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Hepatotoxicity Induced by Microcystin-LR in Rat

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ABSTRACT. Microcystin-LR (MC-LR) is a cyanobacterial hepatotoxin mainly produced by *Microcystis aeruginosa*. The current study examined the effects of a single intraperitoneal dose of MC-LR in rats. Female Sprague-Dawley rats were intraperitoneally injected with MC-LR (100 μg/kg body weight) and they were sacrificed at 0, 20, 40, 80, 160 min, or 12 h after injection. Clinically, animals showed lethargy and had ruffled hair beginning at 40 min post injection. In the gross findings, liver was enlarged and its color was changed into dark red beginning at 40 min post injection. Microscopically, dissociation of centrilobular hepatocytes and hemorrhage was observed in the hepatic central regions and such pathological changes were then extended to the portal regions of liver by time course manner. Interestingly at 80 min after MC-LR injection, the entrapped eosinophilic materials that may be necrotic fragments of dissociated hepatocytes were found in the capillaries of lung and renal glomerulus. Ultrastructurally, microvilli of the hepatocytes were disrupted or lost at all time points. Furthermore, the Disse space and gap junctions were widened beginning at 40 min post injection. These results suggest that liver is the major target organ of MC-LR and isolated hepatocytes by the effects of such hepatotoxin may secondarily reduce the physiological function of lung and kidney.

Keywords: Microcystin-LR, Rats, Liver, Hepatocytes, Lung, Microvilli, Kidney.

INTRODUCTION

Microcystins are cyclic heptapeptide hepatotoxins produced by several genera of blue-green algae (cyanobacteria) including species of *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc* (Carmichael *et al.*, 1988; Harada *et al.*, 1988). Algal blooms that produce microcystins occur worldwide in temperature lakes and rivers, and their incidence may be increasing due to eutrophication (Hallegraeff, 1993). Clinical signs of microcytin induced toxicosis typically include weakness, prolonged capillary refill time, pale mucous membrane, diarrhea, shock, and death (Beasley *et al.*, 1989). Therefore, ingestion of water contaminated by *Microcystis* has been implicated in potential public health hazards (Carmichael and Falconer, 1993; Yu, 1989).

Microcystin-LR (MC-LR) is the most commonly encountered and one of the most toxic members of the algal hepatotoxins (Stotts *et al.*, 1993). Based on previous reports, liver is the most severely affected organ by MC-LR, characterized histologically by dissociation of hepatocytes, disruption of sinusoids, and extensive hemorrhagic necrosis in a large number of species (Miura *et al.*, 1989). The hepatic specificity of such toxin is believed to be due to its selective uptake via a bile acid carrier complex present in hepatocytes (Runnegar *et al.*, 1981).

Morphological changes in liver induced by MC-LR appear to be caused by alterations in the hepatocyte cytoskeleton, as reflected in redistribution of actin microfilaments, cytokeratin intermediate filaments, and cytoplasmic microtubules. Such pathologic cytoskeletal changes are thought to be initiated by MC-LR-induced inhibition of protein phosphatases since MC-LR is known as a potent and specific inhibitor of serine/threonine protein phosphatases type 1 and 2A (Yoshizawa *et al.*, 1990; Runnegar *et al.*, 1993). Inhibition of protein phosphatases is believed to result in increased phosphorylation of several cytosolic and cytoskeletal pro-

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Abbreviations: MC-LR, Microcystin-LR

teins inducing disruption of normal cytoskeletal architecture, bleb formation, loss of cell-to-cell adhesions, and finally necrosis (Khan *et al.*, 1995). Furthermore, MC-LR has been also shown to be a potent liver tumor promotor (Nishiwaki-Matsushima *et al.*, 1992).

In view of toxicopathological effects of MC-LR, current study was performed to examine the MC-LR induced pathologic alterations and to compare the sequential effects of lethal doses of such hepatotoxin. This study may help to understand the mechanism of hepatotoxicity induced by MC-LR.

