Microcystin Extracts Induce Ultrastructural Damage and Biochemical Disturbance in Male Rabbit Testis

Ying Liu, Ping Xie, Tong Qiu, Hui-Ying Li, Guang-Yu Li, Le Hao, Qian Xiong

Donghu Experimental Station of Lake Ecosystems, State Key Laboratory of Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, The Chinese Academy of Sciences, People's Republic of China

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ABSTRACT: In the present research, the changes of ultrastructures and biochemical index in rabbit testis were examined after i.p. injection with 12.5 μg/kg microcystin (MC) extracts. Ultrastructural observation showed widened intercellular junction, distention of mitochondria, endoplasmic reticulum, and Golgi apparatus. All these changes appeared at 1, 3, and 12 h, but recovered finally. In biochemical analyses, the levels of lipid peroxidation (MDA) and H_2O_2 increased significantly at 1 h, indicating MC-caused oxidative stress. Finally, H_2O_2 decreased to the normal levels, while MDA remained at high levels. The antioxidative enzymes (CAT, SOD, GPx, GST) and antioxidants (GSH) also increased rapidly at 1 h, demonstrating a quick response of the defense systems to the oxidative stress. Finally, the activity of CAT, SOD, and GPX recovered to the normal level, while the activity of GST and the concentration of GSH remained at a high level. This suggests that the importance of MCs detoxification by GST via GSH, and the testis of rabbit contained abundant GSH. The final recovery of ultrastructure and some biochemical indexes indicates that the defense systems finally succeeded in protecting the testis against oxidative damage. In conclusion, these results indicate that the MCs are toxic to the male rabbit reproductive system and the mechanism underlying this toxicity might to be the oxidative stress caused by MCs. Although the negative effects of MCs can be overcome by the antioxidant system of testis in this study, the potential reproductive risks of MCs should not be neglected because of their wide occurrence. Environ Toxicol 25: 9–17, 2010.

Keywords: microcystin extracts; rabbit; testis; ultrastructure; biochemical index

INTRODUCTION

Arising with eutrophication of lakes, the most serious problem is toxic cyanobacteria bloom. Among various toxins, microcystins (MCs) are the most frequently occurring and widespread groups (Carmicheal, 1996). MCs are produced by various species, such as *Microcystis aeruginosa*, *Anabaena* spp. and *Planktothrix* and *Nostoc*. More than 80 structural variants of MCs are currently known (Dietrich and Hoeger, 2005). The MCs are cyclic heptapeptides characterized by a molecular weight of 800–1100. The general structure of MCs is (D) Ala- (L) X- (D) MeAsp- (L) Z- Adda- (D) Glu- Mdha (Carmichael, 1992; Dawson, 1998). The chemical structure of MCs and the variants MC-LR, MC-RR, and MC-YR are shown in Figure 1. MCs are the most toxic cyanotoxins. MCs have been recognized to cause stomach and intestinal inflammation, liver cancer, and disease of spleen in humans who drink water containing MCs (Kuiper-Goodman et al., 1999). The World Health Organization has set a provisional guideline of 1 μg/L MCs in

Correspondence to: P. Xie; e-mail: xieping@ihb.ac.cn

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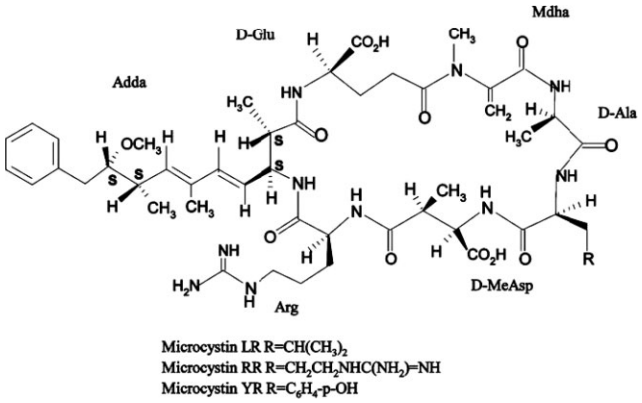

Fig. 1. Chemical structures of MCs used in the study.

freshwater. Up to now, the most tragic incident associated with MCs was the death of over 50 patients in Brazil resulting from the presence of MCs in their dialysis water in 1996 (Azevedo et al., 2002). Generally, MCs act by blocking protein phosphatases 1 and 2a, causing toxicity at the hepatic level, as they use bile carrier to pass through cell membranes. Acute MCs poisoning in experimental mammals is characterized by disruption of hepatic architecture due to phosphorylation of cytoskeletal proteins, massive intrahepatic hemorrhage, and death in a few hours (Rao and Bhattacharya, 1996; Nidhi et al., 2003; Zhao et al., 2008).

