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Genotoxicity of crude extracts of cyanobacteria from Taihu Lake on carp (*Cyprinus carpio*)

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Abstract Genotoxicity of crude cyanobacteria extracts (CBE) from blooms in Taihu Lake, China toward common carp (*Cyprinus carpio*) was measured. The primary extracellular product was determined by HPLC to be Microcystin-LR (MC-LR, L for leucine and R for arginine) with an average concentration of 2.4×10^2 μg MC g^{-1} dry weight of cyanobacteria. Acute toxicity to carp, expressed as the 72-h $LC_{50,}$ was 53 mg, dw cyanobacteria L^{-1}. Genotoxicity, as determined by the micronucleus (MN) and comet assays were both dose- and time-depended. Deformities of cellular organelles in liver and gill were observed by use of transmission electron microscopy (TEM). The results showed that MC-LR from cyanobacteria from Taihu Lake could induce genotoxic response and tissue-level morphological changes in common carp.

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Introduction

As one of the primary producers in aquatic ecosystems, microalgae play an important role in biogeochemical cycling (Morel and Price 2003). Algae can release materials into the surrounding water and approximately 40 microalgae species can secrete toxic extracellular products (Sournia et al. 1991). Some of the most commonly produced cyanotoxins are microcystins (MCs) (Chorus and Bartram 1999). MCs are toxic heptapeptides with a basic cyclic structure which share a common moiety composed of seven amino acids. They are named according to their variable L-amino acids and nearly 80 different MCs have been identified (Hoeger et al. 2005; Djediat et al. 2010). MCs can be produced by several species of cyanobacteria in the genera *Microcystis*, *Anabaena* and *Planktothrix* especially under conditions of eutrophication (Chorus and Bartram 1999) and they can be released from lysed cells during natural senescence, physical pressure, or exposure to herbicides (Dietrich and Hoeger 2005; Ross et al. 2006; Gérard et al. 2009). The most toxic of the MCs is Microcystin-LR (MC-LR, L for leucine and R for arginine; Chorus and Bartram 1999) which can cause (1) strand breaks of DNA in different types of cells (Mankiewicz et al. 2002; Zegura et al. 2003), (2) oxidative damage of DNA in hepatocytes (Maatouk et al. 2004), and (3) formation of micronuclei in human lymphoblastoid cells (Zhan et al. 2004).

MCs are specific inhibitors of serine/threonine phosphatases (Runnegar et al. 1995) and thus can produce reactive oxygen species (ROS) which subsequently can cause DNA damage (Ding et al. 1998), and thus pose a

potential hazard to birds and mammals as well as aquatic animals (Puschner et al. 1998; Gulland et al. 2002; Shumway et al. 2003; Schnetzer et al. 2007). Traditionally, MCs have been considered to be hepatotoxic due to their accumulation in hepatocytes by organic anion transporting polypeptide (OATP) type specific transporters (Fischer et al. 2005). However, OATP are not only expressed in the liver, but also in the gastrointestinal tract, kidney, brain, and across the human blood–brain barrier (Dietrich and Hoeger 2005; Gaudin et al. 2008).

The comet assay and micronucleus assay (MN assay) have been used to investigate DNA damage and chromosomal damage, respectively (Steinert 1999; Fenech et al. 2003). Both of these assays are routinely used as indices of effects on chromatin because they are easy to perform and sensitive (Steinert 1999; Fenech et al. 2003). The alkaline comet assay, also known as the Single Cell Gel Electrophoresis (SCGE) assay, is used to detect single- and double-strand breaks, alkali-labile sites, and DNA–DNA and DNA–protein cross-links in individual cells (Tice et al. 2000; Gaudin et al. 2008). In the comet assay, several endpoints can be used. The Olive tail moment (OTM), which is defined as the product of the tail length and the percentage of tail DNA, has been recommended as an effective index of DNA damage. OTM is an indication of the relative fluorescent intensity in the head and tail (Lovell and Omori 2008). The MN assay is used to detect micronuclei which are formed by chromosome fragments or whole chromosomes that are not reassembled into the nucleus during cell division (Heddle et al. 1991).

