In vivo studies on the toxic effects of microcystins on mitochondrial electron transport chain and ion regulation in liver and heart of rabbit

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A B S T R A C T

This study examined the toxic effects of microcystins on mitochondria of liver and heart of rabbit in vivo. Rabbits were injected i.p. with extracted microcystins (mainly MC-RR and -LR) at two doses, 12.5 and 50 MC-LReq. µg/kg bw, and the changes in mitochondria of liver and heart were studied at 1, 3, 12, 24 and 48 h after injection. MCs induced damage of mitochondrial morphology and lipid peroxidation in both liver and heart. MCs influenced respiratory activity through inhibiting NADH dehydrogenase and enhancing succinate dehydrogenase (SDH). MCs altered Na+–K+–ATPase and Ca2+–Mg2+-ATPase activities of mitochondria and consequently disrupted ionic homeostasis, which might be partly responsible for the loss of mitochondrial membrane potential (MMP). MCs were highly toxic to mitochondria with more serious damage in liver than in heart. Damage of mitochondria showed reduction at 48 h in the low dose group, suggesting that the low dose of MCs might have stimulated a compensatory response in the rabbits.

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1. Introduction

Microcystins (MCs), a group of cyclic heptapeptide compounds with specific hepatotoxins produced by cyanobacterial species, have received worldwide attention in recent years (Cohen, 1989; Carmichael, 1997). Many researches have demonstrated the adverse effects of MCs on different kinds of life forms, including humans (Puschner et al., 1998; Pouria et al., 1998). To date, more than 80 different structural analogues of MCs have been identified (Sivonen and Jones, 1999), with microcystin-LR being the most toxic (Miura et al., 1989). It has been elucidated that MCs cause damage to different organs, and liver is the main target (Robinson et al., 1989), characterized by fulminant intrahepatic hemorrhage and death of animals (Carmichael, 1994). One of the most extensively studied mechanisms is that MCs increase protein phosphorylation (Yoshizawa et al., 1990). In addition to its potent hepatotoxicity, MCs also appear to have tumor promotion activity. Recently, Kujbida et al. (2008) report that neutrophil infiltration in the liver might play an important role in triggering toxic injury and tumor development, although the mechanisms are still unclear.

Previous studies implied that MC-dependent damage was accompanied by oxidative stress and programmed cell death (Gehringer et al., 2003; Zhang et al., 2007). Recently, Nong et al. (2007) demonstrated the critical role and possible sources of ROS generation in MC-LR-induced cyrogenotoxicity in a human hepatoma cell line and a series of consequence induced by ROS in cells. MCs induce a series of ultrastructural changes of hepatocytes such as swollen mitochondria, whirling of the rough endoplasmic reticulum, vacuolated cytoplasm, and condensed chromatin (Li et al., 2005, 2007). Recent studies strongly suggest that mitochondria play a critical role in MC-induced apoptosis though the detailed mechanism remains unclear (Ding and Ong, 2003). Mitochondria are highly sensitive to the stimulation of various toxins, whereas there have been only limited in vivo studies focusing on the toxic effects of MCs on mitochondrial ultrastructure and certain important enzymes.

It has been demonstrated that microcystins bind to ATP synthase, an important component in the mitochondria (Mikhailov et al., 2003). Therefore, it is suggested that the disruption of ETC may be associated with MC-induced apoptosis (Ding and Ong, 2003). It is also known that oxidative stress can inhibit Na+–K+–ATPase and other ATPases (Bertorello and Katz, 1995). MCs influence in vitro ion regulation in fish gills through inhibiting the phosphatase activity, which may be responsible for massive animal death induced by MCs (Vinagre et al., 2003).

Therefore, the main purpose of this study was to examine the toxic effects of microcystins on mitochondrial ETC and ion regulation in...
liver and heart of rabbit with the progress of apoptosis induced by MCs through an in vivo experiment.

2. Materials and methods

2.1. Toxin

The cyanobacterial material used in this experiment was collected from surface blooms of Lake Diaochang, Yunnan, China. Freeze-dried crude algae were extracted three times with 5% acetic acid and suspended in distilled water for the toxic experiment. Quantitative analysis of MCs was performed using a reverse-phase high-performance liquid chromatography (HPLC, LC-20A, Shimadzu Corporation, Kyoto, Japan). MC 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 136.5 μg/mL MC-RR and 22.7 μg/mL MC-LR.

