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Effect of microcystin-LR on Oxidative Damage in the Heart of Male Sprague-Dawley Rats

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Abstract: This study aims to investigate the oxidative damage effect of microcystin-leucine arginine (MC-LR) on rat heart tissue. Studies have found that MC-LR can cause cardiac histopathological changes, but the specific mechanism that causes cardiotoxicity is still unclear. In this study, 12 adolescent Sprague-Dawley (SD) rats were randomly divided into a control group (normal saline) and an experimental group (MC-LR, 40μg/kg), and they were injected intraperitoneally for 14 days. The pathological changes of SD rat heart tissue were detected by HE staining. In addition, we also detected the content of malondialdehyde (MDA) and the activity of superoxide dismutase (SOD) in rat myocardium. The study found that mast cell infiltration appeared in the myocardial tissue of SD rats in the experimental group, and more common ventricular venous congestion was observed. No abnormality was observed in the control group. Compared with the control group, the MDA content and SOD activity of lipid oxidation products in the heart tissue of SD rats increased. These results indicate that MC-LR may damage the heart of SD rats by altering the oxidation of the heart tissue of SD rats.
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Key words: Microcystin-LR; Cardiotoxicity; Oxidative stress

1. Introduction

Due to the rapid development of modern industry and agricultural production and the frequent use of high-phosphorus synthetic substitutes such as washing powder and detergent in daily life, the discharge of a large number of wastewater containing nitrogen, phosphorus, and other nutrients results in eutrophication of the water body, which causes the severe cyanobacteria blooms. Cyanobacteria blooms destroys the ecological balance of hydrosphere, and cyanobacterial toxins are also released into water bodies when cyanobacteria cells rupture [1]. Microcystins (MCs) are the most widely distributed and the most toxic cyanobacterial toxins that pose a serious threat to human health. It has been recognized by the World Health Organization (WHO) as an emerging public health issue [2].

MCs are cyclic peptides consisting of seven amino acids. The chemical properties of MCs are very stable, and they degrade slowly in nature, so conventional treatment cannot effectively remove MCs in drinking water. So far, more than 200 variants of MCs have been discovered. Among all the variants, MC-LR is the most toxic and widely distributed [3-5]. Studies have shown that MC-LR has hepatotoxicity, nephrotoxicity, reproductive toxicity, pulmonary toxicity and other organ toxicity, and it also has potential carcinogenicity. It has been reported that MC-LR is a strong promoter of liver cancer and has bioaccumulation in vivo [6].

It is generally believed that the direct cause of death caused by the acute toxicity of MC is hemorrhagic shock [7]. However, recent studies have shown that MCs can accumulate in the heart and exert toxic effects. It has been reported that following a lethal dose of MC-LR, the heart rate and blood pressure of the rats decreased sharply at the same time, suggesting that dysfunction of the normal compensatory responses to hypotension in the heart and vasculature. At the same time, the continuous decrease of cardiac output and stroke volume in rats indicated that MCs could damage the pump function of the heart [8]. In vivo studies had shown that the electrophysiological study of isolated rat hearts, the application of cyanobacterial toxins could reduce heart rate and myocardial contractility [9]. In addition, systolic arrest occurred in isolated frog hearts after exposure to cyanobacterial toxins [10]. Histopathological changes in acute and chronic toxicology experiments have also confirmed the cardiotoxicity caused by MC-LR exposure [11, 12]. Therefore, cardiotoxicity may be another potential cause of death from MCs.

