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Toxicity of microcystins in the isolated hepatocytes of common carp (*Cyprinus carpio* L.)

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Abstract

The toxicity of hepatotoxic microcystins produced mainly by *Microcystis aeruginosa* in mammals and fishes was well studied in recent years. However, there were scarcely reports in toxic effects of microcystins on isolated hepatocytes of fishes, especially investigation of microcystin-induced apoptosis and/or necrosis in carp hepatocytes. In the present study, the isolated hepatocytes of common carp were exposed to various concentrations of microcystins (0.01, 0.1, 1, 10, 100, 1000 $\mu g\,L^{-1}$) for 2, 4, 8, 16 and 24 h, respectively, and cytotoxicity of microcystins in the toxin-treated cells was determined. Results of this study showed that cytotoxicity of microcystins on carp hepatocytes was time and dose-dependent, and the approximate LC_{50} of microcystins in carp hepatocytes was 169.2 $\mu g\,L^{-1}$. The morphological changes typical of apoptosis, such as blebbing of cell membrane, condensation and fragmentation of cell nucleus were observed in the hepatocytes exposed to microcystins (1, 10 and 100 $\mu g\,L^{-1}$) using fluorescence and differential interference contrast microscopy. Agarose gel electrophoresis of DNA demonstrated a typical apoptotic "ladder pattern" in microcystin-treated hepatocytes after 16 h of exposure. Results of the present study indicated that the form of cell death in microcystin-treated hepatocytes depend on the exposure dose of toxin. When lower concentration of microcystins (10 and 100 $\mu g\,L^{-1}$) was used for exposure, carp hepatocytes died in apoptosis while, when higher one used (1000 $\mu g\,L^{-1}$), they died in the form of necrosis.

Keywords: Microcystins; Common carp; Hepatocyte; Toxicity; Apoptosis

1. Introduction

Microcystins are a family of hepatotoxic heptapeptides produced by some freshwater cyanobacterial species, primarily *Microcystis* (Carmichael, 1994). Their general structure is cyclo (–D-Ala–L-X–erythro-β-methy l-D-isoAsp–L-Y–Adda–D-isoGlu–*N*-methyldehydro-Ala). The amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4, 6-dienoic acid) is considered responsible for the hepatotoxicity of microcytins (de Figueiredo et al., 2004). There are more than 60 microcystin isoforms in part due to the variable L-amino acids *X* and *Y*, but the most frequent variant is microcystin-LR with the variable amino acids leucine and arginine (Codd, 2000). Toxicologically, it appears that microcystins caused damage when they were taken up by hepatocytes and inhibited serine/threonine protein phosphatases 1 and 2A (Eriksson et al., 1990; Runnegar et al., 1993). The resulting imbalance in protein phosphorylation disrupted liver cytoskeleton, which led to massive hepatic haemorrhages that caused death in rodents and fish (Rabergh et al., 1991; Dawson, 1998; Li et al., 1999, 2001a; Fischer and Dietrich, 2000; de Figueiredo et al., 2004; Jos et al., 2005; Pichardo et al., 2005).

There were many reports about the toxicity of microcystins in hepatocytes of rodents (Mereish and Solow, 1990; Ding et al., 1998, 2000) and in cell lines of mammal (Chong et al., 2000; Zegura et al., 2003); information regarding toxic effects of microcystins on hepatocytes of freshwater fish was relatively scarce (Mankiewicz et al., 2001; Li et al., 2001a, b, 2004; Malbrouck, et al., 2004; Li et al., 2005; Pichardo et al., 2005; Xie et al., 2005).

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According to several authors (Eriksson, et al., 1990; Runnegar et al., 1993; Dawson, 1998; de Figueiredo et al., 2004), hepatocytes were the main target of microcystins because the uptakes of these toxins require the action of multispecific transporter bile acids.

