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Kidney Histology of Mice After Seven Days Oral Intake of Cyanobacterial Extract

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ABSTRACT

At the end of the dry season, during October 2004, a dense cyanobacteria bloom was observed in Monjolinho Reservoir (São Carlos city, São Paulo State, Brazil), and a great amount of scum accumulated on the water surface. Samples were collected in order to identify phytoplankton species and to determine its toxicity. *Anabaena circinalis* and *Anabaena spiroides* were the dominant species in this bloom. Initially, mouse bioassays (LD₅₀-24 h) and analysis by ELISA were performed, to detect toxic compounds in crude extracts of the natural samples. After toxicity was confirmed, an oral chronic toxicity test was carried out using three doses of cyanotoxins, in male *Swiss albino* mice, for 7 days. To prepare the extract, cyanobacterial cells were broken by repeated freeze and thaw cycles. Mice received the following doses of crude cyanobacterial extract diluted in drinking test solutions: 0.5 mg L⁻¹; 1.0 mg L⁻¹ and 2.0 mg L⁻¹. Mortality, clinical signs, body weight and weight gain were recorded during the study. In addition, histological slides were prepared to detect toxic injuries to the kidney. Toxin analysis by ELISA revealed 1.77 to 6.12 µg L⁻¹ total microcystins in the mouse drinking water. This study indicated that administration of low doses of cyanotoxins in mouse drinking test solutions did not result in histological injury in the kidney of animals exposed to cyanotoxins, as observed by optical microscopy.

Key words: cyanobacterial blooms, *Anabaena circinalis*, *Anabaena spiroides*, chronic tests, oral toxicity, kidney histology.

RESUMO

Histologia renal de camundongos após sete dias de ingestão oral de extrato de cianobactéria

No final da estação seca, durante o mês de outubro de 2004, ocorreu densa floração de cianobactérias na comunidade fitoplanctônica do reservatório do Monjolinho (São Carlos, SP, Brasil), com grande quantidade de biomassa algal se acumulando na superfície da coluna d'água. Amostras de água foram coletadas para determinação da densidade e abundância relativa das espécies e para determinação da toxicidade. *Anabaena circinalis* e *Anabaena spiroides* foram as espécies dominantes na floração. Inicialmente foram realizados bioensaios com camundongos (LD₅₀-24 h), sendo feita a quantificação de toxinas pelo método ELISA para detectar a concentração total de microcistinas. Após confirmação da toxicidade foram realizados testes crônicos em camundongos da linhagem *Swiss albino*, testando-se três concentrações de toxinas, em experimento com duração de 7 dias. Para a preparação do extrato, as células das cianobactérias foram submetidas a um ciclo de repetidos congelamentos e descongelamentos. Os camundongos receberam as seguintes doses de extrato bruto diluído em água mineral: 0,5 mg L⁻¹; 1,0 mg L⁻¹ e 2,0 mg L⁻¹. A mortalidade, sinais clínicos, peso corporal e ganho de peso foram registrados. Em adição, cortes histológicos foram realizados para análises do rim. A análise de toxinas pelo método ELISA indicou concentrações entre 1,77 e 6,12 µg L⁻¹ de microcistina total na água ingerida pelos camundongos. Este estudo indica que a administração de baixas concentrações de cianotoxinas via ingestão oral não resultou em toxicidade nem em injúria histológica no rim dos animais expostos às cianotoxinas.

Palavras-chave: florações de cianobactérias, *Anabaena circinalis*, *Anabaena spiroides*, testes crônicos, toxicidade oral, histologia renal.