MATERIALS AND METHODS

Animals

6~8 weeks old female Sprague-Dawley rats (150~200 g) were obtained from Bio-Safety Research Institute, Chonbuk National University, maintained under 12 hr light-dark cycle, and had free access to feed and water.

Treatment of toxin

MC-LR isolated from *Microcystis aeruginosa* was purchased from Sigma Chemical Co.. In a time range finding study, rats (n = 3 per group at each time point) were given a single intraperitoneal injection of MC-LR at 100 μg/kg or vehicle (saline). At 0, 20, 40, 80, 160 min, or 12 h, animals were observed for clinical signs and body weights were recorded before euthanasia.

Pathological observation

After euthanasia, animals were weighed and immediately necropsied. In the sequential studies, livers and kidneys (without adrenals) were weighed. For light microscopy, sections of liver (left lateral lobe), kidney (left), lung, spleen, thymus, and heart were fixed by immersion in 10% neutral buffered formalin. Tissues were then routinely processed, embedded in paraffin, sectioned at 5~6 μm, stained with hematoxylin and eosin (H&E), and examined by light microscopy. For transmission electron microscopy, sections of liver from the left lateral lobe were cut into small pieces (around 1 mm cubes) and were fixed in cold 2.5% glutaraldehyde with 0.1 M isotonic cacodylate buffer (pH 7.4) and 3% sucrose (pH 7.2). The tissues were washed in cold phosphate buffered saline, post-fixed in cold 1% osmium tetroxide phosphate buffer, dehydrated through a graded series of ethanol, and processed routinely for embedding in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. They were observed under a transmission electron microscope (JEOL JEM 1010, Japan).

Statistical Analysis

Significant differences between groups were evaluated by using the Student's t test. $p < 0.05$ was regarded as a significant difference between time points.

RESULTS

Clinical and gross pathologic findings

MC-LR-treated rats became slightly less responsive at 20 min post injection and they showed lethargy, inactiveness, and ruffled hair at 40 min after MC-LR exposure (Fig. 1A). All animals survived until they were sacrificed. The livers from MC-LR-treated rats were tinged dark red, markedly swollen, and severely congested at 40 min (Fig. 1B). The severity of lesions was increased by time dependent manner.

Relative liver and kidney weights

As shown in Fig. 2A, relative liver weights (as % of body weight) were markedly increased at 40 min after

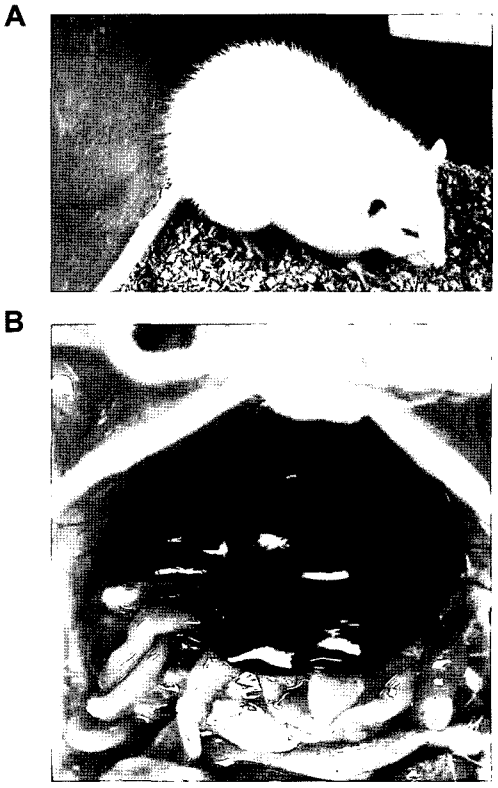

Fig. 1. Clinical and gross pathologic findings of MC-LR dosed rats and their liver. Rats (n = 3) were given a single intraperitoneal injection of MC-LR (100 μg/kg). MC-LR treated rats showed lethargy, inactiveness, and ruffled hair at 40 min post injection (A). The liver was tinged dark red and swollen at 40 min after dosing (B).

