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Full Length Research Paper

Exposure to crude microcystins via intraperitoneal injection, but not oral gavage, causes hepatotoxicity in ducks

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Recently, large-scale cyanobacterial blooms have occurred in fishponds near the suburbs of Xinxiang City in China. The present study aimed to identify the cyanobacterial blooms in the fishpond and evaluate their toxicity on ducks via intraperitoneal injection or oral exposure (gavage) of crude microcystins obtained from the scum of cyanobacterial bloom. The results of the acute toxicity tests showed that intraperitoneal injection of crude microcystin solution caused hepatotoxicity in ducks and ducklings, but oral exposure failed to do so. This result confirms the observation of no duck intoxication by a natural way of oral exposure in the fishponds during the periods of blooms. In addition, subchronic exposure of microcystins by intraperitoneal injection significantly inhibited the growth of ducklings.

Key words: *Microcystis* bloom, microcystins, duck, toxicity.

INTRODUCTION

In recent years, cyanobacterial blooms have occurred frequently in various kinds of freshwater, such as rivers, lakes, reservoirs and ponds, in China. The blooms have been a serious threat to the drinking water supply for local people because they can greatly decrease the quality of drinking-water resources. In 2007, a drinking-water crisis took place in the city of Wuxi in China due to a toxin-producing cyanobacterial bloom of *Microcystis* spp. in Lake Taihu, leaving approximately two million people without clean drinking-water for at least one week (Qin et al., 2010). Cyanobacterial blooms also can bring about intoxication events of aquatic animals and even humans due to the highly toxic microcystins (MCs) produced mainly by *Microcystis aeruginosa* (Jochimsen et al., 1998; Laurent et al., 2008).

There have been many reports on the hepatic toxicity of MCs in fish and wild animals (Li et al., 2007; Kotut et al., 2010; Lance et al., 2010), but the literature regarding MC toxicity in birds is relatively limited (Krienitz et al., 2003; Skocovska et al., 2007; Paskova et al., 2008). Matsunaga et al. (1999) reported that about 20 spot-billed ducks died in Shin-ike pond in Nishinomiya, Hyogo Prefecture, Japan, during the summer of 1995, and the suspected cause was the sudden appearance of a toxic freshwater bloom of *M. aeruginosa.* Autopsies of the birds revealed that the livers were necrotic and severely jaundiced with a dark green color, suggesting the toxicity of MCs contained in the cyanobacterium. Park et al. (2001) and Krienitz et al. (2008) also reported that MCs contributed to the repeated bird losses in Pakowki Lake and other lakes in Canada and found a similar pattern of waterfowl poisonings and mysterious deaths of lesser flamingos at Lake Bogoria, Kenya. Additionally, Skocovska et al. (2007) and Paskova et al. (2008) confirmed the toxicity of the cyanobacterial toxins via acute oral exposure of natural cyanobacterial biomass to Japanese quails.

There are many large-scale fishponds located in the suburbs of Xinxiang, Henan Province in China, and these ponds are the main aquatic product supply for the local people. During the warmer seasons (April to September),

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serious cyanobacterial blooms occur in most ponds and cause fish death, leading to a large financial loss for fishers. Ducks are also fed in these ponds, and they usually ingest some amount of bloom scum when they are looking for food in the water during the periods of cyanobacterial blooms. We have conducted primary acute toxicity tests using a mouse bioassay and have found that the bloom is hepatotoxic. However, no intoxication or death of ducks due to cyanobacterial bloom has occurred according to our inquiry of local fishers. We have also found that gavage of the bloom scum to ducks at satiation causes no acute toxicity or hepatotoxicity via histopathological examination. The present study aims to identify the cyanobacterial blooms in the fish ponds and to further evaluate the toxicity of the blooms in ducks via oral exposure and intraperitoneal (i.p.) injection of the crude microcystins extracted from the bloom scum.

MATERIALS AND METHODS

Bloom scum collection and cyanobacterium identification

Cyanobacterial bloom scum was collected from the fishponds of Dabeinong Fishery Company in the suburbs of Xinxiang City, Henan Province, China, using a phytoplankton net (13$^{\#}$, 112-μM diameter mesh). Large foreign matter was removed, and then the scum was stored at -20°C. Cyanobacterial classification was carried out under a microscope according to the method of Watanabe (1996).