Taihu Lake, the third largest freshwater lake in China, is located in the Yangtze River delta of southeastern China. It has a large human population density and some of the greatest agricultural and industrial production in China. Eutrophication of the lake over the past few decades has lead to the occurrence of cyanobacteria blooms. Average concentrations of MCs in Taihu Lake range from 0 to 16 μg L^{-1}, with the greatest concentrations occurring in Meiliang Bay (Xu et al. 2008), and the most common type of MCs in Taihu Lake is MC-LR (Qi et al. 2009). The current study evaluated the genotoxicity of cyanobacteria extracts (CBE, mainly MCs) from Taihu Lake on the common carp (*Cyprinus carpio* Linnaeus; Cyprinidae) by use of the MN and comet assays. Transmission electron microscopy (TEM) was also used to evaluate the effects of MCs on the ultrastructure of carp liver and gill cells.

Materials and methods

Chemicals and animals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Standard MC-LR material was purchased from the Institute of Hydrobiology, Chinese Academy of Science (Wuhan, China).

150 carp with an average length of 10.4 ± 0.7 cm and average weight of 8.0 ± 2.3 g were purchased from the same supplier in a local fish market (Nanjing, China). All fish had the same histories and were acclimated under laboratory conditions at 18 ± 1°C, 12/12 h dark/light cycle for 7 days before testing. During the acclimation period, all fish were placed in the same container (40 L) and fed with fish food that had been shown previously to contain no MCs. One-fourth of the water in the container was changed every day during acclimation and air was bubbled through the container continuously. Fish were not fed during the experimental period. Tap water used in this study was dechlorinated for 3 days before use.

Collection of cyanobacteria and identification and quantification of MCs

Cyanobacteria were collected from Meiliang Bay, Taihu Lake during a bloom that occurred in early autumn 2008. Cyanobacteria were filtered from water and lyophilized (FreeZone12, Labconco) to produce dry cyanobacteria products. The dry cyanobacterial powder was then stored at −20°C. Dry cyanobacteria powders were mixed with dechlorinated tap water and sonicated at 150 W for 5 min in an ice-water bath with an ultrasonic processor (JY-250, Zhejiang, China) to release MCs into solution and to attain a CBE solution. Microscopic examination of supernatants indicated more than 90% of the cyanobacteria cells were lysed. The extract was centrifuged at 10,000×*g* for 30 min and filtered. The solution was then evaporated and pulled through a Sep-Pak C18 column and washed with 20% methanol. MCs were eluted with 90% methanol containing 0.1% TFA according to previously published methods (Harada et al. 1988; Aranda-Rodriguez et al. 2005; Qi et al. 2009). Concentrations of MCs in the extracts were analyzed by high performance liquid chromatography (HPLC) (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) equipped with a Zorbax Eclipse SB-C18 column (250 mm × 4.6 mm, 5 μm, Agilent Technologies). The mobile phase consisted of water (0.05% TFA) as solvent A and acetonitrile (0.05% TFA) as solvent B. The gradient elution program was performed as follows: % solvent A/solvent B, 10/90 at 0 min, 35/65 at 20 min, 35/65 at 25 min. The flow rate was 1.0 mL min^{-1} and the injection volume was 20 μL. Chromatograms at 238 nm were recorded with an Agilent 1100 Series PDA detector. Quantification was based on external calibrations of standard MC-LR. Recovery experiments were conducted in quadruplicates by adding standard MC-LR in Milli-Q water to 0.5 μg L^{-1}. The recovery rate was 86.6% ± 4.9%.

Concentrations of MCs were reported as μg g^{-1} dry cyanobacteria.

Acute toxicity

Forty common carp were randomly assigned to four groups (ten carp $group^{-1}$) and each group was placed in one of four 10-L fish tanks. Carp groups were exposed to 0, 37.5, 75, or 150 mg CBE L^{-1}. The 72 h median lethal concentration (72 h LC_{50}) was determined by the method previously reported by Fawell et al. (1999). The calculated 72 h LC_{50} was used to determine fish exposure concentrations in subsequent toxicity assays.

