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The profound effects of microcystin on cardiac antioxidant enzymes, mitochondrial function and cardiac toxicity in rat

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ABSTRACT

Deaths from microcystin toxication have widely been attributed to hypovolemic shock due to hepatic interstitial hemorrhage, while some recent studies suggest that cardiogenic complication is also involved. So far, information on cardiotoxic effects of MC has been rare and the underlying mechanism is still puzzling. The present study examined toxic effects of microcystins on heart muscle of rats intravenously injected with extracted MC at two doses, 0.16LD_{50} (14 μg MC-LReq kg^{-1} body weight) and 1LD_{50} (87 μg MC-LReq kg^{-1} body weight). In the dead rats, both TTC staining and maximum elevations of troponin I levels confirmed myocardial infarction after MC exposure, besides a serious interstitial hemorrhage in liver. In the 1LD_{50} dose group, the coincident falls in heart rate and blood pressure were related to mitochondria dysfunction in heart, while increases in creatine kinase and troponin I levels indicated cardiac cell injury. The corresponding pathological alterations were mainly characterized as loss of adherence between cardiac myocytes and swollen or ruptured mitochondria at the ultrastructural level. MC administration at a dose of 1LD_{50} not only enhanced activities and up-regulated mRNA transcription levels of antioxidant enzymes, but also increased GSH content. At both doses, level of lipid peroxides increased obviously, suggesting serious oxidative stress in mitochondria. Simultaneously, complex I and III were significantly inhibited, indicating blocks in electron flow along the mitochondrial respiratory chain in heart. In conclusion, the findings of this study implicate a role for MC-induced cardiotoxicity as a potential factor that should be considered when evaluating the mechanisms of death associated with microcystin intoxication in Brazil.

1. Introduction

Public health concerns about toxic cyanobacteria have recently increased in many countries, owing to the frequent occurrence of cyanotoxins in both drinking and recreational waters. Among cyanotoxins, microcystins (MCs) are the most common all over the world, with molecular weights ranging between 900 and 1000 Da. To date, more than 80 structural variants have been identified (Sivonen and Jones, 1999), differing primarily in the two variable L-amino acids. So far, human illnesses attributed to cyanobacterial toxins can be categorized into gastroenteritis and related diseases, allergic and irritation reactions, and liver diseases (Bell and Codd, 1994; Chorus et al., 2000; Hitzfeld et al., 2000). In Brazil, tragic deaths of 60 hemodialysis patients are confirmed as a result of contamination of cyanobacterial toxins in the water supply used in hemodialysis unit (Jochimsen et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002).

It is widely considered that hypovolemic shock leads to deaths from microcystin intoxication (Theiss et al., 1988). However, recent studies suggest that there may be also an important cardiogenic component involved. Leclaire et al. (1995) report simultaneous sharp decreases in both heart rate and blood pressure in rats administrated with MC-LR at a lethal dose, suggesting a fundamental dysfunction of the normal compensatory responses of the heart and vasculature to hypotension. Meanwhile, the sustained decreases in both cardiac output and stroke volume in the treated rats indicate that microcystins impair the blood-pumping function of heart. In accordance with in vivo findings, the electrophysiological studies on isolated rat heart indicate that cyanotoxins administration decreases both heart rate and myocardial force contraction (Mason and Wheeler, 1942). Moreover, systolic arrest in the isolated frog heart is recorded after cyanotoxins exposure (Ostensvik et al., 1981). Recently, histopathological alterations in both acute and chronic toxic experiments also verify the cardiotoxicity from MC-LR exposure (Zhang et al., 2002; Milutinović et al., 2006). Therefore, the cardiotoxicity could be another potential contributing factor to the deaths associated microcystin intoxication.

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Cardiovascular diseases are well known to be directly or indirectly related to oxidative damage. Myocardium possesses several features such as abundant mitochondria and rich polyunsaturated fatty acids in the mitochondrial and plasma membranes, all of which make the myocardium vulnerability to free radical attack (Kowaltowski and Vercesi, 1999). Oxidative stress is associated with the pathophysiology of many cardiomyopathies, such as anthracycline-mediated cardiomyopathy (Singal et al., 1997; Xu et al., 2001) and alcoholic cardiomyopathy (Edes et al., 1986). Nowadays, accumulating evidences imply that MC-dependent damage is accompanied by oxidative stress in liver, kidney and intestinal mucosa in mammals (Botha et al., 2004; Ding et al., 1998, 2001; Moreno et al., 2003, 2005). Moreover, studies strongly indicate that mitochondria are the vulnerable target of MC. To satisfy the huge energy demands of heart, cardiac mitochondria occupy about 40% of total intracellular volume of cardiomyocytes, and mitochondrial defects indeed lead to cardiomyopathy and heart failure (Goffart et al., 2004). It seems that oxidative damage and mitochondrial dysfunction are probably associated with the cardiotoxic effects from MC exposure. In the present experiments, we studied the acute responses of antioxidant system in heart and the physiological changes in mitochondria electron transport chain (ETC), with discussion on the underlying mechanism of MC-induced cardiotoxicity.

We conducted intravenous injection of extracted MC in rats at two doses, and our main purposes were: (1) to monitor the clinic characteristics of heart from MC intoxication, (2) to evaluate the roles of oxidative stress and mitochondrial dysfunction in cardiotoxic effects by MC. In this study, we determined MC contents in liver and heart, monitored heart rate, blood pressure, and clinic biomarkers, and examined pathological alterations after exposure of MC. Also, activities and mRNA transcription levels of main antioxidant enzymes (GSH-Px, GST, SOD and CAT), and levels of GSH and MDA (as a measurement of LPO) were measured in cardiac muscle; while activities of complex I, II, III in ETC and MDA levels were measured in mitochondria.