Crude MC isolation and high performance liquid chromatography (HPLC) analysis

Crude MC extraction from the bloom scum were prepared by the method of Spoof et al. (2010). Briefly, the scum was first incubated in boiling water for 15 min, cooled on ice and then centrifuged at 12 000 × *g* for 10 min. The supernatant was collected and then extracted by 70% methanol at 4°C for 12 h. After extraction, it was centrifuged at 12000 × *g* for 15 min and the supernatant was obtained. Then the supernatant was evaporated to dryness in a rotary evaporator to remove the methanol. Following this, the dried supernatant was suspended in phosphate buffered saline (PBS) and passed through a C_{18} ODS cartridge (Sep-Pak® Plus, Waters). The crude MC was eluted with 20 and 100% methanol. The elution was then evaporated to dryness to remove the methanol, and finally the crude toxin were resolved in PBS and stored at -20°C.

Quantitative analysis of MC content in the crude toxin was performed by the ultraviolet high-performance liquid chromatography (UV-HPLC) method (Spoof et al., 2010). Samples were analyzed using a reversed phase Nova-Pak C_{18} column (3.9 × 150 mm, 10 μL injection volume). The mobile phase was methanol/50 mM phosphate buffer pH 3.0 (52:48 v/v) with a flow rate of 1 ml min^{-1}. MCs were detected by absorbency at 238 nm due to the conjugated diene absorption of the Adda moiety and identified by co-elution with standards (MC-LR, MC-RR, and MC-YR, Wako Chemical Co. Japan). MC content in the crude product was expressed as μg MCs mL^{-1} crude toxin in PBS.

Acute toxicity of crude MCs on ducks and ducklings

Female ducks (*Anas Platyrhynchos* var. domestica) with body weights of 1.5 ± 0.25 kg were provided by the Institute of Henan Science and Technology, China. They were raised in birdhouses (3 to 5 m^2 in area) in which the temperature was kept at 16 ± 1°C, the humidity was 60 ± 5% and the photoperiod was a 12:12 dark/light cycle. All ducks were allowed free access to standard commercial pellet diet and tap water. Ducklings (7 days after hatching, body weight 40 ± 0.5 g) were fed in a similar way to that of ducks. The animals were handled following the guidelines in the China Law for Animal Health Protection and Instructions for Granting Permit for Animal Experimentation for Scientific Purposes (Ethics approval No. SCXK (YU) 2005- 0001). Acute toxicities of crude MCs in ducks or ducklings were determined according to their lethal effects on animals after 24 h of i.p. injection or oral exposure (gavage) to the crude toxin. The exposure doses of i.p. injection were 75.2, 79.6, 84.3, 89.3, and 94.6 μg kg^{-1} bodyweight for ducks, and 54.5, 59.1, 64.2, 69.7, and 75.6 μg kg^{-1} bodyweight for ducklings, respectively according to the results of primary acute toxicity tests. The design of the acute toxicity tests were conducted by the method of Spearman-Karber (Kärber 1931). There were five treatment groups with five ducks or ducklings per group, and no food was provided during the test. Acute toxicity was expressed as the median lethal dose (LD_{50}), which was the dose that killed 50% of the tested animals. The LD_{50} values were calculated by the method of Spearman-Karber (Kärber 1931) using SPSS 11.5 for Windows.

Subacute toxicity in female ducks

Twenty egg-producing ducks were randomly divided in two groups (ten ducks in each group); one group received MC treatment, and another served as a control. The ducks in the treatment group were administered an i.p. injection of the crude MC solution (in PBS) daily at a dose of 16.96 μg kg^{-1} (1/5 LD_{50}) for 10 days. The control ducks received the same volume of 0.9% PBS for the same duration. During the treatment, all of the ducks were fed as before and received human care. The time, frequency and quantity of egg-production by the ducks in the treatment and control groups were also recorded until the termination of the test. After 10 days of exposure, the ducks were weighed and anaesthetized with 100 mg kg^{-1} body weights of ketamine by intramuscular injection and then dissected for histological observation. The livers from each duck were removed and weighed separately. The relative weight of the liver was expressed as liver weight/100 g body weight.