Recently, a research found that MCs may exert negative effects on reproductive system of male mice (Ding et al., 2006). Histological damages were found in the testis of mice administered i.p. injection of *Microcystis* cell extract, with decline in the quality of mature sperm in the seminiferous tubules. These suggest that MCs are toxic to male mammalian reproductive system. However, the underlying mechanisms are still unclear.

Previously, reactive oxygen species (ROS) formation was found to be closely associated with the disruption of cytoskeleton organization and consequent hepatotoxicity induced by MC-LR *in vitro* (Ding et al., 2001). Mammalian testis are highly sensitive to oxidative stress and lipid peroxidation (LPO), significant increase of which will cause etiology of male infertility (Rajeswary et al., 2007). Oxidative stress will occur when the activities of cellular antioxidant defense systems decrease or ROS increases (Moreno et al., 2005). ROS can damage DNA, proteins and lipids, and affect enzyme activity and genetic machinery (Winston and Di Giulio, 1991; Lopez-Ongil et al., 2000). Testes are rich in unsaturated lipids, thus may be vulnerable to peroxidative damage. In view of these considerations, we hypothesize that testicular toxicity of MCs might be closely relevant with ROS formation. To determine the involvements of ROS formation in MCs-induced testicular toxicity, the present study analyze the changes in antioxidants, prooxidants, and ultrastructure in testes of rabbit i.p. injected with MCs extracts.

MATERIALS AND METHODS

Toxin

The cyanobacterial material used in this experiment was sampled by phytoplankton net from surface blooms in Lake Dianchi, Yunnan province, China. Crude MCs in the cyanobacteria were extracted three times with 75% (V/V) methanol and were suspended in distilled water for the toxic experiment. Quantitative analysis of MCs were performed using a reverse-phase high-performance liquid chromatography (HPLC, LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) equipped with an ODS column (Cosmosil 5C18-AR, 4.6 × 150 mm, Nacalai, Japan) and a SPA-10A UV–vis spectrophotometer set at 238 nm. 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 MC content was 80.5 μg MC-LRequivalent/mL, among which MC-RR and -LR were 167.7 and 47.0 μg/mL, respectively. Crude MCs extracts were finally suspended in salt solution water (0.9% NaCl).

Microcystin Exposure and Tissue Collection

Healthy and immature male Japanese White Rabbits (1.6 ± 0.2 kg) were obtained from a commercial breeder in Wuhan City, China. The animals were housed three per cage and maintained in a mass air-displacement room with a 12-h light–dark cycle at 20–26°C with a relative humidity of 50–70%. The animals were fed with commercial rabbit food at a rate of 2.0% of body weight per day and offered with drinking water. They were housed in standard anodized aluminum sheet cages and allowed to acclimate for at least 7 days prior to experimentation. Rabbits were randomly divided into three groups, and received treatments as follows. Two groups were administered an i.p. injection of 1 mL microcystin extracts at a dose of 12.5 (low dose) and 50 μg MC-LRequivalent/kg body weight (high dose), respectively. Each group contained 15 rabbits. Another group of 18 rabbits, served as control group, was injected with the same volume of 0.9% saline solution.

Tested rabbits in the MCs-treated groups were sacrificed at 1, 3, 12, 24, and 48 h postinjection, respectively. The control groups were sacrificed at 0, 1, 3, 12, 24, and 48 h postinjection. The testes samples were quickly excised and divided into two parts: one was immediately frozen in liquid nitrogen before being stored at −80°C for biochemical analysis, and the other one was fixed for microscopic examination. This study was performed in accordance with the *Guiding Principles in the Use of Animals in Toxicology* adopted by the Society of Toxicology. All experimental procedures for animals were approved by the local Animal Care and Use Committee at Chinese Academy of Sciences.

Biochemical Analysis

The testes of the rabbit were dissected free of surrounding connective tissues, and then homogenized (1:10, w/v) in a cold (4°C) buffer solution containing tris base (20 mM), EDTA (1 mM), dithiothreitol (1 mM; Sigma), sucrose (0.5 mM), KCl (150 mM), and phenylmethylsulfonyl fluoride (1 mM; Sigma, Saint Louis, USA), with a pH adjusted to 7.6. Homogenates were centrifuged at 5000 × *g* (4°C) for 10 min, keeping the supernatants. Then, they were centrifuged at 10 000 × *g* (4°C) for 15 min. The supernatants of the final centrifugation were used as enzyme source.