2. Material and method

2.1. Toxin

The cyanobacterial material used in this experiment was collected from surface blooms (phytoplankton cells) of Lake Dianchi, Yunnan in China during May and June 2006. According to microscopic examinations, the predominant species was *Microcystis aeruginosa*. Methods on MC extract and the analyses of the cyanobacterial material were according to Li et al. (2005). Crude extract 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). Extracted crude microcystins were finally suspended in distilled water. Acute 24 h LD_{50} of MC-LR was determined by Dixon's up and down method (Dixon, 1965). The selected low dose ($0.16LD_{50}$) in this experiment was the only dose without death in a series of doses of toxins in the LD_{50} assay.

2.2. Animals and treatment protocol

Male Wistar rats weighing 180–200 g were supplied by Hubei Laboratory Animal research Center (Hubei, China). The rats were housed under controlled conditions of 12 h light/dark cycle, 50 ± 5% humidity and 23 ± 1 °C. Animals were allowed free access to food and water. Five rats were administered by intravenous injection (i.v.) with $0.16LD_{50}$ (14 μg MC-LReq kg^{-1} body weight) and ten rats with $1LD_{50}$ (87 μg MC-LReq kg^{-1} body weight), respectively. An equivalent volume of saline solution was applied to five controls. Twenty-four hours after the administration, the rats were sacrificed. The blood were collected from the carotid artery and centrifuged at 850 × *g* for 10 min. Serum was stored at −80 °C for clinic chemicals analysis. The hearts and livers were rapidly extirpated, frozen in liquid nitrogen, and maintained at −80 °C until being processed for analyses. Samples for histopathological observation were collected at the same time. During the experiment, autopsy was promptly performed on dead rats. All animal procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory.

2.3. Extraction and determination of MC in tissues

Extraction and analysis of the MC in liver and heart of rat basically followed the method of Xie et al. (2004). Qualitative and quantitive analysis of MC in the toxin-containing fraction was performed with a Finnigan LC–MS system comprising a thermo surveyor auto sampler, a surveyor MS pump, a surveyor PDA system, and a Finnigan LCQ-Advantage MAX ion trap mass spectrometer equipped with an atmospheric pressure ionization fitted with an electrospray ionization source (LC–ESI-MS).

2.4. Blood pressure and heart rate assay

Systolic blood pressure (*S*) and diastolic blood pressure (*D*) were measured in conscious rats by tail-cuff blood pressure analysis in a blinded fashion with a multichannel acquisition system for physiological signals (RM6240BD). The mean arterial pressure (MAP) was calculated as follow: $MAP = D + (S - D)/3$. Heart rate was counted by Acuson sequoia 512 ultrasound systems (Siemens, Mountain View, CA, USA).

2.5. Clinic chemicals assays

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) levels were determined on the Abbott Aeroset analyzer according to the manufacturer's recommendations. Creatine kinase (CK) was assessed by enzymatic colorimetry methods (Diasys Diagnostic Technology Co., Ltd., Germany) according to manufacturer instructions. Troponins I (cTnI) levels were determined with the Bechman Coulter AccuTnI kit on the ACCESS 2 (Beckman Coulter, Fullerton, CA).

2.6. Isolation of subcellular fractions

Mitochondria and cytosol of cardiac muscle subcellular fractions were prepared according to the procedure described elsewhere (Yang and Cortopassi, 1998; Aureliano et al., 2002; Soares et al., 2007) with modifications. Briefly, tissues were homogenized in ice-cold homogenization medium (0.25 M sucrose, 5 mM HEPES, 1 mM EDTA, pH 7.5) by homogenizer. The crude homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C, and the resultant supernatant was centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant from the final centrifugation contained the cytosol fraction, while the mitochondria pellet was washed twice and resuspended in 0.5 mL of respiratory buffer (70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.5 mM EDTA, pH 7.4). Protein content was determined by Bradford method (Bradford, 1976). All isolation procedures were accomplished at 0–4 °C.

2.7. Assays for antioxidant enzyme activities and the GSH level in cytosol of cardiac muscle

Catalase activity (CAT) was determined by measuring the rate of disappearance of H_2O_2 at 240 nm for 3 min according to Claiborne (1985). Superoxide dismutase activity (SOD) assay was based on the method described by Bayer and Fridovich (1987). Glutathione S-transferase activity (GST) was measured according to the method of Habig et al. (1974) and Habig and Jakoby (1981) by evaluating the conjugation of GSH (1 Mm, Sigma) with the standard model substrate 1-chloro-2,4-dinitrobenzene (CDNB) (1 mM, Sigma). Glutathione peroxidase activity (GPx) was determined according to Drotar et al. (1985), using H_2O_2 as substrate. GSH content was measured according to Griffith (1980). Each assay was conducted by triplicate.

2.8. Assays for activities of mitochondrial respiratory complexes

The suspensions underwent three cycles of freezing at −20 °C and thawing. Then they were used for the measurement of enzymatic activity. 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 (Schneider et al., 1980). Succinate dehydrogenase (SDH, complex II) activity was determined according to kit protocol (Nanjing Jiancheng Bioengineering Institute, China). $CoQH_2$-Cytochrome *c* reductase (complex III) activity was measured according to Fang et al. (2001) with slight modifications. The reaction mixture was in 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 2 mM NaN_3, with 15 mM succinate as substrates and 50 μM cytochrome *c* as electron acceptor. Reactions were monitored at 550 nm. Each assay was conducted by triplicate.

2.9. Lipid peroxidation assay

Lipid peroxidation was determined according to a kit protocol (Nanjing Jiancheng Bioengineering Institute, China) based on the thiobarbituric acid (TBA) method (Ohkawa et al., 1979).