MN assay and comet assays

One hundred common carp were randomly assigned to two groups (50 carp each) and exposed to CBEs for 3 or 7 days. Within each of the exposure periods, subgroups of ten common carp, each, were exposed to 0, 6.25, 12.5, 25.0 or 50.0 mg CBE L^{-1}. At the end of the exposure period, peripheral blood was collected from the caudal vein and smeared onto pre-cleaned slides. Three fish were randomly selected from each concentration of each exposure period and three slides were prepared for each fish. After fixation in pure methanol for 10 min, the slides were allowed to air-dry and then stained with 10% Giemsa solution for 25 min. All slides were coded and scored blind. Five thousand cells were scored from each concentration (Zhu et al. 2004) when observed under ×1,000 magnification (Chen et al. 2006). Small, nonrefractive, circular or ovoid chromatin bodies that display the same staining and focusing pattern as the main nucleus were classified as micronuclei (Al-Sabti and Metcalfe 1995).

The alkaline comet assay was performed according to the methods of Tice et al. (2000) with some modifications. Carp kidneys of three fish randomly selected from each of the exposure groups were removed and rinsed twice with ice-cold phosphate-buffered saline (PBS). Kidneys were then cut into pieces, transferred into glass tubes, homogenized using a glass rod for 1 min in an ice bath and passed through a 110 mesh sieve to remove suspended materials. The kidney cell suspensions were centrifuged at 1,500×*g* for 10 min (Beckman J2-MC), and collected cells were stored at 4°C for comet assay (Li et al. 2004). Frosted microscope slides, on which cells were embedded in an agarose sandwich, were immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% *N*-Lauroyl Sarcosine Na, adjusted to pH 10 with NaOH, 1% Triton X-100 and 10% DMSO). To remove cellular proteins, slides were stored in the dark at 4°C for at least 1 h. To allow DNA unwinding before electrophoresis, slides were placed on a horizontal gel electrophoresis unit filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA) at 20°C for 30 min. Electrophoresis was conducted at 20°C under 20 V and 200 mA for 20 min. To avoid DNA damage, the above steps were conducted under red light. After electrophoresis, the slides were washed three times with a neutralizing buffer (0.5 M Tris, pH 7.5), then DNA was stained with 50 μg mL^{-1} ethidium bromide (EB) and the slides were examined with a fluorescence microscope (BX41, Olympus, Japan). Each group consisted of at least five parallel samples and at least 50 cells were analyzed for each slide. Photos were taken with a digital camera (Olympus C-5050 ZOOM) and images were analyzed according to Collins et al. (1997) with the comet assay software project CASP (1.2.2).

TEM analysis

In the 7 day exposure group, livers and gills of controls or carp exposed to 50.0 mg L^{-1} were cut into pieces, fixed in 2.5% glutaraldehyde and made into paraffin-embedded sections following Datta et al. (2007). Sections were examined under TEM (Hitachi H7650, Japan) to observe changes in cell organelles.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Differences between control and treated samples were analyzed by one-way ANOVA using Origin Pro 7.0 software. For significant ANOVAs, a Student's *t*-test was used to determine which groups were significantly different from each other. A $p < 0.05$ was considered statistically significant.

Results

MCs concentrations and acute toxicity

The mean concentration of MC-LR from Meiliang Bay, Taihu Lake was 244.26 μg g^{-1} dry cyanobacteria. Based on this relationship, concentrations of MC-LR in the treatment solutions of 0, 6.25, 12.5, 25 and 50 mg CBE L^{-1} contained 0, 1.5, 3.0, 6.0 and 12.0 μg MC-LR L^{-1}, respectively. The 72-h LC_{50} for common carp was 50 mg CBE L^{-1}. After 72 h exposure, no fish died in the control group and all fish died in the group exposed to 150 mg CBE L^{-1}.