In recent years, heavy *Microcystis* bloom frequently occurred in the eutrophic Dianchi Lake, and the dominant species is *Microcystis aeruginosa*, which produced microcystins (Li et al., 2001a, 2003). Unpleasant odors and tastes were consequences of this bloom, and the bloom also caused poisoning of livestock and wildlife. Dianchi Lake is an important resource for agriculture and drinking water to people in Kunming, Yunnan province of China. So, there is much concern about using this lake as a source of drinking water and the possible effects of this water on human health. Meanwhile, common carp is major ingestor of *Microcystis* in this lake, and they are often captured as food supply for citizens of Kunming. Therefore, research on the behavior of microcystins in the freshwater food chain and the possible hazard to human health is necessary (Carbis et al., 1997; Li et al., 2004).

The present study aimed to determine the cytotoxicity of microcystins in the isolated hepatocytes of common carp after the cells were exposed to various concentrations of microcystins (0.01, 0.1, 1, 10, 100, 1000 $\mu g\,L^{-1}$) for different time intervals to evaluate the potential risks for human health.

2. Materials and methods

2.1. Toxin and reagents

Microcystins used were crude toxin isolated and purified from *Microcystis* bloom from Dianchi Lake according to the method of Harada et al. (1988) with minor modifications. High performance liquid chromatography (HPLC) analysis revealed that microcystin-LR was the dominant variant in the crude microcystins, and its concentration was 380.67 μg/mg dry weight of crude toxin. The concentrations of both microcystin-RR and microcystin-YR in the crude microcystin were below the limits of detection by HPLC analysis.

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin and streptomycin were obtained from GIBCO BRL (Grand Island, NY 14072, USA). Hoechst 33258, proteinaseK and RNaseA were bought from Sigma (St. Louis, MO, USA).

2.2. Preparation of hepatocytes

Common carp (4 female fishes, mean body weight 1000 ± 16 g) were purchase from the local market, and were maintained in a 400-L tank containing drinking water. The fish were fed ad libitum with commercial carp food at a daily-rate of 1.5% of body weight. Water temperature was maintained at 20 ± 1 °C and dissolved oxygen values were maintained between 7.1 and 7.6 $mg\,L^{-1}$. Fish were exposed to a 12-h light/12-h dark photoperiod, and the tank water was changed once every 3 days.

After acclimatization for two weeks, fish were killed by a sharp blow on the head, and their individual livers were pooled. Pooled livers were washed with cold phosphate buffered saline (PBS, pH 7.2) to eliminate as much blood as possible. Hepatocytes were isolated by 0.25% trypsin digestion (Li et al., 2001b), and then cells were suspended in DMEM and washed with the same medium for three times to eliminate trypsin and blood cell. Isolated hepatocytes were resuspended in DMEM supplied with penicillin (100 IU/ml), streptomycin (100 μg/ml), 10% fetal bovine serum at a concentration of 1×10^6 cells/ml, and were incubated in the 25 ml cell culture bottles at 25 °C until cytotoxicity assay (8–12 h later). Cell number and viability were determined from trypan blue exclusion (Li et al., 2001b).

2.3. Cytotoxicity assay

According to the dinking water standard of microcystins by WHO (Falconer et al., 1994), the isolated hepatocytes were treated in the culture bottles with microcystins (0.01, 0.1, 1, 10, 100, 1000 $\mu g\,L^{-1}$) in DMEM at 25 °C for 2, 4, 8, 16, and 24 h, respectively. Every treatment was conducted in triplicate. After incubation, toxin was removed by washing the cell cultures with DMEM for three times, and survival viability of cell was measured by trypan blue staining. The control cells were cultured in the same conditions without toxin, and viability was also determined by the same method as the treatments.

2.4. Cell analysis by microscopy

During the period of toxin-exposure described as above, 50 μL of hepatocytes were taken from the culture bottles and stained with DNA-specific dye Hoechst 33258 (final concentration = 1 $\mu g\,mL^{-1}$) for 5 min at 25 °C. After washing with PBS to eliminate the dye, cells were resuspended in DMEM and analyzed using Nikon Eclipse E600 microscopy with fluorescence light and differential interference contrast.

2.5. Analysis of DNA

DNA from samples of control and microcystin-treated hepatocytes was isolated according to the method of Zhao (1997). Electrophoresis was carried out for 2 h in 1.3% agarose gel. A *Hind*III and *Eco*RI digest of λ phage DNA was served as molecular size standard.