LPO (as malondialdehyde, MDA) was determined by the thiobarbituric acid (TBA) method (Ohkawa et al., 1979) with minor modification. Briefly, 3.1 mL of thiobarbituric reactant solution containing 15% trichloracetic acid (w/v), 0.375% TBA (w/v), and 2.5% hydrochloric acid (w/v) was added to 0.1 mL of 10% (w/v) testis homogenates. After stirring, tubes were incubated in boiling water for 40 min, cooled under tap water, and then subjected to centrifugation at 4000 × *g* (4°C) for 15 min. The absorbency of the supernatant was determined at 532 nm. The concentration of MDA was expressed as nanomole MDA per milligram protein.

The activity of hydrogen peroxide (H_2O_2) was determined by using commercially available kits (Jiancheng bioengineering, Nanjin, China), according to the manufacturer's instructions. The concentration of H_2O_2 was expressed as micromole H_2O_2 per milligram protein.

CAT was measured according to Claiborne (1985) with slight modifications. The reaction mixture in a total volume of 3 mL contained 67 mM sodium phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reaction was initiated by the addition of 0.02 mL of enzyme extract. CAT activity was determined by measuring the rate of disappearance of H_2O_2 at 240 nm for 2 min. SOD activity was assayed according to the method of Bayer and Fridovich (1987). One unit of the enzyme activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of nitro blue tetrazolium reduction measured at 560 nm. GST activity was measured using 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-dinitroben-zene (1 mM, Sigma). GPx activity was determined according to Drotar et al. (1985), using H_2O_2 as substrate. GSH content was measured according to Griffith (1980). The activity or the content of antioxidants was calculated in terms of the protein content of a sample. Each assay was carried out in triplicate.

Transmission Electron Microscopy

Specimens of testis were diced into 1 mm^3, prefixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) solution, followed by three 15-min rinses with 0.1 M phosphate buffer (pH 7.4). 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 of 50–100% and then embedded in Epon 812. Ultrathin 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.

Statistic Analyses

The data from MC-treated and control rabbits were presented as mean ± SE. The data were tested for statistical differences by one-way ANOVA followed by Duncan's multiple comparison test using SPSS11.0 to compare data of control rabbits and the ones exposed to extracted MCs. Statistical differences were determined at the $P < 0.05$ and $P < 0.01$ levels for all analyses.

RESULTS

In the high dose group (50 μg/kg MCs), all rabbits died after 3-h exposure. Therefore, the results concerning high dose group are not shown.

Ultrastructural Observations

Spermatogonia

In the control group, the spermatogonia were surrounded with Sertoli cells. The mitochondria were spherical or ovoid, and their cristae and matrix were clear [Fig. 2(A,B)]. The Sertoli–spermatogonia junction was normal [Fig. 2(B)].

In the low dose group (12.5 μg/kg MCs), the lightly stained mitochondria lost cristae and matrix with hydropic changes, and the endoplasmic reticulum was vesicular and dilated after 1-h exposure [Fig. 2(C)]. At 3 h, the endoplasmic reticulum was dilated and the Golgi apparatuses were slightly dilated [Fig. 2(D)]. The widening of Sertoli–spermatogonia cell junction was observed, and the mitochondria were still swelling, and the number of dilated Golgi apparatus increased at 12 h [Fig. 2(E)]. At 24 h, the damaged mitochondria showed a slight recovery. However, the Golgi apparatuses were still dilated badly and the dilated Golgi apparatus increased in number [Fig. 2(F)]. At 48 h, the ultrastructure of spermatogonia recovered, while the mitochondria and endoplasmic reticulum returned to normal state [Fig. 2(G)].

Sertoli Cells

In the control group, mitochondria, endoplasmic reticulum, Golgi apparatus, and the intercellular junction were all normal in Sertoli cells [Fig. 3(A,B)].