MN assay

Both dose- and time-dependent responses were observed in MN formation (Fig. 1). MN frequency was directly

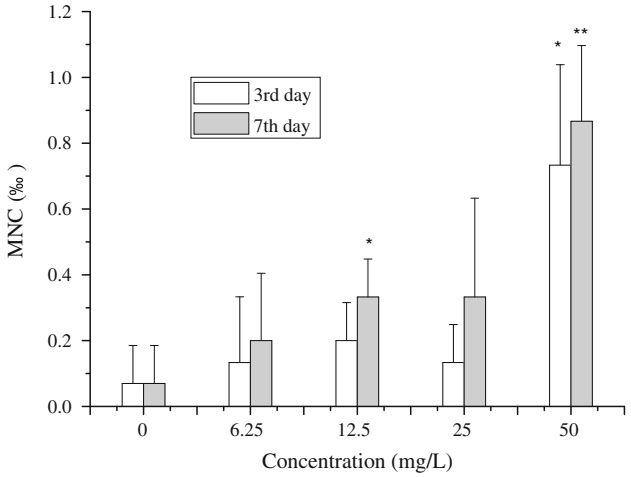

Fig. 1 Frequency of micronuclei in peripheral blood cells of carp exposed to CBE for 3 or 7 days, respectively. Values are shown as mean ± SD. Analysis of variance (ANOVA): $*p < 0.05$; $**p < 0.01$

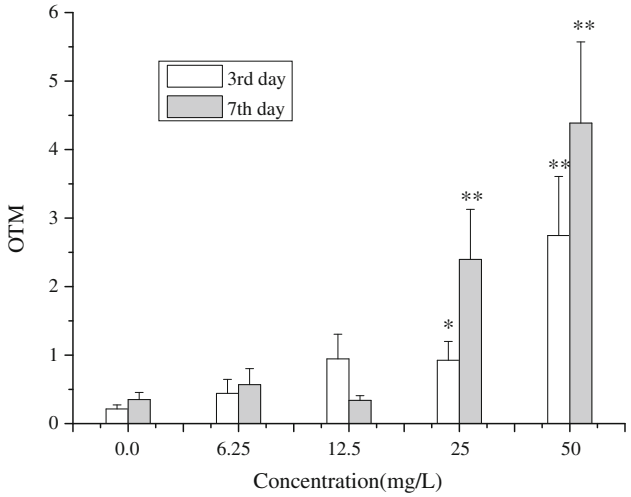

Fig. 2 Olive tail moment (OTM) values of kidney cells of carp exposed to CBE for 3 or 7 days, respectively. Values are shown as mean ± SD. Analysis of variance (ANOVA): $*p < 0.05$; $**p < 0.01$

proportional to concentrations of cyanobacteria equivalents. After 3 days exposure, there were significantly more MN in blood cells of carp exposed to 50 mg L^{-1} than in blood cells of carp not exposed to CBE. After 7 days exposure, there were significantly more MN in blood cells of carp exposed to 12.5 and to 50 mg L^{-1}, but not to 25 mg L^{-1} ($p < 0.005$) than in blood cells of unexposed carp (controls). The greater concentrations of CBE also caused damage to chromosomes. Frequencies of MN were 10- and 12-fold greater than the control when exposed to the greatest concentration of CBE for 3 and 7 days, respectively.

Comet assay

Dose- and time-depended effects of CBE on OTM of kidney cells were observed in the comet assay (Fig. 2). The OTM was proportional to concentration of CBE except for the 12.5 mg L^{-1} in the 7 days group. The OTM after 7 days exposure was greater than 3 days exposure. After 3 days exposure, the OTM of kidney cells from carp exposed to 25 or 50 mg CBE L^{-1} were significantly greater than that of the unexposed control carp. After 7 days exposure, DNA damage, as measured by the OTM was 5.8- and 11.5-fold greater than that of controls for the two greatest concentrations, respectively.