2.10. Total RNA extraction, reverse transcription and real-time quantative PCR

Total RNA in cardiac ventricle of rats treated with $1LD_{50}$ was isolated using Trizol reagent (Invitrogen) and quantified by determination at OD260. The purified total RNA (2 μg) was then reverse transcribed. Reverse transcription was

performed with oligo (dt) 18 primer using first strand cDNA synthesis kit (Toyobo, Japan). Real-time quantative RT-PCR was conducted by amplifying 1.0 μL of cDNA with the SYBR Green qPCR kit (Finnzymes, Finland) on a Chromo 4 Real-Time Detection System (MJ Research, Cambridge, MA) as described previously (Li et al., 2008). One commonly used housekeeping gene GAPDH was analyzed in samples. Based on the results obtained, GAPDH level was stable in the present experiments and therefore it was used as the internal control gene for the quantitative RT-PCR assay. Sequence of each primer are following: F-5′-ATACCTGTGAACTGTCCCTACCGT-3′ and R-5′-AATGTCCGCACCTGAGTGACG-3′ (CAT); F-5′-TCGCTTACAGATTGCCGCCTGCT-3′ and R-5′-ACTGAAGATAGTAAGCGTGCTCCCA-3′ (SOD); F-5′-CAGTTCGGACATCAGGAGAAT-3′ and R-5′-AGAGCGGGTGAGCCTTCT-3′ (GPX); F-5′-TGCTACTGACACAGACCAGAGCCAT-3′ and R-5′-TCTCTCCTTCAGGT-CCTTCCCATAC-3′ (GST); F-5′-ATGGAGAAGGCTGGGGCTCACCT-3′ and R-5′-AGCCCT-TCCACGATGCCAAAGTTGT-3′(GAPDH).

The specification of each pair of primers was confirmed by randomly sequencing six clones, and further confirmed by the melting curve analysis using real-time PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid and only primers with similar amplification efficiency were used in this experiment. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in three tubes, and PCR reactions without the addition of the template used as blanks. After completion of the PCR amplification, data were analyzed with the Option Monitor software 2.03 Version (MJ Research, Cambridge, MA). Relative transcript quantities were calculated using the $\Delta\Delta C_t$ method with GAPDH as the endogenous reference gene amplified from the samples.

2.11. Light microscopy and electron microscopy observation

Myocardial infarction was determined by TTC staining. The excised hearts of control rats and dead rats were cut transversely into four 1.5-mm-thick slices. Each slice was then incubated in a 2% solution of triphenyltetrazolium chloride (TTC) in phosphate buffer for 30 min at 37 °C. In the presence of intact dehydrogenase enzyme systems, TTC forms red-colored precipitates, while areas of necrosis lack dehydrogenase activity and therefore do not stain. Tissue slices were photographed after the staining, and the percentage of necrotic tissue (TTC-negative staining) was determined using Image Pro Plus, version 6.0.

Tissues were processed in a standard fashion for histological examination. Samples were first fixed in 10% buffered formalin for 24 h at 4–8 °C, dehydrated, paraffin embedded, and achieved. Sections of 4 μm were mounted. Following deparaffinization, the sections were rehydrated, stained with hematoxylin and eosin, and subsequently subject to pathological assessment.

For transmission electron microscopic study, specimens were prefixed in 2.5% glutaraldehyde solution, diced into 1 mm^3, followed by three 15 min rinses with 0.1 M phosphate buffer (pH 7.4). Post-fixation 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 of 50–100% 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.

2.12. Statistic analyses

Data are expressed as the mean ± S.D. and the differences between the control and treatment were analyzed by one-way analysis of variance (ANOVA). When significance was found, independent two-tailed *t*-test was used and differences were considered significant from $p < 0.05$.

3. Results

3.1. Animal deaths

No mortality occurred in both the $0.16LD_{50}$ dose and the control groups. In the $1LD_{50}$ group, five rats died, while the other five survived at 24 h post-exposure. The MC-treated rats could be distinguished from the controls by reduced motor activity and reduced resistance at handling.

3.2. MC contents in liver and heart of rats

In the control group, no MC were detectable in liver and heart. Table 1 shows MC contents in liver and heart of the dead rats and the survived ones in the $1LD_{50}$ dose group. In the dead rats, MC content in liver was up to 19.8 ng/g dry weight, while in heart there was only a half (10 ng/g dry weight). At 24 h post-injection, MC content in liver dropped to 3.3 ng/g dry weight, while heart had a similar MC content (4 ng/g dry weight).

Table 1
Microcystin (MC) contents in livers and hearts of the control rats, the dead rats and the survived ones after i.v. injection with $1LD_{50}$ dose.

Parameter	Control	The dead rat	The survived rat
Micocystin (MC) contents in liver, ng/g dry weight	0	19.8 ± 3.0	3.3 ± 4.0
Micocystin (MC) contents in heart ng/g dry weight	0	10.0	4.0 ± 2.0

3.3. Clinical characteristics

At 24 h post-injection, heart rate (HR) and blood pressure (BP) of rats were monitored (Table 2). Compared with the control ones, both HR and BP decreased sharply in the survived rats in the high dose group. At the $0.16LD_{50}$ dose, MC treatment did not affect HR, but significantly reduced BP.

Table 2 shows the alterations in biochemical indicators of blood plasma in rats. In the $1LD_{50}$ dose group, ALT, AST, LDH, CK and cTnI levels were significantly elevated. Compared with the control group, the average ALT activity increased more than 8 folds, while the average cTnI concentration showed an increase of 4 folds. In the $0.16LD_{50}$ dose group, serum ALP, AST and CK activities were prominently enhanced, and the average CK activity showed an increase of 141.3% compared with the control one. We recorded the maximum activities in ALP, AST, LDH and CK in the first dead rat, while cTnI concentration of all the dead rats, ranging from 0.43 to 0.53 ng/mL, showed an increase of more than 20 folds compared with the control rats.

3.4. Antioxidant enzyme activities, GSH content and LPO level in the cytosol of cardiac muscle

Fig. 1 shows activities of antioxidant enzymes, GSH and MDA levels in the cytosol of cardiac muscle cells at 24 h post-exposure. At $0.16LD_{50}$, MC administration evidently enhanced GST activity, but exerted no discernible effects on the activities of CAT, SOD and GPx. GSH in the cytosol maintained at its basal level throughout the experiment. There was a slight increase in cytosolic MDA level at 24 h post-administration. In the $1LD_{50}$ dose group, there were significant increases in activities of all antioxidant enzymes at 24 h post-exposure: activities of CAT, SOD, GPx, GST were respectively elevated to 210.1%, 129.7%, 138.7% and 315.1%, when compared with the controls. GSH in the cytosol was increased nearly 2-fold, and MDA content increased 149.8% compared with the controls.