2.6. Statistical analysis

ANOVA and the Student's *t*-test were used to analyze differences of cell viability between the treated and control samples. Statistical significance was indicated by *P*-values <0.05.

3. Results

3.1. Cytotoxicity of microcystins

Viabilities of isolated carp hepatocytes incubated with various concentrations of microcystins for different periods of time were shown in Table 1. Cytotoxicity of microcystins on carp hepatocytes was in a time and dose-dependent manner. Cell viability decreased with the time of incubation. At the early periods of exposure, no difference was found in cell viability of all treated groups. After 16 h of exposure, however, viability of hepatocytes incubated with 1000 $\mu g\,L^{-1}$ of microcystins was much lower than that of control ($P<0.001$). Moreover, obvious differences were also detected in cell viability of treatments (10 and 100 $\mu g\,L^{-1}$) and the control after 24 h of incubation ($P<0.05$).

The toxic effect of microcystins on carp hepatocytes was also dose-dependent. There was no significant change in viabilities of cells treated with low concentrations of microcystins (0.01, 0.1 and 1 $\mu g\,L^{-1}$) during 24 h of

Table 1
Viabilities of carp hepatocytes exposed to various concentrations of microcystins for different time intervals

Time (h)	Concentrations of microcystins ($\mu g\,L^{-1}$)						
	0	10^{-2}	10^{-1}	1	10	10^{2}	10^{3}
0	96.3±0.59	95.4±0.9	94.5±1.2	94.8±0.8	94.3±1.4	93.9±1	93.8±1.6
2	96.3±0.59	95.4±0.9	94.5±1.2	94.8±0.8	94.3±1.4	93.9±1	93.8±1.6
4	95.8±1	93.5±0.9	93.1±2.1	94.2±1.2	92.5±1.7	94.4±0.4	91.7±1.1
8	93.5±0.9	92.5±0.6	92±0.3	91.4±0.7	90.4±1.2	88.7±2	88.1±1.3
16	81.5±1.7	77.9±4.1	78.3±3	78.1±2.3	77±0.9	75.7±1.5	4.6±0.8***
24	71±3.1	69.4±2	68.2±2.9	61.9±4.9	44.5±3.9*	38.7±2.5*	0***

Cell viability was determined by trypan blue exclusion.
Values are the mean±SD for triplicate. *$P<.0.05$, ***$P<0.001$.

exposure. In contrast, viabilities of hepatocytes incubated with higher concentrations of toxins (10 and 100 $1\,\mu g\,L^{-1}$) decreased significantly compared with that of control, and highest concentration of microcystins in this test (1000 $1\,\mu g\,L^{-1}$) caused 100% of cell death after 24 h of incubation. The approximate LC_{50} of microcystins in carp hepatocytes was $169.2\,\mu g\,L^{-1}$ as determined after 24 h of exposure.

3.2. Cytological alterations

Morphological changes in hepatocytes were examined by means of fluorescence and differential interference contrast microscopy. First cytological alteration typical of apoptosis was detected after 4 h of incubation with microcystins (1, 10, and $100\,\mu g\,L^{-1}$, respectively). These changes noted were condensation and margination of chromatin, shrinking and deformation of nuclei (Fig. 1B), and blebbing of cell membrane (Fig. 1C). No cytological changes were observed in cells treated with low concentrations of microcystins (0.01 and $0.1\,\mu g\,L^{-1}$, respectively) duration of 24 h exposure. Cell necrosis was found in hepatocytes treated with $1000\,\mu g\,L^{-1}$ of microcystins after 16 h of exposure (Fig. 1D).

3.3. DNA damage

Agarose gel electrophoresis was used to detect the biochemical changes in DNA. After 2, 4, and 6 h incubation of the cells with 1, 10 and $100\,\mu g\,L^{-1}$ of microcystins, respectively, no change was observed. DNA damage (DNA strand breaks) was first found after 8 h of exposure at $10\,\mu g\,L^{-1}$ of microcystins, and this damage became more and more serious as the time of incubation, as shown in Fig. 2. This agarose gel electrophoresis of DNA demonstrated a typical apoptotic "ladder pattern" (Fig. 2, lanes 6–8). Instead of DNA ladder, $1000\,\mu g\,L^{-1}$ of microcystins after 16 h incubation caused DNA smear characteristic for necrosis (Fig. 2, lane 9).