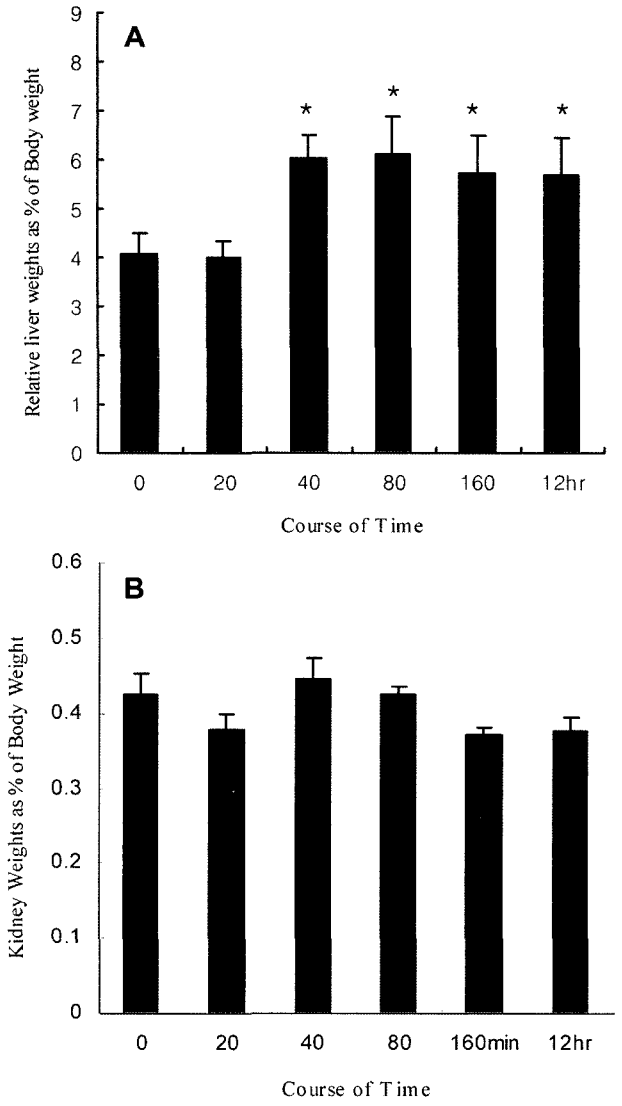

Fig. 2. Relative liver and kidney weights after MC-LR treatment. After MC-LR exposure, animals were weighed at different time points. In the sequential studies, livers (A) and kidneys (B) were weighed at each time point. Results are expressed as the mean ± SE of three separate mice and graphs are representative of three similar experiments. Statistically significant differences in relative liver weights (* $p < 0.05$) were observed.

MC-LR treatment and were significantly higher until 12 h post injection compared to MC-LR non-treated control group ($p < 0.05$). Relative kidney weights (as % of body weight) were not significantly changed (Fig. 2B).

Light microscopic changes

Light microscopic changes progressed in lesion severity and extent with increasing MC-LR exposure times. Centrilobular hepatocytes of MC-LR-treated rats were mildly dissociated beginning at 20 min post injection. At 40 min, hemorrhages around central veins were more severe than those around portal regions (Fig. 3A).