3.5. Activities of respiratory chain enzymes and LPO level in mitochondria

Fig. 2 shows activities of mitochondrial respiratory complexes and MDA level in mitochondria. The activity of complex I showed a dose-dependent reduction at 24 h post-exposure: 25.9% and 75.8% at the $0.16LD_{50}$ and $1LD_{50}$ doses, respectively, when compared with the controls. No discernible effects were observed on the activities of complex II at both doses. The complex III activity showed 44.2% and 55.4% reductions in the low and high dose groups, respectively. MDA level showed an increase of 180.3% and 158.3% in the low and high dose groups, respectively.

Table 2
Alterations in physiological parameters and plasma biochemical indicators of rats treated with different doses of MC.

Parameter	0 μg kg^{-1}	14 μg kg^{-1}	87 μg kg^{-1}	Dead rats
Mean arterial pressure, mmHg	90.9 ± 3.3	83.6 ± 0.4*	33.1 ± 5.2**	–
Heart rate, bmp	419.5 ± 80.6	350.0 ± 50.5	159.3 ± 59.8**	–
Plasma ALT activity, IU/L	40.5 ± 1.5	40.0 ± 2.2	335.3 ± 145.8**	255.7[a]
Plasma ALP activity, IU/L	270.0 ± 10.0	305 ± 23.0*	271.3 ± 49.4	338.0[a]
Plasma AST activity, IU/L	144.5 ± 28.5	190.0 ± 24.9*	395.3 ± 117.7**	433.9[a]
Plasma LDH activity, IU/L	1316.0 ± 138.0	1337.7 ± 204.5	1667.7 ± 446.2**	2178[a]
Plasma CK activity, IU/L	2151.0 ± 116.0	3041.0 ± 198.3**	3475.7 ± 228.9**	6126[a]
Plasma cTn I content, ng/mL	0.02 ± 0.01	0.02 ± 0.01	0.1 ± 0.04**	0.43–0.53[b]

The values are expressed as mean S.E. ($N = 5$). The significance levels observed are $p < 0.05$ (*) and $p < 0.01$ (**) in comparison to control group values.
[a] The plasma biochemical indicators of the first dead rat.
[b] The range of cTn I content of all the dead rats.

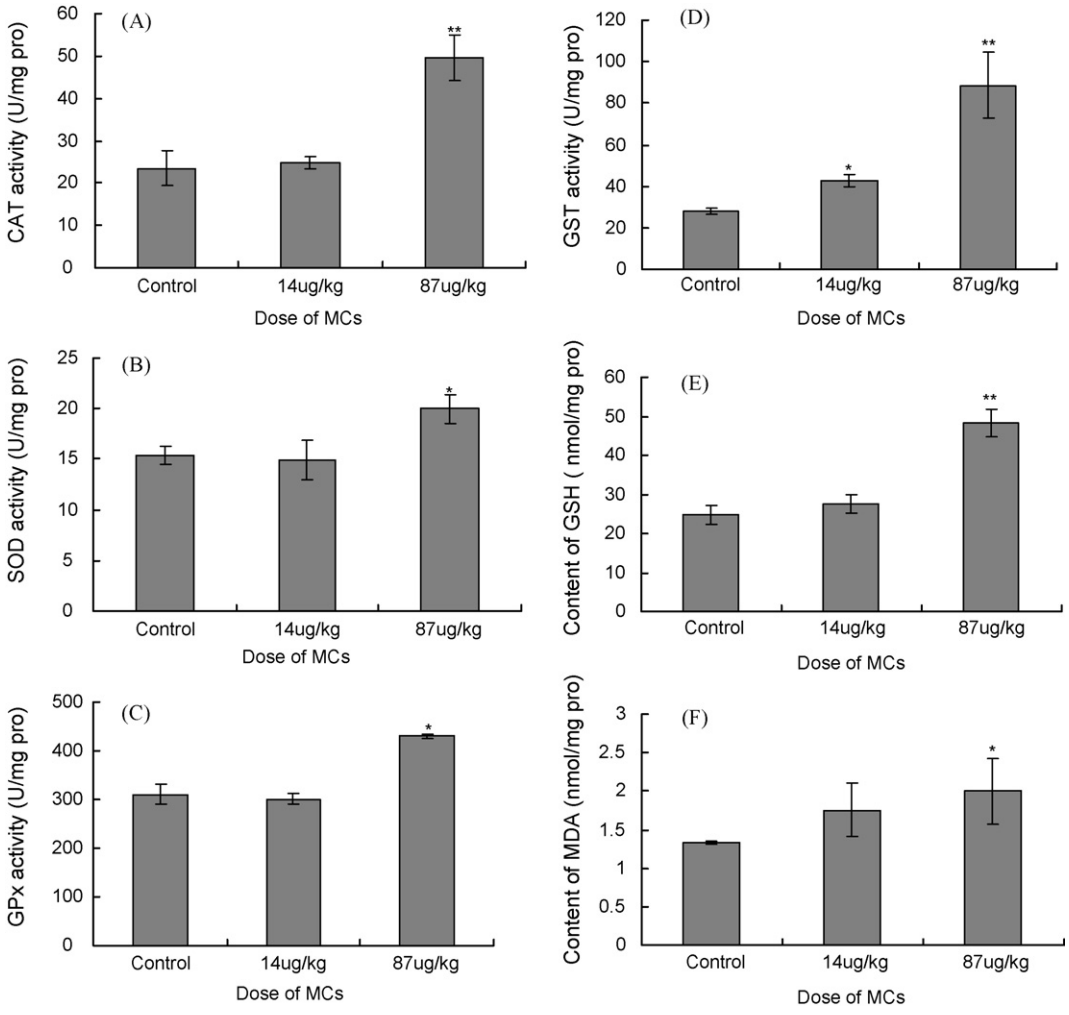

Fig. 1. The activities of antioxidant enzymes (CAT, SOD, GPx, GST), the level of GSH and the MDA level in cytosol of cardiac muscle in rats after an i.v. injection with extracted MC for 24 h. The values are expressed as mean S.E. ($N = 5$). The significance levels observed are $p < 0.05$ (*) and $p < 0.01$ (**) in comparison to control group values.