Subchronic toxicity in ducklings

Subchronic toxicity test was conducted to determine the effects of longer-term MC-exposure on the growth and development of ducklings. The test was carried out in two groups with ten ducklings (40 ± 0.5 g) in each group ; one group received MC treatment, and another served as a control. Animals in the treatment group were exposed daily to crude MCs at a dose of 12.95 μg kg^{-1} (1/5 LD_{50}) by i.p. injection of the crude MC solution (in PBS) for 21 days. The control ducks received the same volume of 0.9% PBS for the same duration. At the end of the experiment, all of the ducklings in the two groups were weighed and then sacrificed for developmental and histological observation. After dissection, the viscera (mainly including the liver, kidney, spleen and pancreas) of the ducklings were weighed.

Statistical analysis

Data were analyzed by one-way analysis of variance to compare differences between the groups. Statistical analysis was performed using SPSS 11.5 for Windows. The results were expressed as the mean ± standard deviation (SD). A p value < 0.05 was considered statistically significant.

RESULTS

Cyanobacterial bloom and MC content in crude product

Microscopy observation identified the cyanobacterial bloom to be *M. aeruginosa.* HPLC analysis showed that the MC content in PBS solutions of the crude toxin was 146.1 μg mL^{-1} and the main components were MC-LR and MC-YR, accounting for 96.4% and 3.6%, respectively.

Acute toxicity

The results of the acute toxicity tests showed that the 24 h-LD_{50} values of the crude MC for ducks and ducklings were 84.8 μg kg^{-1} bodyweight (95% confidence limits of 82.5 to 87.2 μg kg^{-1}) and 64.7 μg kg^{-1} (62.4 to 67.1 μg kg^{-1}), respectively when exposed via i.p. injection. In oral exposure experiments, the crude MC solution did not cause death in either ducks or ducklings; therefore, the LD_{50} via gavage could not be obtained.

Subacute toxicity

Subacute toxicity tests revealed that MC exposure for 10 days induced a significant decrease in the net gain weight of ducks (Table 1), while the absolute liver weight and relative liver weight of the animals (Table 2) were greater than those of the controls, suggesting that the crude MC may be hepatotoxic to duck. In addition, during the periods of MC exposure, animal behavior in the treatment group was also different from that of the controls; for example, the treated ducks were out of spirits, they liked sleeping instead of moving and ate little but secreted more saliva and tears, indicating symptoms of intoxication. Nevertheless, there was no difference found in the frequency and quantity of egg-production of ducks between treatment and control groups at the terminal of tests.

Subchronic toxicity

After 21 days of subchronic MC exposure, the body weights of the ducklings in the treatment group were obviously less than that of the controls (Table 3), indicating that MC inhibits the growth of ducklings. Also, the absolute liver weight and relative liver weight of the treated ducklings were significantly greater than those of the controls (Table 4).

DISCUSSION

LD_{50} determination is the essential part of an acute toxicity test to identify the toxicity of a compound. In the present study, the LD_{50} values of the crude MC via i.p. injection for ducks and ducklings are similar to the average LD_{50} in mice (75 μg kg^{-1}), according to comparisons with the previous studies reviewed by de Figueiredo et al. (2004). This result suggests that the sensitivity of ducks to MC-toxicity might be similar to that of rodents, although the two kinds of animals are biologically different. Additionally, we also found that ducklings were more sensitive to MC-exposure than ducks, reflecting the poorer ability for detoxification of MC in the developing liver of the ducklings.

Gavage and i.p. injection are the most frequently adopted methods of toxin exposure in acute toxicity tests. However, the oral LD_{50} of MC in the mouse is usually ten to one hundred times higher than the i.p. LD_{50} because toxin intake via i.p. injection is much more direct and faster than by oral exposure. It was reported that the oral LD_{50} of MC in mice was around 500 μg kg^{-1}, while the i.p. LD_{50} was 75 μg kg^{-1} (Ito et al., 2000, 2001). In this study, the i.p. LD_{50} of crude MC for ducks was slightly higher than that for mice, whereas the oral LD_{50} for ducks or ducklings failed to be obtained, even though the ducks and ducklings were satiated with the solution of crude toxins. Thus, we can conclude that the ducks may not be sensitive to oral exposure of MC. It is possible that the gastrointestinal tract of ducks might degrade MC or prevent MC intake. On the other hand, a high enough dose of MC via gavage in the acute test may not have been attained, leading to poisoning-resistance. Therefore, this study indicates that intraperitoneal injection of MC, an unnatural way of exposure, causes hepatotoxicity in ducks rather than oral exposure. This also accounts for the observation that ducks can live well within the scum of *M. aeruginosa* blooms in the fishponds without intoxication.