Oxidative stress is caused by excessive production of reactive oxygen species (ROS) in the body, such as overproduction of superoxide anion radical O^{2-}, hydroxyl radical (•OH), and hydrogen peroxide H_2O_2. ROS can be generated by a variety of biochemical processes, and the body has an efficient system to deal with ROS. However, when the pathological increase of ROS exceeds the body's

antioxidant capacity, it will lead to oxidative damage of proteins, DNA, and biofilm lipids [13]. It is generally believed that exposure to MCs will cause oxidative damage. Studies have shown that MC-LR can induce large amounts of ROS in the liver of mice, up-regulate the expression of Bax and Bid, and lead to the loss of mitochondrial membrane potential, eventually leading to hepatocyte apoptosis and liver injury [14]. Superoxide dismutase (SOD) is a representative substance in the antioxidant system in vivo. SOD can participate in the clearance of ROS in vivo, which plays an important role in reducing oxidative damage. It has been reported that after the treatment of the crucian carp with MC-LR, the level of SOD transcription in liver tissues increased significantly [15]. Other studies have shown that after 48 hours of exposure to MC-RR, the activities of SOD and catalase (CAT) significantly increased [16]. At the same time, malondialdehyde (MDA) as a lipid peroxidation product is widely used to determine the level of oxidative damage and oxidative stress in tissues. It was reported that after administration of MC-LR, the levels of MDA and SOD in various organs of mice increased at different levels [17].

Various studies have shown that heart disease is directly or indirectly related to oxidative stress. The myocardium is rich in mitochondria, and the mitochondrial membrane and plasma membrane are rich in polyunsaturated fatty acids, all of which make the myocardium vulnerable to free radical attack [18]. Oxidative stress is associated with the pathophysiology of many cardiomyopathies, such as anthracycline type 2 mediated cardiomyopathy and alcoholic cardiomyopathy [19-21]. At present, more and more evidence shows that the body damage caused by MCs is accompanied by oxidative stress such as liver, kidney and intestinal mucosa [22]. Therefore, we believe that oxidative damage may be related to the cardiotoxic effects of MCs exposure. In this experiment, we investigated the effect of MCs on oxidative stress in rat hearts and discussed the mechanism of MCs-induced cardiotoxicity.

2. Materials and Methods

2.1 Reagents

Microcystin-LR (purity >96%) was purchased at Beijing MRT Technology Co., Ltd. (Beijing, China). The lipid oxidation (MDA) test kit (product number S0131) and the total SOD activity test kit (product number S0101) were purchased from Beyotime Biotechnology (Shanghai, China). BCA Protein Concentration Assay Kit (P0012S, Bi Yun Tian, China).

2.2 The main equipment

Optical Microscopy (Olympus, Japan). Sunrise Remote microplate reader (Thermo Thermoelectric Instrument Co., Shanghai). High-speed cryogenic centrifuge (Sigma, USA). SMI-F140 ice maker (SANYO, Japan). Pipette (10/200/1000ul) (Eppendorf, Germany). -80 °C refrigerator (SANYO, Japan).

2.3 Experimental animals

Twelve adolescent SD male rats, weighing 200 to 260 g each, were purchased from the Experimental Animal Center of Henan Province, animal license number: SCXK (Yu) 2010-035. Three days before the experiment, the SD rats were housed in two cages. They were given free access to drinking water, natural daylight and nighttime rhythm. The breeding room was often ventilated and regularly sterilized. The room temperature was 20 - 25 °C, and the relative humidity was 50% - 70%.

2.4 Experimental Methods

2.4.1 grouping and model establishment

Twelve SD rats were randomly divided into two groups (experimental group and control group), 6 in each group. SD rats were injected for 14 consecutive days using intraperitoneal injection and injected daily at the same time. The rats in the experimental group were injected with MC-LR at a dose of 40 μg/kg•bw daily, and the rats in the control group were injected with the same amount of normal saline. After the end of the effect, SD rats were killed by intraperitoneal anesthesia with 10% chloral hydrate (3.3 ml/kg). Each group took a fresh heart of SD rats for HE staining. The hearts of the other 5 rats were taken, frozen in liquid nitrogen and placed at -80°C for subsequent experiments.

2.4.2 Calculate rat body weight change, heart index, survival rate

After the SD rats were sacrificed, the hearts of the rats were isolated and weighed. The percent body weight change, heart index, and rat survival rate were calculated according to the following formulas. Percent change in body weight of rats / % = (final weight - initial weight) / initial weight × 100. Heart Index / % = Heart Weight / Final Weight × 100. Survival / % = numbers of surviving rats / numbers of starting rats × 100.