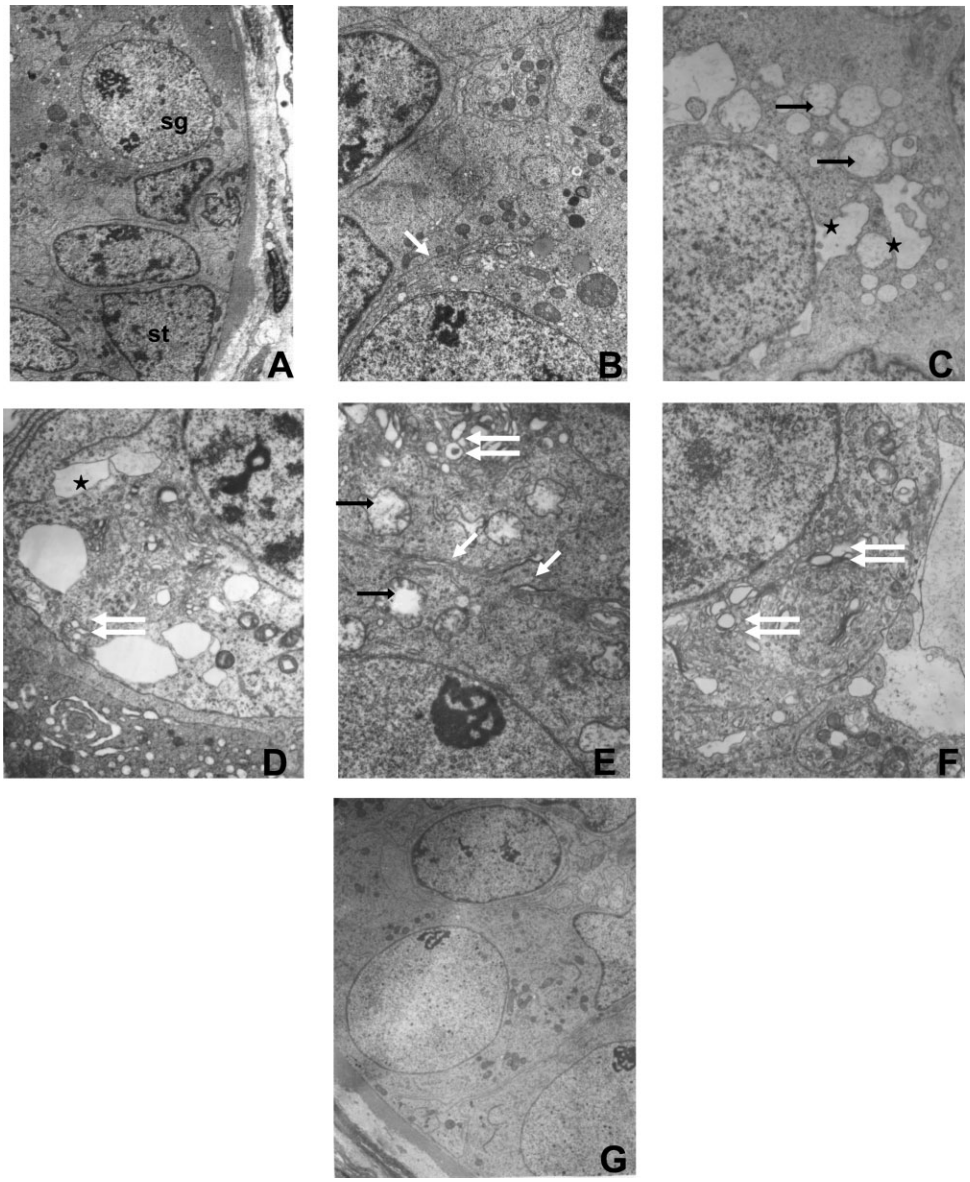

Fig. 2. Toxic effects of MCs on the spermatogonia in testis of rabbits after injection with 12.5 μg MC-$LR_{equivalent}$/kg bw. (A) andspermatogonia of control rabbit, 3500×, (B) showing the normal Sertoli–spermatogonial junction in control rabbit (white arrow), 8000×, (C) showing the swollen mitochondrian (black arrow) and vesicular endoplasmic reticulum (black asterisk) at 1 h, 8000×, (D) showing the dilation of the endoplasmic reticulum (black asterisk) and the Golgi apparatus (double white arrow) at 3 h, 8000×, (E) showing the swollen mitochondria (black arrow), the dilated Golgi apparatus (double white arrow), and the widening of the Sertoli–spermatogonia junctions (white arrow) at 12 h, 8000×, (F) showing the dilation of Golgi apparatus (double white arrow) at 24 h, 8000×, (G) showing the recovery of the spermatogonia at 48 h, 3500×. sg, spermatogonia; st, Sertoli cells.

In the MCs treatment group, mitochondria were swelling and endoplasmic reticulum was dilated after 1-h exposure [Fig. 3(C)]. The endoplasmic reticulum and Golgi apparatus were dilated [Fig. 3(D)], and the Sertoli–Sertoli cells junction widened [Fig. 3(E)] after 3-h exposure. At 12 h, mitochondria were also badly dilated, and lipid droplets highly increased [Fig. 3(F)]. At 24 h, cytoplasmic vacuolation and intercellular vacuolation were much more serious and even formed pool [Fig. 3(G)]. However, mitochondria and endoplasmic reticulum were not badly swollen. At 48 h, structure of Sertoli cells recovered with normal mitochondria, endoplasmic reticulum, and cell junction [Fig. 3(H)].