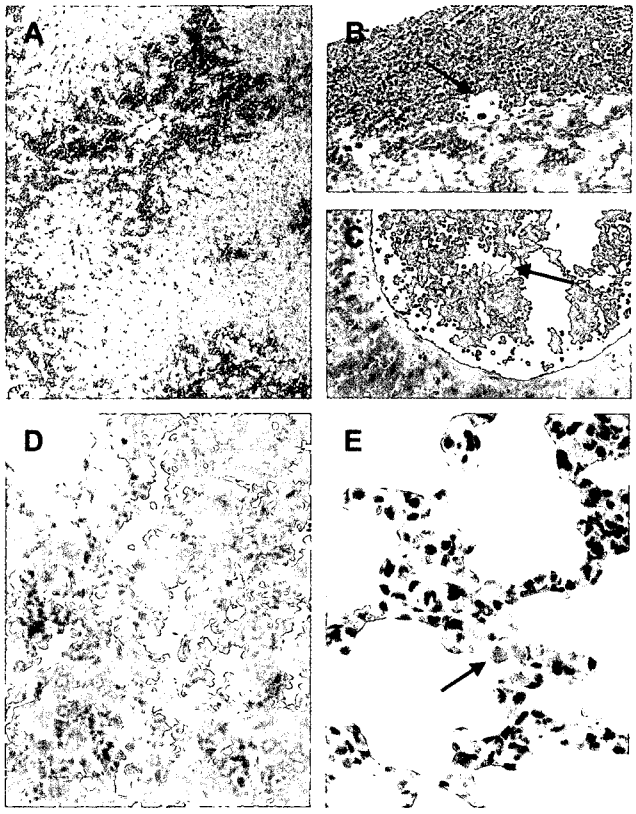

Fig. 3. Light micrography of MC-LR treated rat liver and lung at 40 min post dosing. Hemorrhage around the central vein (A). × 100. Dissociated hepatocytes (arrow) mixed with erythrocytes on the liver surface (B) and in the central vein (C). × 200. Extensive hepatocytic dissociation and hemorrhage were prominent (D). × 400. The entrapped eosinophilic debris (arrow) in the lung capillaries (E). × 400.

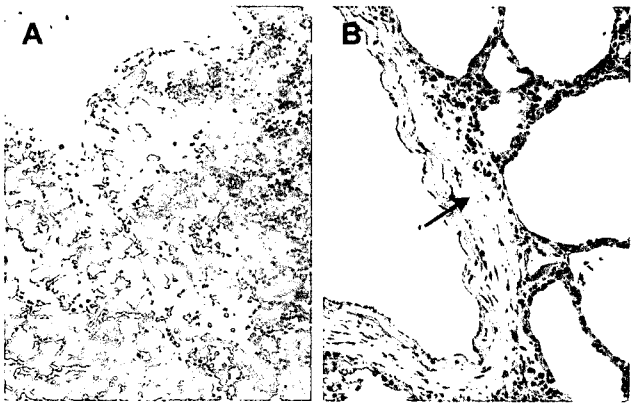

Fig. 4. Light micrography of MC-LR treated rat liver and lung at 80 min post dosing. Loss of centrilobular architecture with destruction of central vein (A). × 200. Edema around vessel of the lung were examined (B). × 200.

Degenerative hepatocytes mixed with hemorrhagic erythrocytes were observed on the surface of liver (Fig. 3B) and some instances, dissociated hepatocytes were examined inner central vein (Fig. 3C). In some regions, extensive hepatocytic dissociation and hemorrhage were prominent (Fig. 3D). Interestingly, entrapped hepatocellular debris were observed in the pulmonary capillaries of animals (Fig. 3E). At 80 min post exposure, the regions of hepatocytic dissociation extended to midzonal regions. Furthermore, loss of centrilobular architecture with destruction of the central vein (Fig. 4A), eosinophilic debris entrapped in the capillaries of glomerulus of the kidney, and edema around the vessels of the lung were examined (Fig. 4B). At 160 min post dosing, many hepatocytes together with inflammatory cells became necrotic. At 12 h post treatment, some regions of necrotic hepatocytes were sloughed off and such pathologic lesions extended to portal regions (Fig. 5A). The entrapped hepatocellular debris were still observed in the pulmonary venules or capillaries (Fig. 5B) as well as the capillaries of glomerulus of the kidney (Fig. 5C). And also the tubular epithelia of kidney were degenerative (Fig. 5D). Further noticible lesions were not observed in other organs.

Ultrastructural Changes

Hepatocyte cytoplasm of control group showed normal architecture of the endoplasmic reticulum, well preserved mitochondria, normal bile canaliculi with microvilli, and intact tight or gap junctions. Ultrastructural alterations were observed beginning at 20 min after MC-LR treatment and became progressively more severe. It was noticible widening of intercellular spaces between centrilobular hepatocytes and the bile canaliculi containing membranous fragments. Such changes of lesions included disrupted microvilli along the sinusoidal face in the perisinusoidal space of Disse (Fig. 6A).