3.6. mRNA expressions of antioxidant enzymes

Fig. 3 shows relative antioxidant mRNA expression in cardiac ventricle of rats exposed to MC. Transcription levels of all four antioxidant enzymes (CAT, SOD, GPx, GST) were elevated at 24 h post-treatment at the $1LD_{50}$ dose, which paralleled the increases of the activities in these enzymes.

3.7. Pathological observations

Fig. 4 presents MC-induced pathological changes in rat myocardium at 24 h post-injection. The architecture of the myocardium was intact with regular myofiber arrangement in the control rats. In hearts of rats treated with MC, disorganizations of cell structure and loss of adherence between cardiomyocytes were pronounced (Fig. 4C, E, F). At the $0.16LD_{50}$ dose, the myocardial damage included enlarged cells with enlarged and often bizarre-shaped nuclei, occasional cytoplasmic vacuolization and partial degenerative muscle fibres (Fig. 4E, a, and b). At the $1LD_{50}$ dose, disarray of myocardial fibres and the degenerative muscle fibres with myocytolysis were the most prominent features (Fig. 4F, a and b). Pathologically, there were picnotic changes in the nuclei, occasional malformation of blood vessel and slight infiltration of lymphocytes. Cardiac ultrastructure was normal with intact and

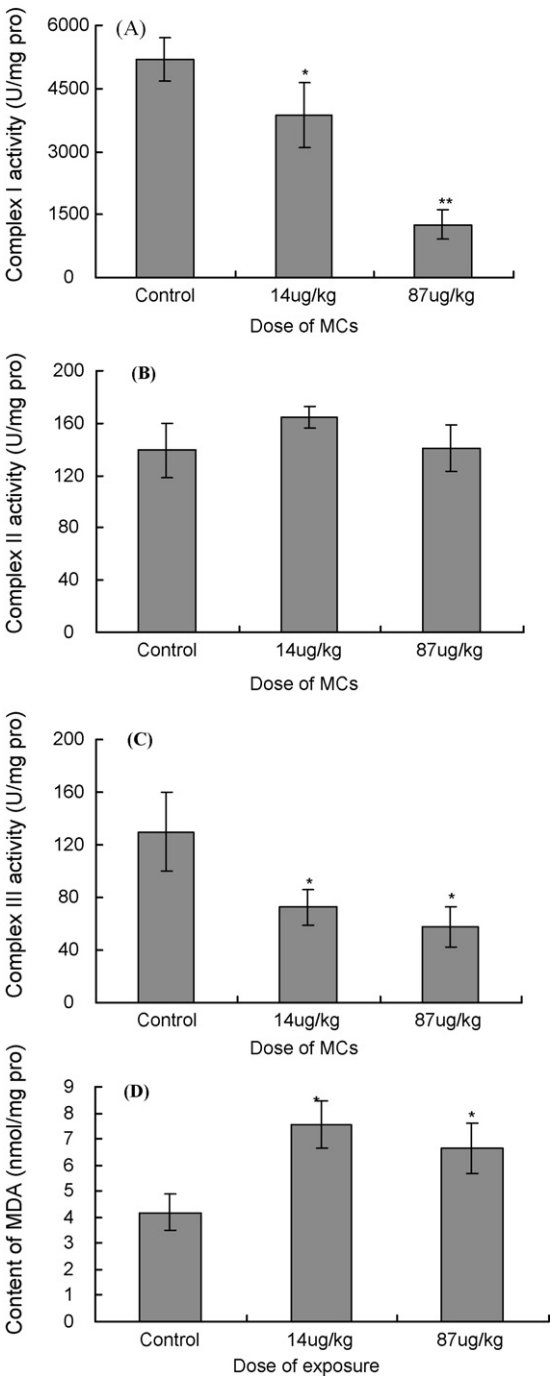

Fig. 2. The activities of mitochondrial respiratory complexes (I, II, III) and the MDA level in mitochondria in heart of rats after an i.v. injection with extracted MC for 24 h. The values are expressed as mean S.E. ($N = 5$). The significance levels observed are $p < 0.05$ (*) and $p < 0.01$ (**) in comparison to control group values.

abundant mitochondria in hearts of the control rats, while most mitochondria were seriously swelling in heart of rats exposed to MC at the $1LD_{50}$ dose (showed with black arrow). Moreover, the swelling of interstice among mitochondria (showed with black star) and some mitochondria with ruptured outer membrane (showed with white arrow) were observed as well.

Positive TTC stain was observed in the control heart, while in hearts of the dead rats treated with MC, the dehydrogenase-deficient infarcted myocardium was remarkable, covering 17–23% of the whole areas (Fig. 5A). Histologically, cytoplasmic vacuolization and degenerative muscle fibres in myocardial cell were obvious in the dead rats (Fig. 5C). Simultaneously, a massive interstitial hemorrhage was observed in liver (Fig. 5E).

4. Discussion

Ostensvik et al. (1981) attribute the drop in mean blood pressure from MC intoxication to sequestration of blood in the liver. However, diminution of the physiological cardiac reserve may compromise the normal response to circulatory insufficiency as well. Leclaire et al. (1995) suggest that there may be an important cardiogenic component involved in the death caused by MC intoxication.