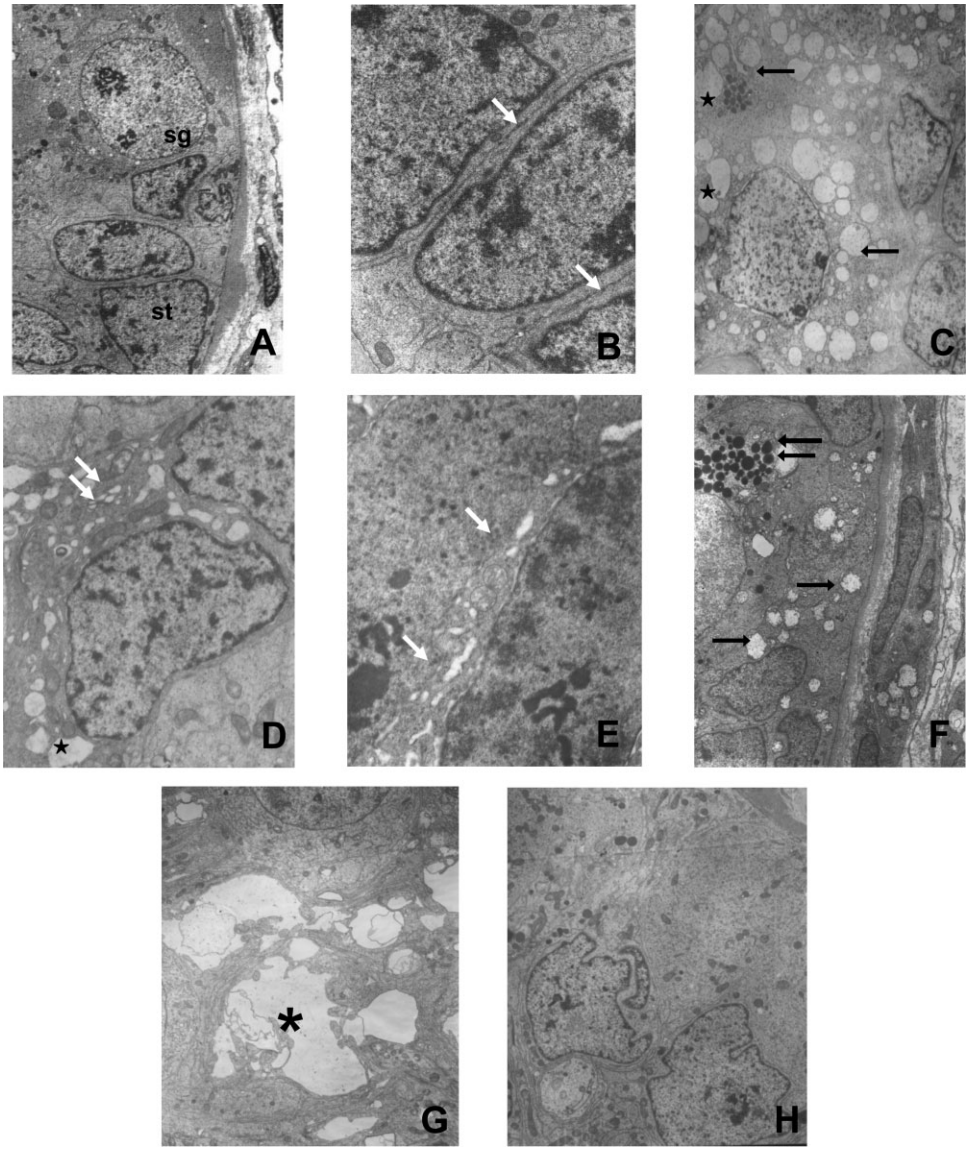

Fig. 3. Toxic effects of MCs on the Sertoli cells in testis of rabbits after injection with 12.5 μg MC-$LR_{equivalent}$/kg bw. (A) Sertoli cells of control rabbit, 3500×, (B) showing normal Sertoli–spermatogonial junctions in control rabbit (white arrow), 8000×, (C) showing the swollen mitochondria (black arrow)and the dilated endoplasmic reticulum (black asterisk) at 1 h, 8000×, (D) and (E) showing the dilation of the endoplasmic reticulum (black asterisk) and the Golgi apparatus (double white arrow), and the widening of the Sertoli–Sertoli cells junction (white arrow) at 3 h, 8000× and 10000×, respectively, (F) showing the swollen mitochondria (black arrow) and the increased lipid droplets (double black arrow) at 12 h, 3500×, (G) showing the serious cytoplasmic vacuolation and intercellular vacuolation (*) at 24 h, 3500×, (H) showing the recovery of the Sertoli cells at 48 h, 3500×. sg, spermatogonia; st, Sertoli cells.

Biochemical Analysis

Levels of LPO and H_2O_2 in rabbit testis are shown in Figure 4. MDA, as a marker of LPO, significantly increased after injection of MCs extracts. The maximum increase in MDA level was observed at 1 h (414%) postinjection, with a slight recovery at 48 h (64%). H_2O_2, a species of ROS, also significantly increased after exposure to MCs, and the maximum increase was observed at 12 h (240%). At the end of the experiments (48 h), H_2O_2 returned to normal level.