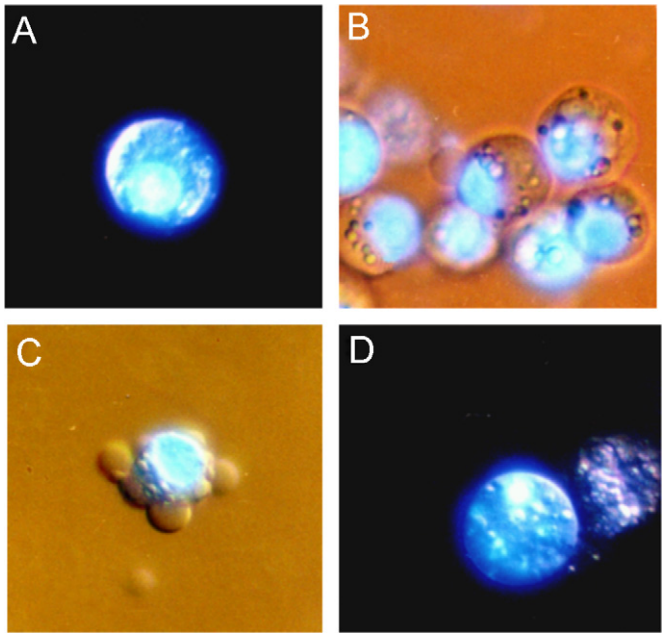

Fig. 1. Microscopic observations in the microcystin-treated hepatocytes of common carp. (A) Control cell, 600× of magnification. (B) Hepatocytes exposed to $100\,\mu g\,L^{-1}$ of microcystins for 4 h, showing the deformation of nuclei, 600× of magnification. (C) Hepatocytes incubated with $100\,\mu g\,L^{-1}$ of microcystins for 16 h, showing the blebbing of cell membrane, 600× of magnification. (D) Showing the necrotic hepatocytes exposed to $1000\,\mu g\,L^{-1}$ of microcystins for 16 h, 600× of magnification.

4. Discussion

The results of this study showed that cytotoxicity of microcystins in the isolated hepatocytes of common carp was dose dependent, and the approximate LC_{50} of microcystins in carp hepatocytes was $169.2\,\mu g\,L^{-1}$. This value was much higher than the LC_{50} in rat hepatocytes obtained by Mankiewicz et al. (2001), where 50% of hepatocytes exhibited morphological changes after 120 min incubation with cyanobacterial extract (EC_{50}) at the range between 50 and 100 nM. In addition to the present study, we also gained similar LC_{50} in silver carp and crucia carp hepatocytes in the separate study (data not shown).