In the present study, accumulation of MC in livers and hearts was identified by LC–MS. In accordance with previous studies, MC led to massive interstitial hemorrhage in liver in the dead rats and increased the activities of certain plasma enzymes (ALT, AST, LDH, ALP, GGT), which were correlated with the MC-induced liver dam-

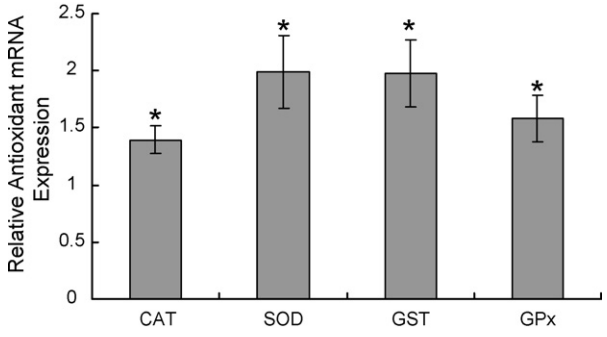

Fig. 3. Relative antioxidant mRNA expression in cardiac ventricle of rats after an i.v. injection with extracted MC for 24 h. The values are expressed as mean S.E. ($N = 5$). The significance levels observed are $p < 0.05$ (*).

age (Billam et al., 2008). An important finding in the current study is that myocardial infarction was confirmed by TTC stain in heart of the dead rats, despite the relatively low MC content. In addition, the significant elevations of serum CK and cTnI levels (two main biochemical markers of heart injury) were in accordance with histopathological observations. The current study indicates a lethal cardiotoxicity in rats exposed to MC.

Histological and ultrastructural observations demonstrated heart injury with MC exposure in the present study. Degenerative muscle fibres with myocytolysis, enlarged cardiomyocytes, and abnormal nuclei were observed, in accordance with previous studies in rats exposed to MC-LR (Zhang et al., 2002; Milutinović et al., 2006). In the present study, disorganization of cell structure and loss of adherence between cardiomyocytes were prominent in the treated animals in a dose-dependent manner, i.e., the damages were more significant in the high dose group. In mammals, the widening of intercellular spaces is presumed to be a critically important effect of MC and is related to the disruption of microtubules, cytokeration intermediate filaments and microfilaments (Wickstrom et al., 1995; Khan et al., 1995). Therefore, the disassociation of cardiomyocytes in the present study indicates the cytoskeleton damage in the heart of rats exposed to MC. So far, many findings in human cardiomyopathic heart tissue have confirmed the association between cytoskeletal changes and contractile dysfunction (Hein et al., 2000). Further studies are needed to examine cytoskeleton alterations in heart by MC exposure. The present study also demonstrated for the first time in hearts of rats after MC exposure serious mitochondrial damage at the ultrastructural level.

Oxidative stress induced by MC exposure is considered to be involved in the toxicity of MC (Ding et al., 1998, 2001). The present studies revealed for the first time that MC exposure not only up-regulated expressions of the main antioxidant enzymes, but also increased activities of the enzymes synchronously. Antioxidant enzymes such as SOD, CAT, and GPx constitute the major defensive system against ROS (Sies, 1993), which alleviates the toxic effects of ROS by scavenging free radicals and ROS. Therefore, activation of antioxidant enzymes indicates the formation of ROS in the target organism (Pinho et al., 2005). On the other hand, oxidative damage in cytosol of cardiac muscle was evaluated with regard to LPO content as well, which is a widely recognized consequence of oxyradical production (Winston and Di Giulio, 1991). In the present study, the significant increment of lipid peroxidation found in hearts not only suggests oxidative stress by MC treatment, but also indicates that the antioxidant response induced by the toxin was insufficient to overcome the oxidative stress. At transcriptional level, significant up-regulation of CAT and SOD induced by MC were recorded in liver of crucian carp (Sun et al., 2008). At variance with our results, Jayaraj et al. (2006) report decreased activities of antioxidant enzymes and stable transcription levels of the enzymes in mice exposed to MC-LR. It seems that antioxidant responses may differ with exposure time, routes, composition of MC and physiological characters of the selected tissues and animals. It is known that increased oxidative stress induces myocyte apoptosis and importantly contributes to the pathophysiology of heart failure (Feuerstein and Young, 2000). Therefore, it is supposed that oxidative stress was involved in the cardiotoxicity of MC in our experiment.

Mitochondrial electron transport chain is considered as a major intracellular source of ROS (Boveris and Chance, 1973; Turrens, 1997). However, the high percentage of polyunsaturated fatty acids in the mitochondria membrane makes it particularly susceptible to damage induced by ROS. In the present study, high oxidative stress in mitochondria of heart in both dose groups is indicated by the increased level of LPO. Mitochondrial membrane lipid peroxidation results in irreversible loss of mitochondrial functions such as mitochondrial respiration, oxidative phosphorylation and ion transport (Vladimirov et al., 1980; Masini et al., 1985; Bacon and Britton, 1990). Indeed, significant inhibition of the complex I and III activities was confirmed in both dose groups in this study. Paradies et al. (2001, 2002) reported that ROS accumulation in mitochondria could affect complex I and III activities through oxidative damage of cardiolipin, which is required for the functioning of complexes. So, we suppose that under MC exposure oxidative damage in mitochondria might have engendered blocks in electron flow along the respiratory chain in heart. What is worse, the impairment of complex I and III activities due to the ROS-induced cardiolipin peroxidation may increase the electron leak from the electron transport chain, generating more superoxide radical and perpetuating a cycle of oxygen radical-induced damage to mitochondrial membrane constituents (Paradies et al., 2002). The ruptured mitochondria in the present study verified the fatal damage of mitochondrial membrane after a high dose MC exposure. Mitochondrial defects lead to cardiomyopathy and heart failure (Goffart et al., 2004). Interestingly, MC exposure at a dose of $0.16LD_{50}$ did not promote antioxidant actions in the cytosol of cardiac muscle in spite of high oxidative stress in mitochondria. It seems that MC exposure firstly induces stronger changes in mitochondrial in heart. The exact mechanism of the mitochondria damage associated with MC-mediated oxidative stress still needs further research.