Figure 5 showed the levels of the antioxidant enzyme CAT, SOD, GPx, and GST in testis of rabbit. CAT

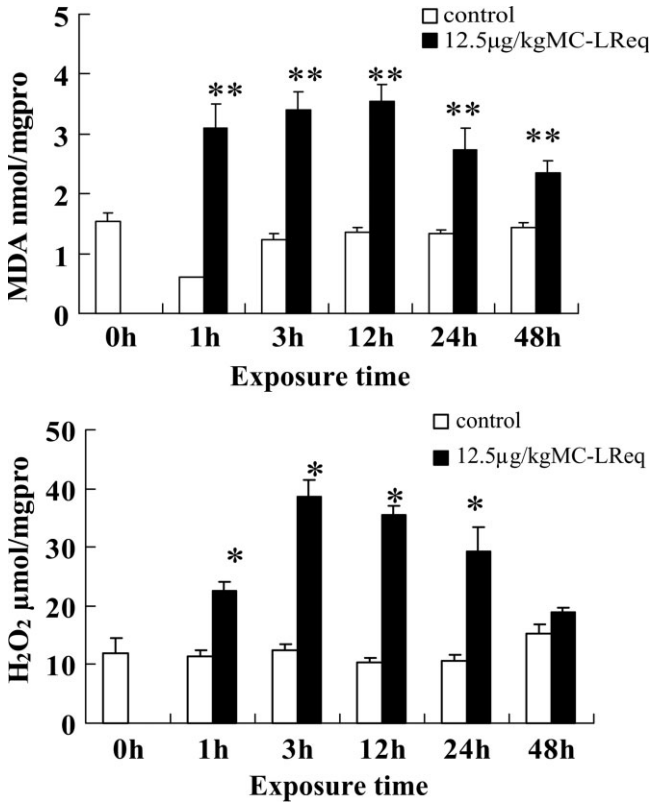

Fig. 4. Effect of 12.5 μg MC-$LR_{equivalent}$/kg bw on rabbit testicular MDA and H_2O_2 levels. Each value is mean ± S. D. $^*P < 0.05$; $^{**}P < 0.01$.

increased significantly at 1, 3, 12, and 24 h, with a maximum at 3 h (189%) and returned to normal level at 48 h. SOD also significantly increased, and the increases peaked at 12 h (183%). GPx activity increased significantly only at 1 and 3 h postinjection of MCs. GST activity increased significantly at all the time postinjection, peaking at 12 h (282%).

GSH content was elevated to 459% at 1-h postinjection of MCs extracts (Fig. 6). At the end of the experiment, GSH content in the MC-treated groups was still significantly higher than that in the control group.

DISCUSSION

Damaged Ultrastructure and Oxidative Stress in Testis

This is the first study to examine both ultrastructural and biochemical responses of mammalian male reproductive system to MC extracts. MC extracts caused prominent degenerative changes in ultrastructure of both spermatogonia and Sertoli cells. It is known that spermatogenesis is dependent upon the normal functions of both cell populations in the seminiferous epithelium: the proliferating and differentiating germ cell population and the nondividing Sertoli cells that create a local environment favorable for germ cell development (Hildebrandt-Stark and Fawcett, 1978). Therefore, the damaged ultrastructures of spermatogonia and Sertoli cells mean the negative effects on the

