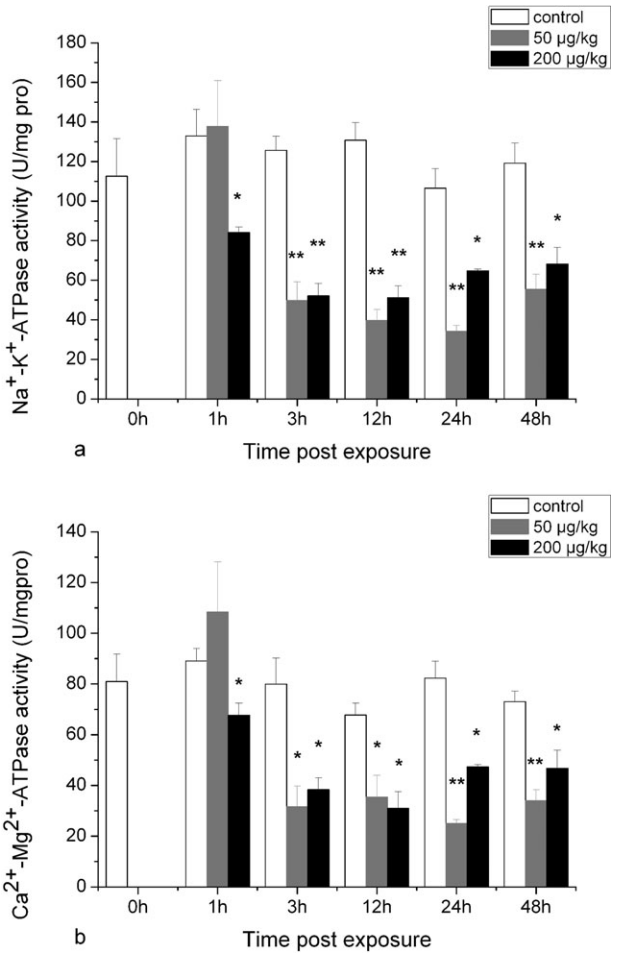

Fig. 5. Na^{+}-K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase activity of mitochondria in liver of control fish and fish exposed to MCs. The values are expressed as mean ± SE. The significance levels observed are $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with control group values.

this damage was reversible in a time dependant pattern. These changes were in accordance with changes in enzyme activities of the complexes of ETC. Li et al. (2005) also observed obvious ultrastructural changes of mitochondria in bighead carp liver after MCs exposure. Our previous study also showed reversible MCs-induced ultrastructural damage to mitochondria in the hepatocytes of rabbits (Zhao et al., 2008). However, rabbits showed more serious ultrastructural changes in mitochondria compared with fish. This demonstrates that both mammalian and fish have a mechanism to resist the external threat and that fish are more tolerant to MCs than mammalians (Xie et al., 2004).

In conclusion, our study showed that the energy generating system of crucian carp was significantly impaired by MCs through the changes in enzymes activities of mitochondrial complexes and mRNA expression of related mitochondrial genes. These impairments might have

contributed to ROS generation, ATP and antioxidant depletion, and consequent dysfunction of the hepatocytes.

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