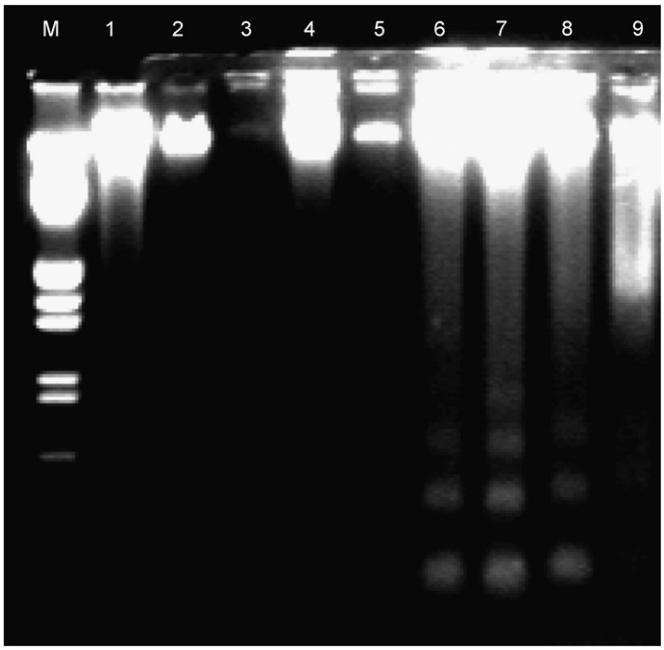

Fig. 2. Agarose gel electrophoresis of DNA from the microcystin-exposed hepatocytes of common carp for different time's intervals. (M) λ phase DNA with *Hind* III and *EcoR*I digest; (1) control cells; (2) hepatocytes exposed to 10 μg L^{-1} of microcystins for 2 h; (3) 4 h of incubation with toxin; (4) 6 h; (5) 8 h; (6) 16 h, (7) 24 h, showing DNA in ladder pattern; (8) 36 h; and (9) hepatocytes exposed to 1000 μg L^{-1} of microcystins for 16 h, showing smear DNA.

Comparing the influence of microcystins on rat and fish hepatocyte, it appears that rat hepatocyte is more sensitive to microcystins than fish cell (Rabergh et al., 1991; Dawson, 1998; Li et al., 1999; Fischer and Dietrich, 2000; Li et al., 2001a, b; de Figueiredo et al., 2004; Malbrouck et al., 2004; Pichardo et al., 2005).

The present study also revealed that toxicity of microcystins in carp cells was time-dependent. First morphological changes and DNA strand breaks in hepatocytes were detected after 4 and 8 h of incubation with microcystins (100 and 1000 μg L^{-1}), respectively. However, statistical difference in cell viability of hepatocytes between the treatment (1000 μg L^{-1}) and control could be found as late as 16 h of exposure (Table 1). Therefore, cytological and biochemical damages caused by toxin can be observed earlier than cell death. Additionally, Mankiewicz et al. (2001) reported that the first toxic effects of microcystins on rat hepatocytes could be detected after 30 min of incubation with the cyanobacterial extract (microcystin-LR = 50 nM), showing a much earlier toxicity time of microcystins in rat cells than that in fish hepatocytes. Sensitivity to microcystins in different species may account for this discordance, and this result also suggested that fish hepatocyte was less sensitive to microcystins than rat cells.

Apoptosis is a mode of cell death, and the typical morphological signs of apoptosis are shrinkage of the cell, blebbing of cell membrane, nuclear condensation, and fragmentation, with the creation of a characteristic "half-moon" structure (Kerr, et al., 1972; Fraser and Evan, 1996). Moreover, internucleosomal fragmentation of DNA with the ladder pattern as a distinguishing characteristic of this process appears in the late phase of apoptosis (Martin and Green, 1995). In this study, all these cytological and biochemistry changes described as above were detected in carp hepatocytes exposed to 10 and 100 μg L^{-1} of microcystins for 24 h. A similar result was obtained by Pichardo et al. (2005) in the microcystin-LR-treated fish cell line PLHC-1. This result was also consistent with the observation previously in rat hepatocytes (Eriksson et al., 1989; Ding et al., 1998) or cell lines of mammal (McDermott et al., 1998; Mankiewicz et al., 2001) and fish (Li et al., 2001a, b, 2003). On the other hand, the mechanisms of microcystin-induced apoptosis and hepatotoxicity have not been fully elucidated until now (Ding et al., 2000). Recently, Ding and Ong (2003) reported that the activation of calpain and Ca^{2+}/calmodulin-dependent protein kinase was believed to be critical in the microcystin-induced apoptotic process. However, further studies on the exact sequence of molecular events by which microcystins induced apoptosis in hepatocytes are necessary.

Generally speaking, hepatocytes of animals died in two ways: either apoptosis or necrosis (Kerr, 1971). Results of the present study indicated that the form of cell death in microcystin-treated hepatocytes depend on the exposure dose of toxin. When lower concentration of microcystins (10 and 100 μg L^{-1}) was used for exposure, carp hepatocytes died in apoptosis, while when higher one used (1000 μg L^{-1}), they died in the form of necrosis.

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