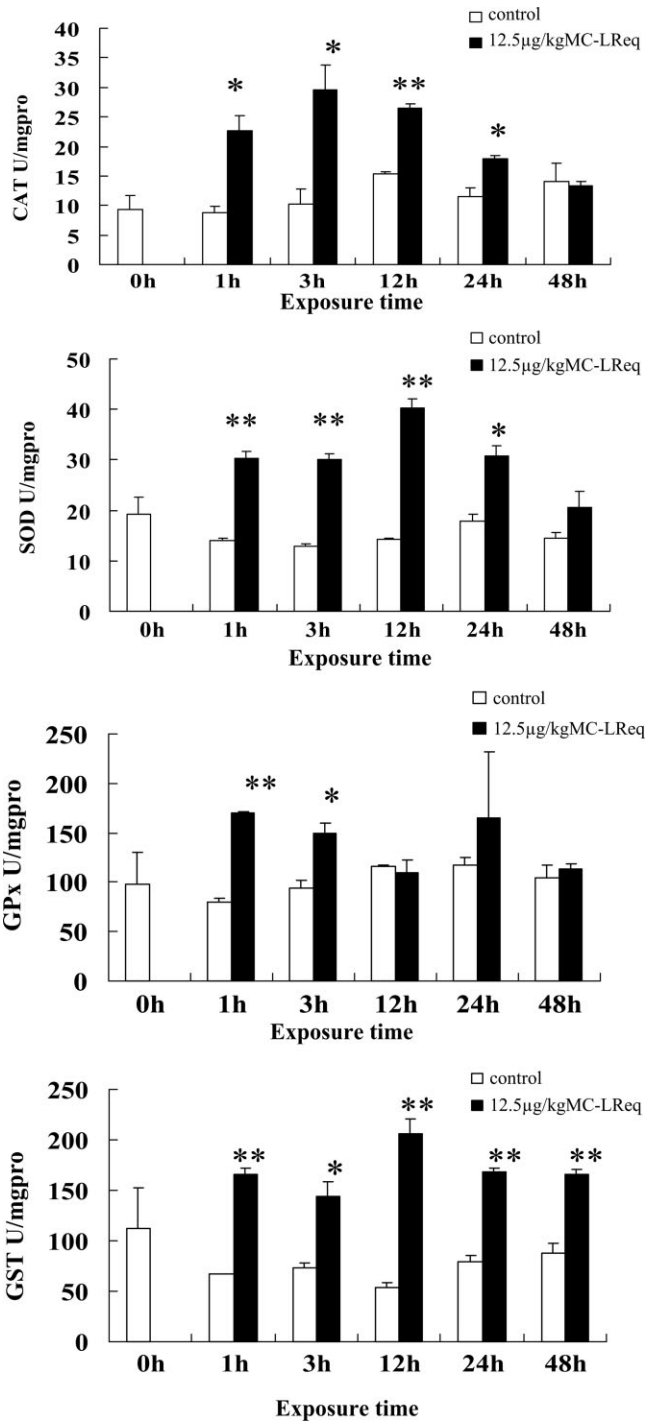

Fig. 5. Effect of 12.5 μg MC-$LR_{equivalent}$/kg bw on rabbit testicular antioxidant enzyme CAT, SOD, GPx, and GST. Each value is mean ± S. D. $^*P < 0.05$; $^{**}P < 0.01$.

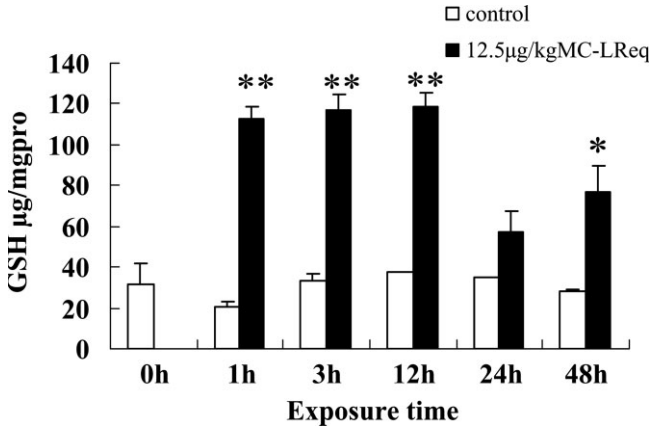

Fig. 6. Effect of 12.5 μg MC-$LR_{equivalent}$/kg bw on rabbit testicular GSH content. Each value is mean ± S. D. $^{*}P < 0.05$; $^{**}P < 0.01$.

spermatogenesis in male mammalian reproductive systems induced by MCs.

In spermatogonia and Sertoli cells, we observed ultrastructural changes in intercellular junction and some organelles, such as mitochondria, endoplasmic reticulum, and Golgi apparatus. MCs act first on the intercellular junctions of hepatocytes in fish (Li et al., 2005). The widening of junction in our study may be due to the fact that MCs are potent and specific inhibitors of PP1 and PP2A, which disturbs the cellular phosphorylation balance and causes hyperphosphorylation of a variety of cytoskeletal proteins, such as actin, talin, and α-actin (Eriksson et al., 1990; Falconer and Yeung, 1992; Runnegar et al., 1993). Wickstrom et al. (1995) and Khan et al. (1996) demonstrated that the widening of intercellular junction caused by MC-LR is related to the disruption of microtubules, cytokeratin intermediate filaments, and microfilaments. Mitochondrial damage is also responsible for the widening of junction, because this damage could impair ATP synthesis which then limits actin polymerization (Cortizo et al., 2000). Besides, oxidative damage might also contribute to the reorganization of actin and disruption of intercellular junctions (Lum and Roebuck, 2001).