In the present study, coincidental falls in HR and BP (both to one third of the levels of the control) were pronounced in rats in the $1LD_{50}$ dose group at 24 h post-administration. Similar results were reported in rats i.v. injected with 100 μg/kg MC-LR (Leclaire et al., 1995). Severe hemorrhage leads to a reflex bradycardia and hypotension. However, lack of reflex tachycardia and the failure of hypotension and bradycardia to respond to isoproterenol or dopamine seemed to suggest that MC-LR could affect the heart of rats (Leclaire et al., 1995). On the other hand, cardiac muscle cells rely heavily on the supply of oxidative energy from mitochondria (Naya et al., 2002). Mitochondria produce 95% of the total adenosine triphosphate (ATP) used by typical vertebrate cells, while large amount of ATP guarantee the beat-by-beat contraction and relaxation of heart. Therefore, even subtle perturbations in mitochondrial content or function can result in cardiac dysfunction. In isolated heart, cyanotoxin exposure also leads to decrease in heart rate and even systolic arrest (Mason and Wheeler, 1942; Ostensvik et al., 1981). It is reported that 3-nitropropionic acid-induced bradycardia in isolated atria is associated with inhibition of mitochondrial respiration and subsequent decreased cardiac ATP content (Lopez et al., 1998). Previous studies identified the binding of ATP synthase by MC in vitro (Mikhailov et al., 2003) and the mitochondrial electron transport chain dysfunction after MC treatment (Zhao et al., 2008), while intracellular ATP depletion was confirmed in microcystin-LR treated lymphocytes from *Carassius auratus* (Zhang et al., 2007). Therefore, obvious inhibition of mitochondrial respiration in the present study might have resulted in

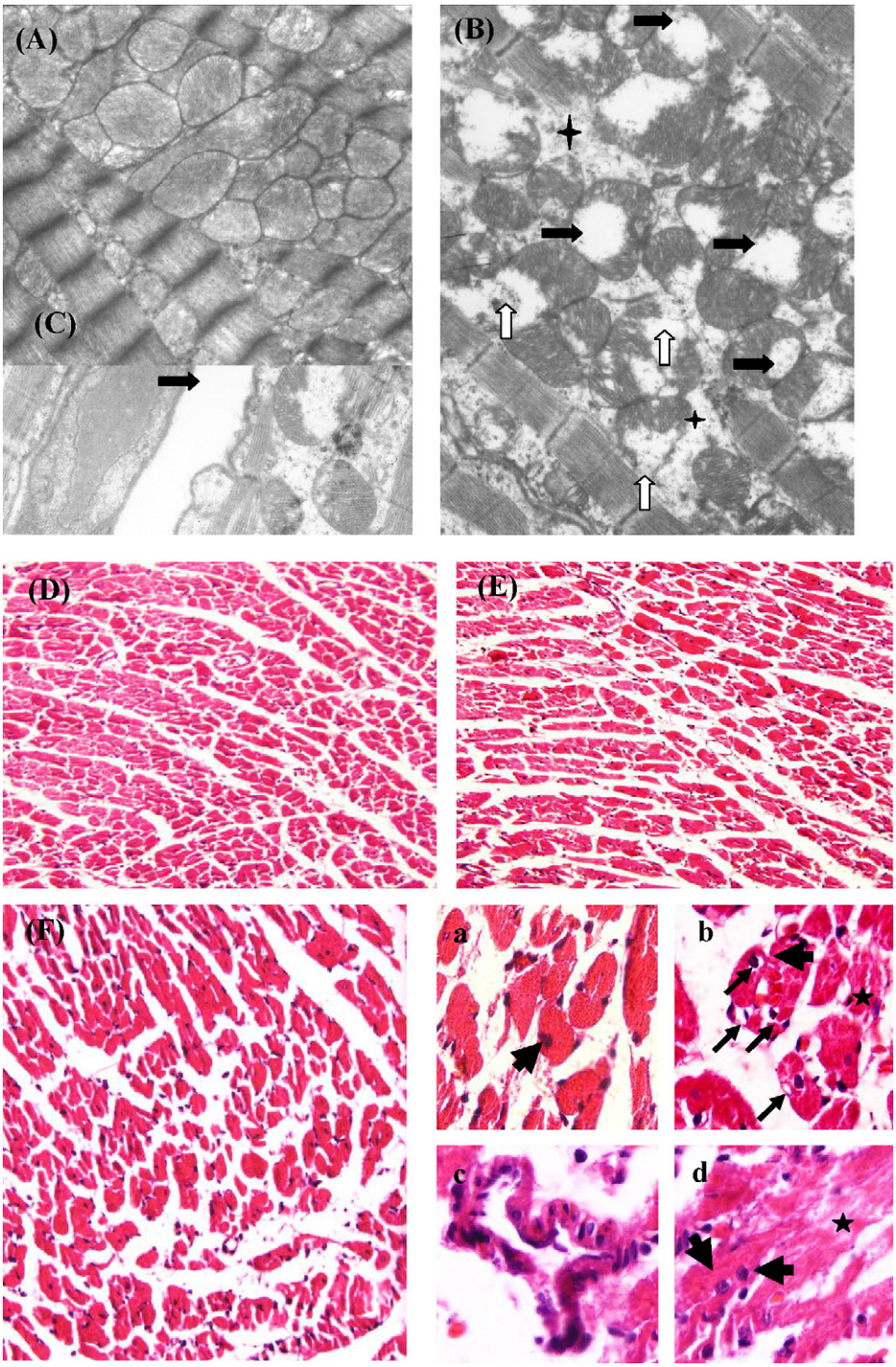

Fig. 4. Pathological alterations in heart of rats after an i.v. injection with extracted MC for 24 h. (A–C) Ultrastructure changes in heart of rats after an i.v. injection with extracted MC for 24 h. (A) control ×10,000; (B) heart of rat i.v. injected with the high dose MC (87 MC-LReq μg kg^{-1} body weight) ×10,000 swollen mitochondria (➡); swollen interstice among mitochondrial (✦); ruptured mitochondria (⇧); (C) dissociated cardiomyocytes. (D and E) Histological changes in heart of rats after an i.v. injection with extracted MCs for 24 h. (D) Control, ×300; (E) heart of rats after an i.v. injection with extracted MC in low dose group: (a) (➡) indicated the bizarre-shaped nuclear in enlarged cells, ×600; (b) (→) indicated the cytoplasmic vacuolization, (★) indicated the degenerative muscle fibers, ×600; (F) heart of rats after an i.v. injection with extracted MCs in high dose group: (c) showed the malformation of blood vessel ×600; (d) (➡) showed picnotic nuclei, (★) showed the degenerative muscle fibers.

the impairment of ATP generation and thereby depletion of the intracellular ATP, consequently leading to bradycardia. Besides the intrahepatic hemorrhage, extremely low heart rate may then cause low blood pressure, which prevents your body from being able to circulate enough blood. The disruption of cellular bioenergetics due to mitochondrial impairment should be responsible for cardiac dysfunction.