2.2. Animals

Healthy male rabbits (Oryctolagus cuniculus) weighing about 1500 to 2000 g were obtained from a local rabbit warren in Wuhan City, China. The animals were housed three per cage and maintained in a mass air-displacement room with a 12-hour light–dark cycle at 20 to 26 °C with a relative humidity of 50 to 70%. Animals were allowed to acclimate for 1 week prior to experimentation and were fed with commercial rabbit food at a rate of 6.0% of body weight per day and offered with water. Feeding was terminated 2 days before initiation of the experiment, and no food was supplied to the rabbits throughout the experiment, but the water offering continued.

2.3. Exposure and sampling

Three acclimated rabbits without administration were expressed as 0 h. A total of acclimated rabbits (N=45) were separated into three groups of fifteen rabbits each according to mean body weight. Rabbits were injected intraperitoneally with crude extracted MCs (mainly MC-RR and -LR) at 50 and 12.5 MC-LReq. μg/kg body weight (bw) (expressed as high and low dose group respectively), and equal volume of distilled water for control group. The 15 rabbits in each dose group and the control were respectively divided equally into 5 rabbit cages. Three rabbits from each group were sacrificed after each injection time (1 h, 3 h, 12 h, 24 h and 48 h).

2.4. Isolation of mitochondria

Mitochondria were isolated from liver and heart of rabbits by standard differential centrifugation according to the method of Yang and Cortopassi (1998) with minor modifications. Livers and hearts excised from rabbit were homogenized in 0.1 g/mL ice-cold homogenization medium (0.25 M sucrose, 5 mM HEPES, 0.1 mM EDTA, 0.1% BSA, pH 7.4) by a homogenizer. Tissue debris and nuclei were separated from the homogenate by centrifugation at 10000 g for 10 min at 4 °C. The supernatant was carefully collected and centrifuged at 10000 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, 0.5 mM EDTA, pH 7.4). All isolation procedures were carried out at 0–2 °C and the final suspended mitochondria were stored at −80 °C for enzyme activity analysis. The mitochondrial protein concentration in each preparation was measured with Bio-Rad protein assay dye (Bradford, 1976; Yang and Cortopassi, 1998) with BSA as the standard.

2.5. Transmission electron microscopic observation

For transmission electron microscopic study, new fresh mitochondria isolated from liver and heart were prefixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), 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.

2.6. Analysis of lipid peroxidation (LPO)

LPO level of mitochondria in liver and heart was determined using the malondialdehyde (MDA) assay kit (Nanjing Jiancheng Bioengineering Institute, China), according to the kit protocol. LPO was calculated as nanomoles of MDA per milligram of protein.

2.7. Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity of mitochondria 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.

2.8. Assays of respiratory chain enzymes

The suspensions underwent three cycles of freezing at −20 °C and thawing before sonication. 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 determination was carried out according to the kit protocol (Nanjing Jiancheng Bioengineering Institute, China).

2.9. Measurement of ATPase activity

Na+-K+-ATPase and Ca2+-Mg2+-ATPase activity of mitochondria were determined by measuring the initial rate of release of Pi from ATP. The assay was measured according to Castilho et al. (2001) with some modifications. The inorganic phosphorus released was determined at 660 nm.

2.10. Statistical analysis

The data from MC-administration 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 STATISTICA software package (Version 6.0, Statsoft, Inc.) to compare data of control rabbits and the ones exposed to extracted microcystins. Statistical differences were determined at the P<0.05 and P<0.01 levels for all analyses.

3. Results

3.1. Animal deaths

Rabbits injected with the high dose of MCs showed high sensitivity to the toxins and all rabbits exposed to the highest dose died after 3 h of exposure.

3.2. Ultrastructural change of mitochondria

In the low dose group, most mitochondria of hepatocytes became compact and concentrated, and obvious vacuolization of mitochondria could be observed (Fig. 1b and c). At 3 h, about 60–70% of mitochondria were abnormal, while this figure declined to about
Fig. 1. Toxic effects of MCs on the mitochondria of liver from rabbits after injection with 12.5 and 50 μg MC-LReq./kg bw, respectively. (a) mitochondria of control rabbits, 20,000×. (b) and (c) showing condensed cristae and matrix in mitochondria in the 12.5 μg MC-LReq./kg dose group at 3 h and 48 h post-injection, respectively, both in 20,000×. (d) showing the swelling and disruption of mitochondria in the 50 μg MC-LReq./kg dose group at 3 h post-injection, 20,000×.