In the present study, MCs caused oxidative stress in the testis of rabbits, indicated by the significantly increased levels of LPO and H_2O_2 after exposure. Although H_2O_2 is less reactive than other ROS in cells, it can be the source of hydroxyl radical formation through the Fenton reaction. Hydroxyl radicals, in turn, initiate LPO (Carubelli et al., 1990; Blokhina et al., 2003). The significant increase of LPO and H_2O_2 demonstrated the excessive generation of ROS in testis caused by MCs. Ding et al. (2000, 2001) found that MCs-LR provoked the formation of considerable amount of ROS in rat hepatocytes. ROS are highly reactive and can react with many intracellular molecules. The main cellular components susceptible to ROS are lipids with subsequent peroxidation of unsaturated fatty acids in membranes, proteins with subsequent denaturation, as well as carbohydrates and nucleic acids (Blokhina et al., 2003; Timofeyev et al., 2006). Therefore, endomembrane systems of spermatogonia and Sertoli cells in our ultrastructural observation are easily damaged by oxidative stress caused by MCs. In our experiments, the simultaneous changes in the LPO and H_2O_2 levels and ultrastructures suggest that oxidative stress might be responsible for the ultrastructural changes of testis in our experiments.

Role of Testicular Defense Systems in Time-Dependent Ultrastructural and Biochemical Changes

Rabbits injected with extracted MCs showed time-dependent ultrastructural changes in both spermatogonia and Sertoli cells of testis within 48-h postinjection: swollen mitochondria was noticed at 1 h in both cell types; at 3 and 12 h, widening of intercellular spaces and distention of endomembrane systems were observed in spermatogonia and Sertoli; at 24 h, mitochondria and endoplasmic reticulum showed slight recovery; at 48 h, most spermatogonia and Sertoli cells returned to a normal state. This time-dependent ultrastructural changes caused by MCs were also observed by Li et al. (2005) in liver of bighead carp i.p. injected with 500 μg/kg MCs. They suggested that the recovery was due to the tolerance of phytoplanktivorous fishes to high MCs from an evolutionary point of view. In our study, the recovery of the testicular ultrastructure was more likely due to the effective antioxidant systems and detoxification systems. When ROS began to generate excessively, testes exhibited a defensive mechanism using various antioxidant enzymes. In the present study, the significantly elevated activity of CAT, SOD, and GPx for 1-h postinjection was concomitant with the increase of H_2O_2 and LPO formation, indicating a rapid response of antioxidative enzymes to oxidative stress. At 48 h, the normal level of H_2O_2 indicated that the excessive H_2O_2 were effectively scavenged by CAT and GPx. Similar results were obtained by previous studies. The activities of SOD, CAT, and GPx increased in the hepatocytes of common carp at 6 h after injection MC-LR *in vitro* (Li et al., 2003). Cazenave et al. (2006) also reported the increased SOD and CAT in liver and kidney, and the increased GPx in kidney of tilapia fish i.p. injected with MCs. These results suggest that the antioxidant enzyme system could be actively induced by the exposure of MCs.

Previous studies have demonstrated that testis is very rich in GSTs (Awasthi et al., 1994). Kaur et al. (2006) suggest that enhanced GST by ROS may represent an adaptive response to detoxify peroxide-containing metabolites generated during oxidative stress. In our study, the enhanced GST activity during the whole experiments might provide a

partial explanation of the reported recovery of ultrastructure in testis and suggest the importance of MCs detoxification by GST via GSH. GSH plays an important role in the protection of cellular constituents against ROS. In the present study, GSH showed increases of 459% at 1 h, and an increase of 172% at the end of the experiment. Such a substantial increase of GSH was consistent with the increasing levels of LPO and H_2O_2 at 1-h exposure. Ding et al. (1998) showed that MC extracts could induce ROS formation in rat hepatocytes within a relatively short exposure time (<1 h). Therefore, the quick and significant increase of GSH at 1 h in our study might be a cellular response to oxidative stress. As we know, cellular responses to xenobiotics often involve changes in GSH content, a result of the balance between its consumption and synthesis. When exposed to MCs, the GSH in testis is consumed by conjugation via GST, or by GSSG formation when GSH reacts with ROS. Then, the initial MC-GSH binding and GSSG may trigger the synthesis of GSH, probably by activating r-glutamylcysteine synthetase, the rate-limiting enzyme for GSH biosynthesis (Meister, 1984). In light of the above discussion, the significantly elevated GSH in testis might be due to the effective synthesis of GSH that might overcome the GSH consumption by conjugation with MCs and the products of oxidative stress. This indicates that the testis of rabbit contained abundant GSH, which could be rapidly synthesized to protect the testis against MCs and oxidative stress.

Summary and Potential Risk

In conclusion, MCs could result in both oxidative stress and ultrastructural change of prepubertal rabbit testis; meanwhile antioxidant and detoxification system effectively started to scavenge the ROS and xenobiotic, and finally succeeded in protecting the testis against damage indicated by gradual recovery of biochemical index and ultrastructure. However, due to the wide occurrence of cyanobacteria containing MCs, the potential reproductive risk caused by MCs should not be neglected in prepubertal animals.

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