TEM analysis

There was severe cell damage in exposed carp. The ultrastructure of both gill (Fig. 3b) and liver (Fig. 4b) cells changed after exposure to CBE compared to unexposed carp (Figs. 3a, 4a). When exposed to MCs, fish liver cells were smaller than those of hepatocytes of untreated carp. The edges of the cell membrane became unclear and mitochondria swelled and some of the contents were missing. Gill cells from unexposed carp had a clear and complete cell structure with obvious cell membrane and nuclei. The mitochondria and endoplasmic reticulum (ER) were intact without any deformation. Gill cells from carp exposed to 50 mg CBE L^{-1} became smaller and were misshapen and membranes were less distinguishable. The nuclei and ER atrophied and nuclear membranes were broken. Mitochondria were swollen and the contents were often missing.

Discussion

CBE from Taihu Lake are genotoxic to carp and can lead to the formation of micronuclei and cause DNA strand breaks in a time- and dose-depended manner. The significant genotoxic effects of CBE observed in the MN and comet studies were consistent with the finding that CBE can cause DNA damage, nuclear condensation and fragmentation in rats exposed to MCs (Ding et al. 1998). MC-LR induce DNA stand breaks, in HepG2 cells, which were similar to the finding reported here (Zegura et al. 2003). Those authors suggested that DNA breaks were transiently present as intermediates formed during DNA repair. It has also been reported that MC-LR can cause genomic DNA fragmentation and DNA stand breaks in mouse liver in vivo (Rao and Bhattacharya 1996). Based on the results of the comet assay, Lankoff et al. (2006) concluded that nodularin, a MC belonging to the same class of MC-LR, that can cause oxidative modifications of DNA in a dose- and time-dependent manner.

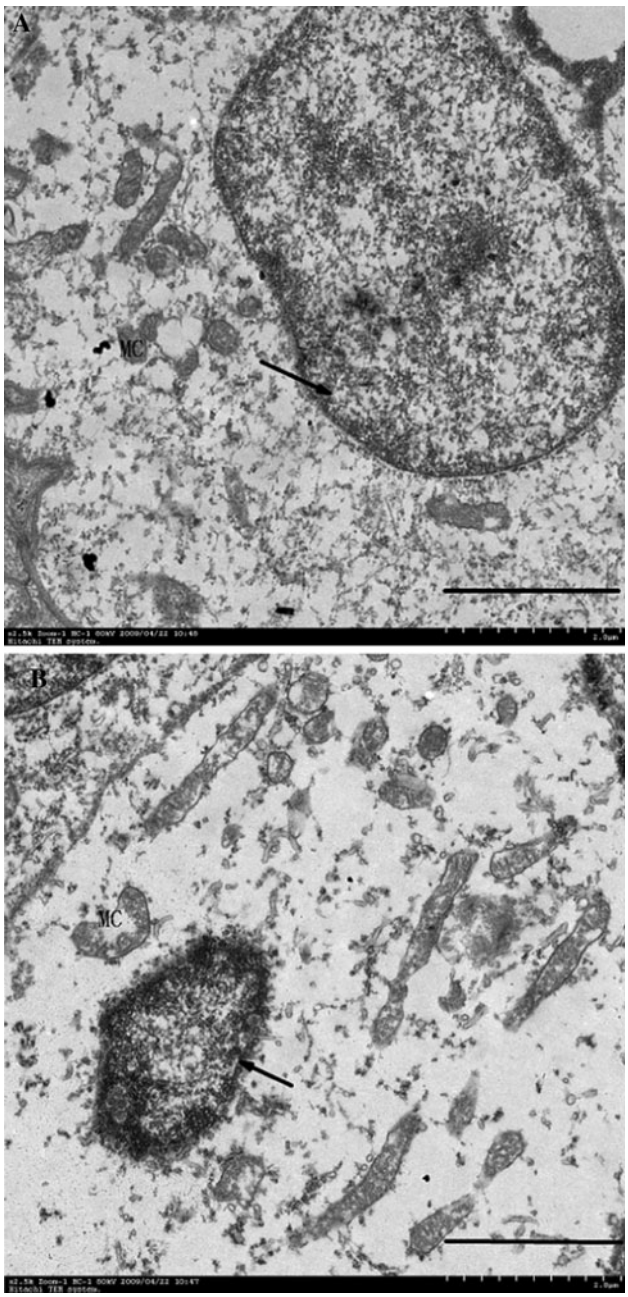

Fig. 3 Gill cell TEM images of carp in control group and 50 mg CBE L^{-1} group in 7-day CBE exposure test. **a** Gill cells of the control group, **b** gill cells of the 50 mg CBE L^{-1} group. ×2,500 magnification; *arrow* nucleus; *MC* mitochondrion; *ER* endoplasmic reticulum; size marker, 2 μm