Fig. 2. Toxic effects of MCs on the mitochondria of heart from rabbits after injection with 12.5 and 50 μg MC-LReq./kg bw, respectively. (a) mitochondria of control rabbits, 10,000×. (b) showing slightly membranous damage in the 12.5 μg MC-LReq./kg dose group at 3 h post-injection, 20,000×. (c) showing various shapes and damage of mitochondria, including the lost of cristae and matrix, swelling and distortion 20,000×. (d) showing the swelling and disruption of mitochondria in the 50 μg MC-LReq./kg dose group at 3 h post-injection, 20,000×.
40–50% at 48 h. In the low dose group, a change of mitochondria in cardiocytes was not as significant as in the hepatocytes. At 3 h, mitochondria only showed slight membranous damage (Fig. 2b). However, at 48 h, about 50% of mitochondria were abnormal with loss of cristae and matrix, swelling and deformation (Fig. 2c). In the high dose group, mitochondria became large and round, and lost their metrical density with highly hydropic changes both in hepatocytes and cardiocytes (Figs. 1d and 2d). The mitochondrial damages of hepatocytes were more prominent and rapid than those of cardiocytes.

3.3 MDA assays

In hepatocytes, MDA content of mitochondria was significantly increased in both dose groups. MDA level increased about 29.9% ($P<0.01$) and 39.5% ($P<0.01$) at 1 h, 3 h and 12 h, respectively in the low dose group, and increased about 23.1% ($P<0.05$) and 46.5% ($P<0.01$) at 1 h and 3 h in the high dose group (Fig. 3a). While in cardiocytes, no significant change of MDA was observed in the low dose group, and an increase of about 46.4% ($P<0.05$) and 47.3% ($P<0.05$) was observed at 1 h and 3 h in the high dose group, in comparison with the control (Fig. 3b).

3.4 SOD activity

SOD activity of mitochondria increased significantly in the hepatocytes at 1 h, about 15.6% ($P<0.05$) and 4.8% ($P<0.01$) increases were observed in the low and high dose groups, respectively (Fig. 4a). However, in the high dose group, reduction in SOD activity was observed at 3 h before their death. There was a constantly significant increase in SOD activity of mitochondria in cardiocytes after 3 h in the low dose group. However, in the high dose group, significant increase of SOD activity was observed at 1 h, but a reduction was noted at 3 h before death (Fig. 4b).

3.5 Respiratory chain enzymes

At 3 h post-MC exposure, liver mitochondrial NADH dehydrogenase activity was decreased by 21.4% ($P<0.01$) and 6.4% ($P<0.01$) in the low and high dose groups, respectively, in comparison with the control (Fig. 5a). No significant change in NADH dehydrogenase activity was observed in cardiocytes (Fig. 5b).

Contrary to NADH dehydrogenase, SDH activity significantly increased in both hepatocytes and cardiocytes. Compared to the
control, mitochondrial SDH activity of hepatocytes in the low dose group increased 49.6% ($P < 0.05$), 84.7% ($P < 0.05$), 134.4% ($P < 0.05$) and 127.4% ($P < 0.05$) at 3 h, 12 h, 24 h and 48 h, respectively, and increased 109.2% ($P < 0.05$) at 3 h in the high dose group (Fig. 6a). In cardiocytes, mitochondrial SDH activity in the low dose group increased 25.9% ($P < 0.01$), 66.3% ($P < 0.01$), 78.8% ($P < 0.05$) and 49.8% ($P < 0.05$) at 3 h, 12 h, 24 h and 48 h, respectively (Fig. 6b).

3.6. ATPase activity

As shown in Table 1, no change occurred in Na$^+$–K$^+$-ATPase activity of mitochondria in both dose groups. In hepatocytes, reduction in Na$^+$–K$^+$-ATPase activity of mitochondria in the low dose group was 34.9% ($P < 0.05$) and 23.3% ($P < 0.05$) at 1 h and 3 h, respectively, and 34.6% ($P < 0.01$) at 3 h in the high dose group. With progress of the experiment, Na$^+$–K$^+$-ATPase activity recovered to normal level.

Ca$^{2+}$–Mg$^{2+}$-ATPase activity of mitochondria was decreased at 1 h ($P < 0.05$, $P < 0.01$) in the low dose group and also recovered to normal level with the progress of the experiment in both hepatocytes and cardiocytes. In the high dose group, Ca$^{2+}$–Mg$^{2+}$-ATPase activity did not differ between treated animals and control animals (Table 1).