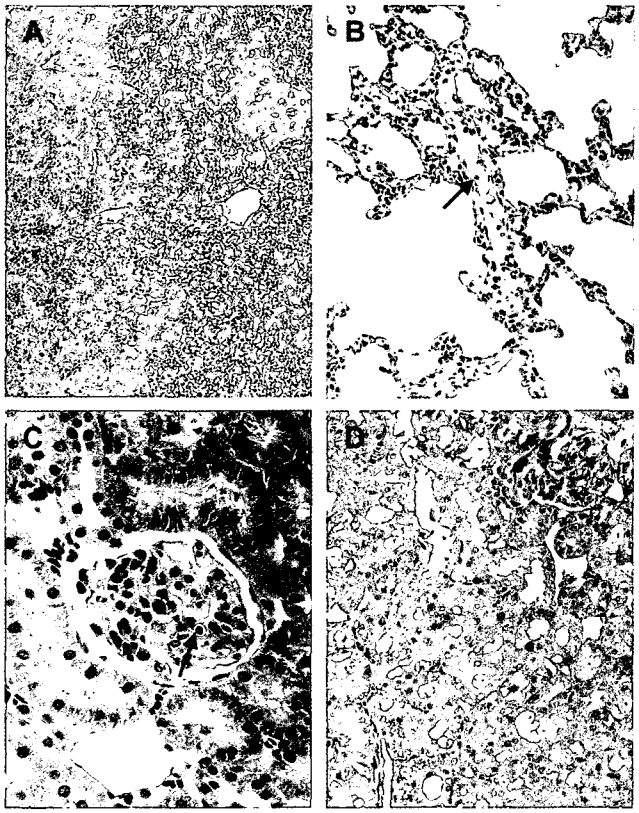

Fig. 5. Light micrography of MC-LR treated rat liver, lung, and kidney at 12 h post dosing. Many hepatocytes became necrotic and this pathologic lesions extended to portal regions. A few hepatocytes around portal area were intact (A). × 100. The entrapped hepatocellular debris (arrow) were shown in the pulmonary venules (B). × 200. The entrapped eosinophilic debris (arrow) on the capillaries of glomerulus were observed (C). × 400. The tubular epithelia of kidney were degenerative (D). × 200.

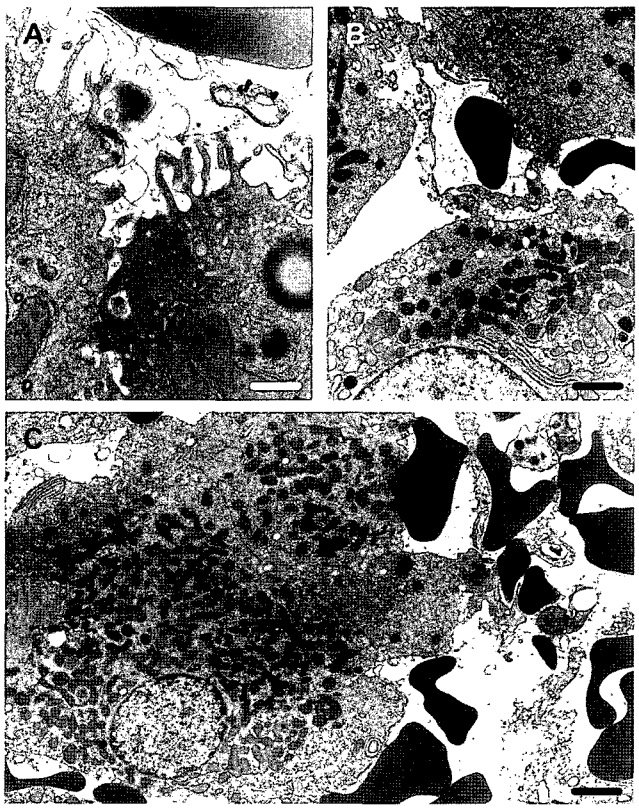

Fig. 6. Transmission electron micrography of MC-LR treated rat hepatocytes. Hepatocyte microvilli were scanty and damaged at 20 min after dosing (A). (Bar = 500 nm, mag. × 15,000). Space of Disse was widened and erythrocytes intermingled with necrotic cell debris were observed at 40 min post dosing (B). (Bar = 1 mm, mag. × 5,000). Dissociation of hepatocytes and erythrocytes intermingled with cell debris, 40 min post dosing (C). (Bar = 2 mm, mag. × 2,500).