2.4.3 HE staining pathological examination

After separating and extracting the heart tissue of each group of SD rats, the fresh tissue was immediately fixed in 4% paraformaldehyde, then embedded in paraffin, and cut into 4 μm sections. The slices were placed in xylene I20 min-xylene II 20 min-absolute ethanol I5 min-absolute ethanol II 5 min-75% alcohol for 5 min, and then rinsed with running water to complete paraffin wax dewaxing. Followed by hematoxylin and eosin staining: the slices were sequentially stained with hematoxylin for 3-5 min-water wash-differentiation differentiation-water wash-back blue-wate rinse, and then the slices were placed into 85% and 95% alcohol dehydration Each 5min, then placed in eosin stain for staining for 5min. Finally, the slices were placed in absolute ethanol I 5min - absolute ethanol II 5min - anhydrous ethanol III 5min - dimethyl

I5min - xylene II 5min transparent, using neutral gum to seal, complete dehydration and mounting after microscopy. Then image acquisition and analysis.

2.4.4 MDA Detection

The myocardium was removed from the -80°C freezer and weighed, added 9 times the volume of pre-chilled PBS and homogenized, 9 times the volume of the tissue weight was added with pre-cooled PBS and homogenized, and then centrifuged at 1000g for 10 min at 4°C. The supernatant was collected for subsequent protein concentration determination and MDA content detection. The MDA content was determined by the lipid peroxidation MDA Assay kit (Beyotime S0131) and following the manufacturer's recommendations. The MDA content is normalized to protein levels.

2.4.5 SOD Detection

The myocardium was removed from the -80°C freezer and weighed. After adding 9 times the tissue weight to the pre-chilled PBS, the mixture was homogenized. After that, 1000 g was centrifuged at 4°C for 10 minutes. The supernatant was collected for protein concentration determination and SOD activity measurement. The SOD activity was measured using a total superoxide dismutase kit (Beyotime S0101), and the experiment was carried out in accordance with the instructions. The principle of this method is based on the WST-8 method and SOD activity is normalized to protein levels.

2.4.6 Statistical Analysis Methods

Experimental data is represented by X±s. SPSS 21.0 software was used for statistical analysis. In this experiment, the homogeneity of variance test and t-test of two independent samples were used to compare the two groups. The test level is α=0.05.

3. Results

3.1 Cardiac coefficient

As can be seen from Table 1, no SD rats died in the control group and MC-LR (40 μg/kg) group during the entire experiment. Compared with the control group, there was no statistical difference in the changes in body weight and cardiac coefficient of SD rats in the experimental group before and after exposure (P>0.05).

3.2 MC-LR causes changes in cardiac histopathology.

In order to explore the cardiotoxicity of MC-LR, the heart tissue sections after HE staining were observed. As showed in Figure 1A, no significant inflammatory cell infiltration was seen in the control group. Under the light microscope, the endocardium, myocardium and epicardium were clearly structured in the control group. Myocardium fibers were evenly stained, the cell demarcation was clear, and no fibrous tissue proliferation was observed. However, some pathological changes were observed in the experimental group. As showed in Figure 1B, mast cell infiltration in the experimental group (black arrow), dotted distribution. The structures of the endocardium, myocardial membrane, and epicardial membrane were clear, the myocardium fibers were uniformly colored, and cell division is clear. Ventricular wall venous congestion was common (blue arrow); Myocardial cells had clear horizontal lines and no abnormal interstitium.

Table 1. Effects of MC-LR on SD Rats and Their Hearts (X±s, n=6)

Groups （%）	Survival rate（%）	Weight change（%）	Cardiac coefficient
Control group	100	25.53±1.90	0.36± 0.02
MC-LR 40 μg/kg	100	27.22±3.09	0.37± 0.05