4. Discussion

Mitochondria are known to be vulnerable targets of various toxins because of their important role in maintaining cellular structures and functions. It is reported that MC-LR could induce morphological changes of mitochondria, uncoupling of mitochondrial electron transport and production of ROS, leading to cell apoptosis in in vitro cultured mammalian and fish cells (Ding and Ong, 2003; Boaru et al., 2006; Xing et al., in press). In the present in vivo study, MC exposure induced two typical ultrastructural changes of mitochondria: concentration of cristae and matrix with obvious vacuolization in the low dose group and loss of their metrical density with highly swollen forms in the high dose group. So far, there have been only a few studies describing ultrastructural changes of mitochondria in mice (Hermansky et al., 1993), rats (Wickstrom et al., 1996) and fish (Li et al., 2004; 2005). When isolated rat livers were perfused with MC-LR at concentrations of 2.0 or 5.0 µg/mL for 10–20 min, mild dilation of mitochondrial cristae was found (Wickstrom et al., 1996). Li et al. (2004) reported obvious vacuoles originating from the toxin-damaged mitochondria in common carp (Cyprinus carpio L.) when the fish were orally exposed to Microcystis at a dose of 50 µg/kg for 28 days. Bighead
demonstrated that liver mitochondria from rats treated with MC-LR were expressed as U/mg protein.

Table 1
Na\(^+-\)K\(^+-\)ATPase and Ca\(^{2+}\)–Mg\(^{2+}\)–ATPase activity of mitochondria in liver and heart of control rabbits and rabbits exposed to MC of two doses (12.5 and 50 µg MC-LR/kg bw)

<table>
<thead>
<tr>
<th>Hour</th>
<th>Na(^+-)K(^+-)ATPase activity</th>
<th>Ca(^{2+})–Mg(^{2+})–ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(U/mg protein)</td>
<td>(U/mg protein)</td>
</tr>
<tr>
<td>Liver</td>
<td>Heart</td>
<td>Liver</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>1.72±0.20</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.69±0.15</td>
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<tr>
<td></td>
<td>3</td>
<td>1.66±0.02</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.48±0.09</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.59±0.21</td>
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<tr>
<td></td>
<td>48</td>
<td>1.61±0.09</td>
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<tr>
<td>12.5 µg/kg</td>
<td>48</td>
<td>1.30±0.07</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.22±0.06</td>
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<tr>
<td></td>
<td>12</td>
<td>1.31±0.19</td>
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<td></td>
<td>24</td>
<td>1.78±0.19</td>
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<tr>
<td></td>
<td>48</td>
<td>1.68±0.26</td>
</tr>
<tr>
<td>50 µg/kg</td>
<td>48</td>
<td>1.26±0.15</td>
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<tr>
<td></td>
<td>3</td>
<td>1.04±0.02</td>
</tr>
</tbody>
</table>

The values are expressed as mean±SE. Na\(^+-\)K\(^+-\)ATPase and Ca\(^{2+}\)–Mg\(^{2+}\)–ATPase activity are expressed as U/mg protein.

\(^{a}\) Significant difference from control, \(P<0.05\).

\(^{b}\) Significant difference from control, \(P<0.01\).

carp (Aristichthys nobilis) were injected with microcystins at doses of 200 and 500 µg/kg bw, which caused ultrastructural changes of mitochondria in liver (Li et al., 2005). The doses of MCs used in the present study for rabbit were much lower than the ones used for bighead carp. However, rabbit showed more serious ultrastructural changes in mitochondria compared with bighead carp. It appears that fish are much more tolerant to high microcystins (Xie et al., 2004). In addition, in the present study, the recovery of mitochondrial damage was observed with prolonged MCs exposure. There were few reports about the ultrastructural recovery of mitochondria in hepatocytes of mammals exposed to MCs. Li et al. (2005) reported that most mitochondria of bighead carp showed considerable recovery at 48 h when the fish were exposed to 500 µg/kg MCs. This recovery suggests that both mammalian and fish have a mechanism to degrade or bind MCs actively to resist the external threat. In the present study, damages of mitochondrial ultrastructure were more prominent and rapid in hepatocytes than in cardiocytes, and the mechanisms for such a difference still remain unclear.

Many studies have demonstrated the increase of LPO in tissues of different organisms as a result of MCs exposure (Moreno et al., 2005; Prieto et al., 2007; Weng et al., 2007). Our results showed a high oxidative stress in mitochondria of rabbit indicated by the increased level of MDA. The enhancement of SOD activity in mitochondria might be a defensive response of rabbit to oxidative stress induced by MCs. This was in accordance with the change of SOD activity in different tissues of tilapia (Oreochromis sp.) after exposure to 500 µg/kg MC-LR (Prieto et al., 2006). Mitochondrial electron transport chain is considered as a major intracellular source of ROS, mainly at the levels of NADH dehydrogenase and coenzyme Q (Turrens and Boveris, 1980). It is supposed that under MC exposure a block in electron flow along the respiratory chain results in an increased production of free radicals, and LPO products, which can functionally damage selectively mitochondrial enzymes and proteins (Turrens, 2003).