The gap or tight junctions adjacent to bile canaliculi appeared intact at this time point. At 40 min post dosing, widening of the Disse space with leakage of red blood cells into the space (Fig. 6B), separation of adjacent hepatocytes (Fig. 6C), loss of sinusoidal architecture, and widening of the gap or tight junctions were prominent.

DISCUSSION

In the present study, the hepatic lesions induced by MC-LR were similar to those observed in another study (unpublished) using mouse model. There was a tendency of lesions toward a centrilobular distribution. Such lesions extended from central to portal regions and the severity was increased by time dependent manner. Furthermore, the ultrastructural observation demonstrated that MC-LR induced progressive hepatic damage on the time dependent. Especially, surface structures of hepatocyte, like space of Disse, microvilli, tight and gap junctions were mainly affected by MC-LR exposure.

Relative liver weights of MC-LR treated animals were markedly increased at 40 min post dosing, but after 80 min, relative values were slightly decreased until 12 h. Based on these data, we could speculate that slight decrease of relative liver weights from 80 min might be caused by the extensive hemorrhage from the liver and loss of hepatocytes, resulted from hepatocytic dissociation and circulation of necrotic hepatocytes through vascular system to lung, kidney, and other organs. In the current study, extensive hemorrhage and hepatocytic dissociation was prominent and entrapped intravascular eosinophilic debris were considerably observed in the pulmonary and glomerular capillaries. Furthermore, dissociated and circulative necrotic hepatocytes mixed with erythrocytes were examined in the liver surface and pulmonary venules.

Secondary effects of MC-LR may possibly affect the function of lung and kidney by emboli of necrotic hepatocytes within pulmonary and glomerular capillaries. Also, recent work with rats exposed to MC-LR has suggested a decrease in cardiac reserve that may compromise the normal response of the heart to circulatory insufficiency associated with severe intrahepatic hemorrhage (LeClaire *et al.*, 1995). In the current study, we could not find degenerative lesions from cardiac muscles of rats, but cardiac muscles of mice exposed to MC-LR (100 μg/kg) were degenerative (unpublished data).

The hepatotoxic mechanisms by MC-LR have been extensively studied (Stotts *et al.*, 1993; Miura *et al.*, 1989; Runnegar *et al.*, 1981). This toxin is mainly transported to the liver through a bile acid carrier and induces hepatocyte membrane damage by alteration of phosphorylation of cytoskeletal enzymes, resulting in cell necrosis or apoptosis (Yoshizawa *et al.*, 1990; Runnegar *et al.*, 1993). Recently, it was reported that MC-LR induces mouse hepatocyte apoptosis by two independent apoptosis pathway induced by various doses of MC-LR (50~70 μg/kg of body weight) (Chen *et al.*, 2005). In the current study, however, injection of MC-LR with higher dose (100 μg/kg of body weight) induced hepatocytes dissociation within 40 min post injection rather than apoptosis. Although some necrotic hepatocytes in the liver and other organs including kidney and lung were observed, it is not clear in the present study whether such cell death was caused by direct effects of MC-LR or by loss of cell to cell contact induced by MC-LR exposure. Therefore, additional study using lower dose of MC-LR may clearly show the morphologic changes of apoptotic hepatocytes and this issue is under investigation.

In conclusion, our findings demonstrated that MC-LR is primarily hepatotoxic which microscopical and ultrastructural changes were mainly observed in liver, and also can secondarily affect other organs including kidney, lung, and heart. Therefore, MC-LR is an environmental cytotoxin that has to be considered in water-related illness that can occur in both human and animals.

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