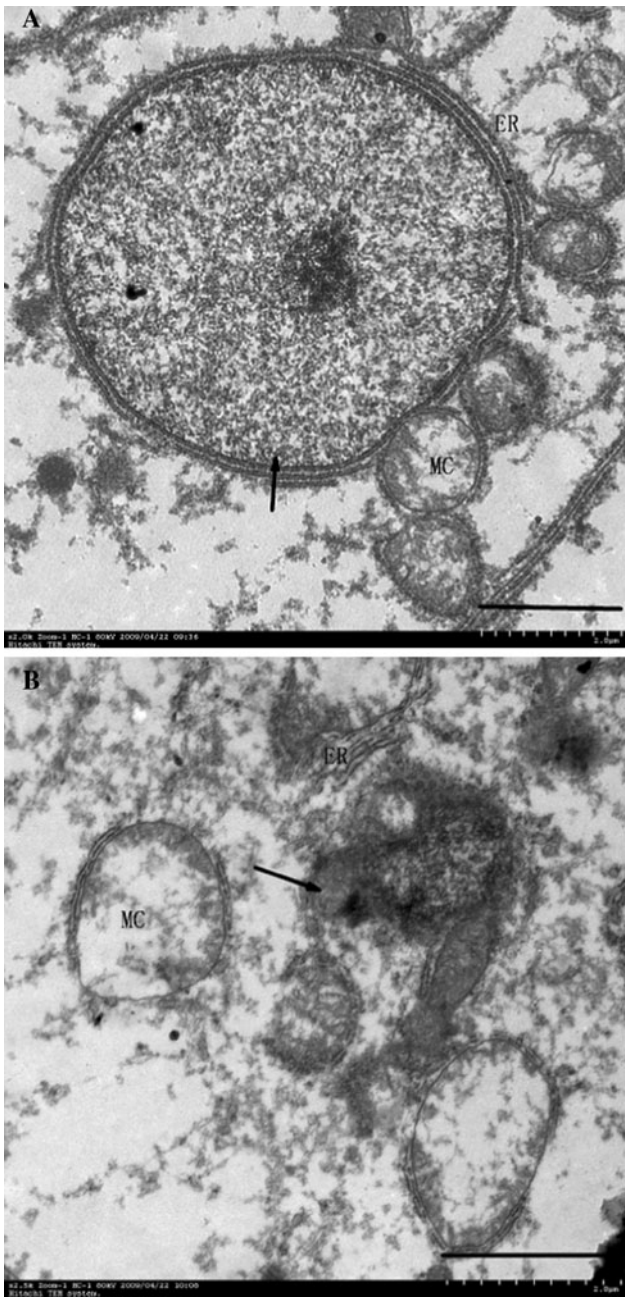

Fig. 4 Liver cell TEM images of carp in control group and 50 mg CBE L^{-1} group in 7-day CBE exposure test. **a** Liver cells of the control group, **b** liver cells of the 50 mg CBE L^{-1} group. ×2,500 magnification; *arrow* nucleus; *MC* mitochondrion; *ER* endoplasmic reticulum; size marker, 2 μm

According to Bolognesi et al. (2006) and Binelli et al. (2009), peripheral erythrocytes are the most appropriate tissues for evaluating the formation of MN in fish. While the MN test was able to demonstrate effects of CBE on fish cells, a disadvantage of using peripheral blood is that blood cells are generally less responsive to effects of genotoxic agents (Buschini et al. 2004). Also, it has been suggested that the comet assay, which is a sensitive indicator, should be combined with other biomarkers to determine DNA damage and genotoxicity (Mitchelmore and Chipman 1998). It was determined that the simultaneous use of the MN and comet assays was a useful battery of tests to determine the genotoxicity of CBE to the common carp.