to test the hypothesis, we employed several mitochondrial enzymatic assays to examine the effect of MCs on individual redox reactions of mitochondria. Miura et al. (1989) have previously demonstrated that liver mitochondria from rats treated with MC-LR showed alterations in appearance which was correlated with a loss of coupled electron transport. La-Salete et al. (2008) found a significant effect of MC-LR on mitochondrial oxidative phosphorylation of isolated mitochondria from kidney of rat. Paradies et al. (2002) reported that ROS accumulation in mitochondria could affect the NADH dehydrogenase activity through oxidative damage of cardiolipin which was required for the functioning of this multisubunit enzyme complex. As to MCs, the exact relationship between the oxidative stress and electron transport chain in mitochondria still needs further research.

Few in vivo studies have documented the effect of MCs on ion regulation in mitochondria of mammals. The present study focused on the change of Na\(^+-\)K\(^+-\)ATPase and Ca\(^{2+}\)–Mg\(^{2+}\)–ATPase activity on the membrane of mitochondria in hepatocytes and cardiocytes which are important in maintaining Na\(^+/\)K\(^+\) gradient and Ca\(^{2+}\) homeostasis of mitochondria. It is known that ATPase can be influenced through two different mechanisms by oxidative stress (Bertorello and Katz, 1995; Bury et al., 1996). The indirect mechanism is to promote generation of LPO, consequently altering the membrane fluidity. The direct mechanism is to act through the direct oxidizing action of ROS on sulfhydryl groups of ATPase (Rauchová et al., 1999; Vinagre et al., 2003). Al-Jassabi (2004) reported that Na\(^+-\)K\(^+-\)ATPase in hepatocytes of mice was significantly lower in MC-treated groups than in control ones, while no change in LPO level was observed in the hepatocytes after exposure to the toxin. He concluded that indirect mechanism was not responsible for the Na\(^+-\)K\(^+-\)ATPase inhibition in hepatocytes of mice by MCs. It appeared that the decreased ATPase in mitochondria in our study could be explained by the indirect mechanism due to the increased level of LPO.

It is now known that damage caused by MCs is related with apoptosis and that mitochondria may work as a central executioner in the apoptotic signaling pathway (Ding and Ong, 2003). In the present study, the decrease of NADH dehydrogenase activity and the binding of ATP synthase (Mikhailov et al., 2003) by MCs might have reduced ATP production, consequently causing mitochondria dysfunction. Disruption of electron transport, oxidative phosphorylation, and ATP production are known as one of the general mechanisms in triggering apoptotic and nonapoptotic cell death by mitochondria (Green and Reed, 1998). Wu et al. (1990) considered that a loss of mitochondrial membrane potential (MMP) might occur only when supplies of ATP were depleted. So we confirmed that the impairment of ETC might be one of the very earlier events of apoptosis induced by MCs. On the other hand, Ding and Ong (2003) demonstrated that MCs could induce ROS formation and lead to onset of mitochondrial permeability transition (MPT) which provoked irreversible dissipation of MMP in apoptosis. Therefore, the loss of MMP and onset of MPT are known as the very crucial steps in apoptosis caused by MCs. However, not all MMP losses are induced by MPT. For instance, Yu (2003) pointed out that disturbance of mitochondrial K\(^+\) homeostasis reduced MMP which were responsible for MPT. Zhang et al. (2007) have also demonstrated the critical role of Ca\(^{2+}\) concentration in mitochondria in apoptosis caused by MCs. Mitochondrion possesses a large capacity for the Ca\(^{2+}\) uptake and functions as an intracellular buffer for the intracellular Ca\(^{2+}\) homeostasis (Duchen, 1999). An early study has shown that microcystin purified from a cyanobacterial bloom enhanced intracellular Ca\(^{2+}\) (Falconer and Runnegar, 1987). Therefore, it is likely that the change of ATPase activity on mitochondrial membrane possibly causes the break of ion regulation in mitochondria, consequently being partly responsible for the loss of MMP in MC-induced apoptosis.

In conclusion, our in vivo study indicates that the toxic effect of MCs was through influencing the mitochondrial respiratory chain and phosphorylation system, ultimately leading to disruption of cellular bioenergetics and metabolism, and that MCs promoted reactive oxygen species formation, which might be responsible for the change of ion regulation in mitochondria, consequently leading to loss of
MMP and mitochondrial dysfunction. The recovery of mitochondrial damage in the low dose group suggests that the low dose of MCs might have stimulated a compensatory response in animal to overcome the threat. Further studies are needed to clarify the detailed mechanisms.

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References