From the subacute toxicity tests, we suggested that the target organ of MC in ducks might be the liver, as indicated by absolute liver weight and relative liver weight. Regarding the mechanism of liver enlargement in MC-exposed animals, it is generally supposed that MC is taken up by hepatocytes via multi-specific bile acid transporters, and can potently inhibit serine/threonine protein phosphatases 1 and 2A (Runnegar et al., 1993). The resulting imbalance in protein phosphorylation disrupts the liver cytoskeleton, which leads to massive hepatic heamorrhages that cause hepatomegaly (Dawson, 1998). This finding is in agreement with other previous investigations into MC-toxicity in other kinds of birds and animals (Lance et al., 2010; Pikula et al., 2010; Damkova et al., 2011; Paskova et al., 2011). However, no harmful effect of MC-exposure on the frequency and quantity of duck egg production was found, the reason for which may be due to the short period of exposure in the subacute test.

The growth and body weight changes of the treated animals are synthetic indices of the endpoints that must

Table 1. The body weights and net-weight gain of ducks in the subacute toxicity test.

Group	Number of animal	Initial body weight (kg)	Final body weight (kg)	Net-weight gain
Control	10	1.47 ± 0.25	1.46 ± 0.28	6.52 ± 0.23
MC	10	1.42 ± 0.29	1.34 ± 0.23*	-0.80 ± 0.30**

Data are the mean ± SD. Asterisks denote a response that is significantly different from controls (*p<0.05, **p<0.01). MC, microcystin.

Table 2. The liver weights and relative liver weights of ducks in the subacute toxicity test.

Group	Number of animal	Absolute liver weight (g)	Relative liver weight (g/100 g)
Control	10	40.26 ± 4.18	0.44 ± 0.03
MC	10	47.34 ± 6.82**	0.51 ± 0.02**

Data are the mean ± SD. Asterisks denote a response that is significantly different from controls (**p<0.01). MC, microcystin.

Table 3. The body weights and net weight gain of ducklings in the subchronic toxicity test.

Group	Number of animal	Initial body weight (g)	Final body weight (g)	Net weight gain
Control	10	39.41± 0.36	79.31± 2.48	39.90 ± 3.23
MC	10	38.63 ± 0.42	71.68 ± 3.52**	33.05 ± 1.85**

Data are the mean ± SD. Asterisks denote a response that is significantly different from controls (**p<0.01). MC, microcystin.

Table 4. The liver weights and relative liver weights of ducklings in the subchronic toxicity test.

Group	Number of animal	Absolute liver weight (g)	Relative liver weight (g/100 g)
Control	10	1.32 ± 0.28	0.026 ± 0.003
MC	10	1.42 ± 0.26*	0.030 ± 0.002*

Data are the mean ± SD. Asterisks denote a response that is significantly different from controls (*p<0.05). MC, microcystin.

be observed in a chronic toxicity test because the inhibited growth in juveniles or obvious body weight loss in adults often reflects intoxication by a toxin. In the present study, 21 days of MC-exposure by i.p. injection not only significantly inhibited the growth of the treated ducklings, but also caused damage to their livers. This result indicates that MC is hepatotoxic in ducks and ducklings. Skocovska et al. (2007) also examined the effects of cyanobacterial biomass from the Brno reservoir, Czech Republic on the Japanese quail by daily oral ingestion of 10 ml of *Microcystis* biomass containing 0.045, 0.459, 4.605 or 46.044 µg MCs, respectively for 10 and 30 days, and they found that no quail mortality occurred throughout the test. However, hepatic histopathological and subcellular observation revealed that MCs caused damage to quail liver and hepatocytes, suggesting that oral exposure to MCs causes hepatotoxicity in quail. On the contrary, in the present study, gavage of crude MC solution in ducks or ducklings showed no acute toxicity. Differences between duck and quail and their environments may account for this difference. Our results also point to the cause of the lack of duck death when cyanobacterial blooms appear in Xinxiang fishponds.

Conclusion

The results of the present study revealed that intraperitoneal injection of crude microcystin solution causes hepatotoxicity in ducks and ducklings, but not oral exposure. Additionally, subchronic exposure of microcystins by intraperitoneal injection significantly inhibited ducklings' growth.

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