The results of the current study indicate the ultrastructure of carp liver and gill cells were changed by exposure to CBE. As reported by Dietrich and Hoeger (2005), MCs

were found to form non-covalent and covalent interactions with the target enzymes PPs (PP1, 2A, 4, and 5). These interactions could then inhibit the catalytic subunits of the PPs and cause disorganization of cellular architecture and degeneration, and usually followed by cell death (Dietrich and Hoeger 2005). A recent study on the histopathology of snails exposed to dissolved MC-LR (Lance et al. 2010) indicated severe pathological changes including cell lysis and necrosis, vacuolization as well as cell shape alteration, in the digestive gland. Those authors also detected MCs in spermatozoids and oocytes of the snails, which suggested MCs may have potential adverse effects on reproduction functions. This is similar to the TEM reported here, that MCs extracted from cyanobacteria in Taihu Lake caused damage to cells of carp and malformations in organelles. MCs led to the formation of digestive vacuoles in cytoplasm, fusion of lysosomes, swelling of the ER and vacuolization of the Golgi apparatus. This is consistent with the results of other studies that have found that ER in hepatic cells of rats and carp were the most sensitive organelle when exposed to crude MC-LR extracted from CBE (Berg et al. 1988; Li et al. 2001). The results of those studies suggested that the structural deformations were related to modifications of the cytoskeleton. ER and the Golgi apparatus have detection and signaling mechanisms that respond to stressors by either activating cell repair or death pathways (Maag et al. 2003; Hicks and Machamer 2005). In another study with ovary and kidney cells, a causal relationship between ultra structural changes of the ER and Golgi in response to CBE was observed (Davidson et al. 1992). The TEM observations made in our study also found that ER and mitochondria in cells of carp swelled and the nucleus became condensed. These results indicated that CBE alter cell structures which will then affect cell functions. All of these observations are consistent with cellular organelles being the intracellular target for effects of crude extracts of cyanobacteria. However, the mechanism for this response remains unknown.

The occurrence of toxic cyanobacterial blooms in eutrophic lakes, reservoirs, and recreational waters is a worldwide problem (Paerl et al. 2001). MCs produced during blooms can be toxic to aquatic animals and can accumulate in the food chain and become hazardous to wildlife and humans. Small concentrations of MC-LR can cause oxidative DNA damage and may be the mechanism through which chronic exposure to MCs contributes to increased cancer incidence (Zegura et al. 2003). A positive association has been reported between the incidence of primary liver cancer and exposure to cyanobacteria-contaminated drinking water (Yu 1995).

To our knowledge, in vitro studies using mammalian cells (Zhan et al. 2004; Lakshmana Rao et al. 1998; Humpage et al. 2000; Zegura et al. 2003; Lankoff et al. 2003, 2004, 2006), bacteria cells (Mankiewicz et al. 2002; Ding et al. 1999), as well as in vivo experiments using mice and brown rats (Lakshmana Rao et al. 1998; Rao and Bhattacharya 1996; Shen et al. 2002; Bouaicha et al. 2005) have reported that MCs are genotoxic. However, most previous studies have used commercially available MCs rather than MCs extracted from cyanobacteria blooms, and these commercially available MCs were less genotoxic than CBE (Mankiewicz et al. 2002). As far as we know, the current study is the first report of in vivo genotoxicity of crude MCs products extracted from a cyanobacteria bloom on fish, especially regarding on organs other than fish liver.

Conclusions

CBE from Taihu Lake are genotoxic to carp and can lead to formation of micronuclei and cause DNA strand breaks in a time- and dose-depended manner. The ultrastructure of carp liver and gill cells organelles including mitochondria, ER, nuclear membranes of organelles were changed by exposure to CBE. These changes on cell structures are supposed to result in changes in cell functions. More studies are needed to find the mechanism and pathways of the genotoxic damages. Eutrophication, cyanobacteria blooms are severe problems in Taihu Lake, and this highlights the need to investigate the corresponding toxicity of cyanobacteria, especially considering the long term toxicity effects.

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