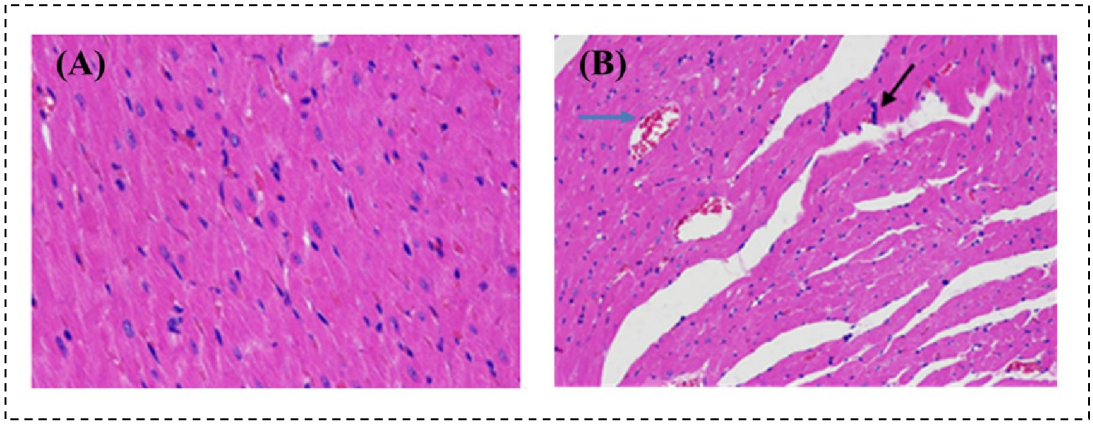

Figure 1. Comparison of HE staining of myocardial tissue in the control group (A) and the experimental group (B).

3.3 MC-LR increases the level of oxidative stress in the heart.

In order to study the mechanism of MC-LR inducing heart damage, we tested the content of malondialdehyde (MDA) content and superoxide dismutase (SOD) activity in the heart tissue of SD rats. Table2 and Figure 2 shows that the changes in MDA content and SOD activity in the heart tissue of SD rats in the experimental group were statistically significant compared with the control group. These results indicate that MC-LR caused an increase in the oxidation level of the heart tissue of SD rats in the experimental group.

Table 2 Comparison of MDA content and SOD activity in heart tissue of SD rats between experimental group and control group ($\bar{X}$±s, n=6)

Groups (%)	MDA content (μM/mg)	**SOD activity** (U)
Control group	0.15±0.02	0.56±0.05
MC-LR 40μg/kg	0.19±0.00*	0.90±0.15*

Abbreviation: *P < 0.05 vs. control group.