In 1996, use of MC contaminated water for patients at a hemodialysis unit in Caruaru led to the deaths of 60 patients. The case patients had a more than 7-fold increase in serum AST con-

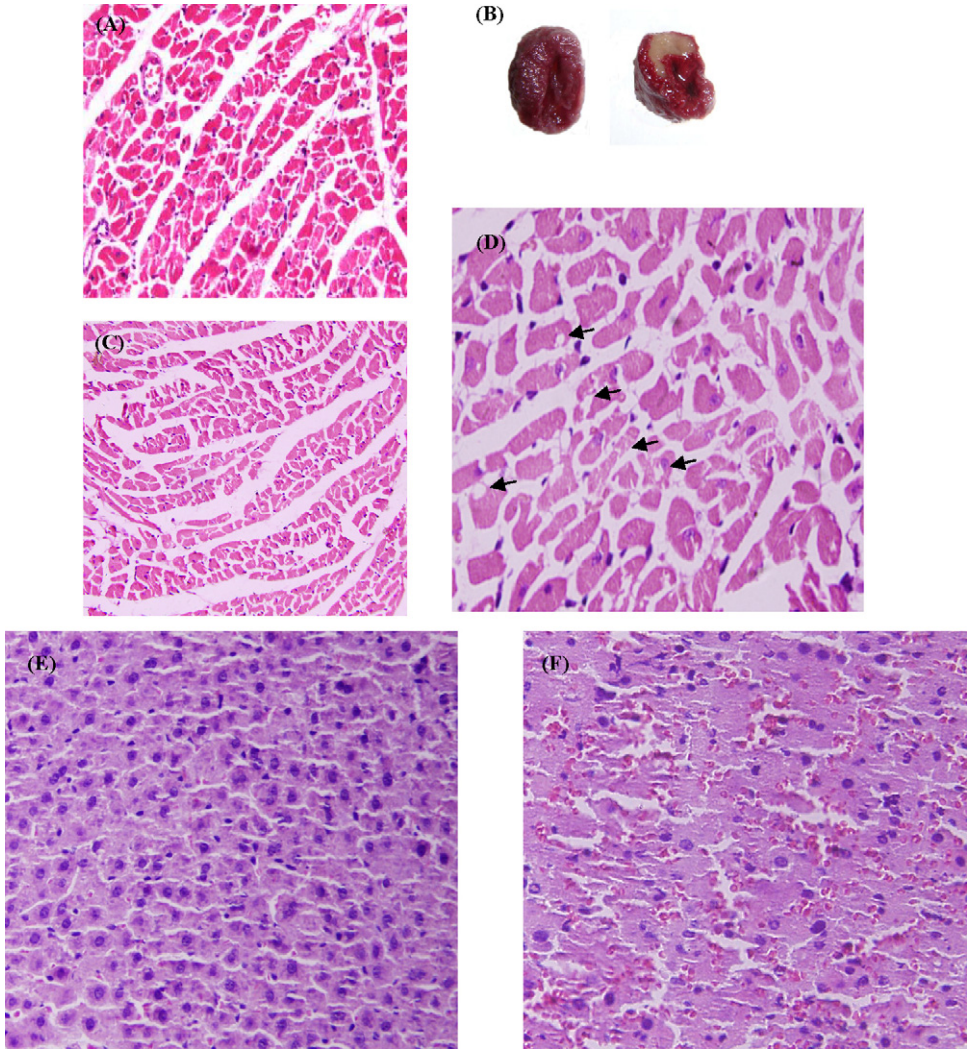

Fig. 5. Pathological alterations in dead rats after an i.v. injection of extracted MC in $1LD_{50}$. (A) Control heart, ×300; (B) positive TTC stain in control heart on the left, and the dehydrogenase-deficient infarcted myocardium without stain in treated heart on the right (→); (C) treated heart with diassociated structure, ×300; (D) treated heart with cytoplasmic vacuolization and degenerative muscle fibres, ×600 (→); (E) control livers, ×300; (F) treated livers with massive interstitial hemorrhage, ×300.

centrations, a more than 4-fold increase in serum concentrations of total and conjugated bilirubin (Jochimsen et al., 1998). However, the most significant augment in AST activity could be an indication of cardiac injury despite of the hepatic damage. The organ with the maximum amount of AST is heart, and then followed by liver, skeletal muscle and kidney. Extremely high levels of AST occur in both severe liver necrosis and acute myocardial infarction. Actually, there was a patient who died from a cardiovascular disturbance (Pouria et al., 1998).

In the present study, after rats were i.v. injected with MC, which is similar to the exposure route of the hemodialysis patients in Brazil, there were sharp falls in HR and BP, indicating abnormal function of the rat heart. Meanwhile, we observed obvious physiological and pathological changes in heart, serious oxidative damage in cardiacmyocytes, and remarkable mitochondrial disturbances after the toxin exposure. Oxidative stress and mitochondrial alterations are both contributing factors to the apoptosis in heart, which is deeply involved in the pathology of almost all types of heart disease and is an important mechanism for dysfunction of individual cardiac myocytes (Takemura and Fujiwara, 2006). The present results suggest that pathological alterations in heart after MC exposure induced cardiac dysfunction, leading to disorders in circulatory system and consequently circulatory insufficiency. It is most likely that cardiotoxic effects of MC aggravate the pathogenesis of hypovolemic shock, and therefore could be a new contributing factor to the patient deaths associated with microcystin intoxication in Brazil.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgments

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