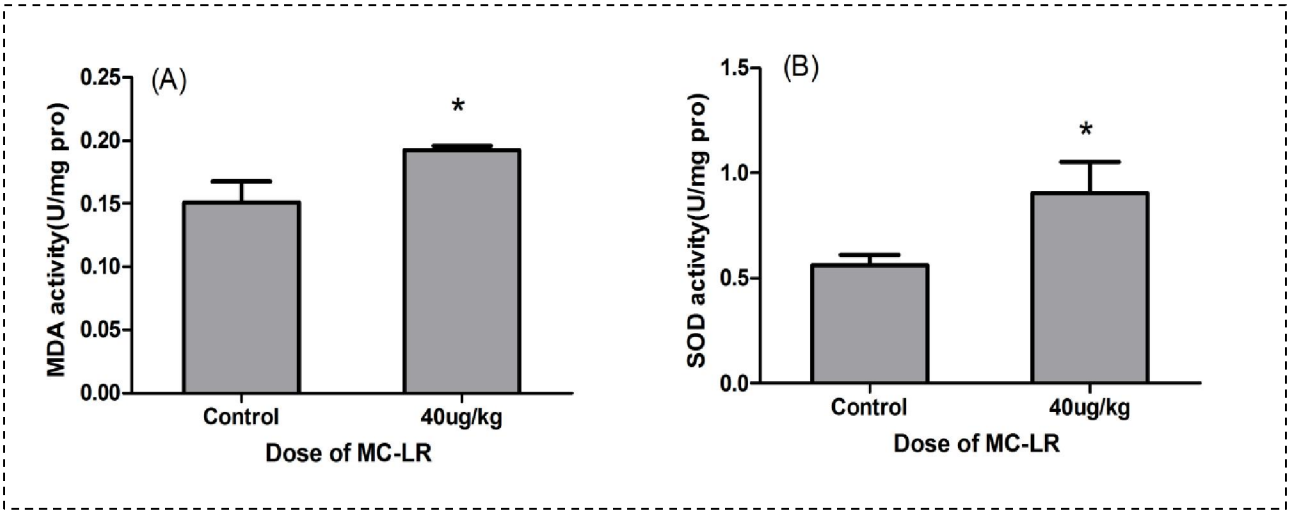

Figure 2. Comparison of MDA content (A) and SOD activity (B) in heart tissue of experimental rats and control SD rats. *P < 0.05 vs. control group.

4. Discussion

At present, there are few studies on the cardiotoxicity and mechanism of MCs. It is generally believed that the death from MCs poisoning was due to hypovolemic shock caused by massive interstitial hemorrhage in the liver [7]. However, some scholars believe that the cause of death caused by MCs poisoning may contain serious cardiogenic factors [23]. In studies of MC-LR acute toxicity in rats, enlarged cardiomyocytes and degenerative muscle fibres with myocytolysis were observed [11, 12]. Recent studies have shown that at lethal doses, MCs have significant lethal toxicity to rat hearts, and the occurrence of myocardial infarction was confirmed by TTC staining, the study also observed the loss of adhesion and mitochondrial rupture between myocardial cells [24]. Furthermore, the HE staining proved that even at a dose of 40 μg/kg, MCs could cause pathological changes in myocardial tissue in SD rats, namely mast cell infiltration, and ventricular venous congestion.

In order to further explore the mechanism of MC-LR-induced cardiotoxicity, we also explored the mechanism of MC-LR-induced cardiotoxicity. Many experiments have shown that oxidative stress may be an important mechanism for MCs to exert toxic effects. For example, it has been reported that MC-LR can induce a large number of ROS production and cause rapid apoptosis of rat hepatocytes through oxidative injury [25]. At the same time, studies had shown that increasing levels of oxidative stress could induce cardiomyocyte apoptosis and contribute to heart failure [26]. To further investigate the mechanism of MC-LR-induced cardiotoxicity, we examined the oxidative stress levels of heart tissues in SD rats after exposure to MC-LR, and observed lipid oxidation products in the heart tissue of SD rats in the experimental group. MDA content and SOD activity were increased compared with the control group, and the difference was statistically significant. This suggests that MCs-induced cardiotoxicity may be generated through oxidative stress. This conclusion is consistent with the study of Qiu et al. They also found that exposure to MCs at a dose of 1LD50 not only enhanced the activity of

antioxidant enzymes, but also up-regulated its mRNA transcription level and GSH content [24]. In conclusion, MCs are likely to produce cardiotoxicity by changing heart oxidative stress levels.

In addition, we also explored possible mechanisms of increased damage to heart tissue with increased levels of oxidative stress. Studies have shown that mitochondria are susceptible targets of MCs. In order to meet the huge energy requirements of the heart, myocardial mitochondria accounts for about 40% of the total volume of myocardial cells, and mitochondrial defects could easily lead to cardiomyopathy and heart failure [27]. MC-LR could induce apoptosis by activating the mitochondrial caspase cascade, and oxidative stress-mediated signaling pathways may contribute to mitochondrial dysfunction [28]. At the same time, studies have shown that oxidative stress may also cause damage to the components of cardiac mitochondrial membranes [29]. The above studies suggest that the oxidative stress induced by MCs may damage the heart by destroying the mitochondrial structure and function. In addition, MC-LR could affect apoptosis-related pathways through oxidative stress, leading to mouse hepatocyte apoptosis [30]. It has been reported that oxidative stress induced by MCs can damage SD rat lymphocytes by destroying DNA [31]. In summary, oxidative stress may damage the heart tissue by disrupting mitochondrial structure and function, attacking DNA and promoting apoptosis.

In summary, MC-LR exposure can induce heart tissue damage in SD rats and increase the level of oxidative stress in the heart, which suggests that oxidative stress may play an important role in the cardiotoxicity of MCs.

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