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INTRODUCTION

Toxic cyanobacteria found in eutrophic water bodies used for public supply are dangerous because they produce lethal toxins that affect wild and domestic animals, sometimes even to humans (Carmichael *et al.*, 1985; Carmichael, 1994; Dawson, 1998; Nasri *et al.*, 2004). It is now common knowledge that species belonging to certain genera of cyanobacteria, such as *Anabaena*, *Microcystis*, *Nostoc* and *Oscillatoria*, produce hepatotoxic peptides, named microcystins, and neurotoxic alkaloids. Chronic exposure to microcystins (MC) contaminating drinking water can induce human liver cancer (Nishiwaki-Matsushima *et al.*, 1992). In Caruaru, Brazil, patients suffered liver failure and death after exposure to microcystins at a Hemodialysis Center, because the water was contaminated (Jochimsen *et al.*, 1998). Chronic treatment of rats with intraperitoneal injections of sublethal doses of microcystins MC-LR and MC-YR revealed collapsed glomeruli with thickened basement membranes and dilated tubuli (Milutinovic *et al.*, 2003). In a preliminary study, the crude extract from a recent cyanobacterial bloom in SE Brazil was seen to cause histological alterations in the liver of *Swiss albino* mice (24 h-LD₅₀ = 297.30 mg kg⁻¹) submitted to acute toxicity test (unpublished data). An oral chronic toxicity test was performed using three doses of cyanotoxins in male mice for 7 days, in drinking water: 0.5 mg L⁻¹; 1.0 mg L⁻¹ and 2.0 mg L⁻¹. No mortality and none histological injury in liver of animals exposed to cyanotoxins were observed. The present study was thus carried out to discovery whether the same concentrations of the same natural cyanobacterial extract could affect the mouse kidney histology.

MATERIALS AND METHODS

Description of the study area

The site of this study is a small and shallow artificial reservoir (Figure 1) located at São Carlos city, São Paulo State, Brazil, formed by the damming of the Monjolinho River (21° 59' 8.4''S and 47° 52' 45''W).

Phytoplankton sampling

Samples used in this study were taken on October 25th, 2004, near the dam. For the quantitative and qualitative studies of the phytoplankton community, samples were collected in Van Dorn bottles and with a 20 µm mesh phytoplankton net, respectively, transferred to flasks of known volume and preserved, respectively, in Lugol's iodine and 4% formaldehyde solution, maintained in a cool and dark place until the time of analysis. The phytoplankton was counted by the inverted microscope technique of Utermöhl (1958). Filaments of *Anabaena* were counted and converted to cell numbers using the mean cell number per filament in the sample (68 cells per filament of *Anabaena circinalis* and 101 for *A. spiroides*).

Preparation of crude cyanobacterial extract

Lyophilized bloom material (collected on October 25th, 2004) was suspended in drinking water, frozen at -20°C and thawed at room temperature. This freeze and thaw cycle was repeated 4 times. After the last cycle, the thawed material was ultrasonicated 3 times for 1 min. Finally, debris were removed by centrifuging at 2800 × g for 5 min.

Animals

Twelve male Swiss albino mice weighing from 20 to 30 g at the beginning of the experiment were used in the study. The mice were housed in standard plastic cages with sawdust cover on the floor. They were maintained on a 12 h light-dark cycle, at room temperature, with free access to pellets chow and tap water. The animals were obtained from Federal University of São Carlos.

Oral toxicity test

Oral toxicity tests were performed during a week. Experimental test solutions at the concentrations of 0.5, 1.0 and 2.0 mg mL⁻¹ of cyanobacterial extract were supplied to the mice whereas the control group received only drinking water. During the 7-days exposure, the test solutions were renewed 3 times per week in order to reduce toxins degradation in the test solutions supplied. All groups were allowed to drink "ad libitum". At the beginning of the experiment toxin concentrations were measured in experimental test solutions. The drinking bottles were covered with aluminum foil to avoid the degradation of toxins by light. Animals were weighted at the beginning and at the end of the experiment and weight gain determined. Mice were fed daily with standard pellet chow and at the end of the experiment they were sacrificed. Kidneys were removed and fixed in Bouin's solution for histological analysis.

Microcystin analysis

Total microcystin concentrations in test solutions were performed by ELISA immunoassay (Enzyme-linked immuno assay) with a Microcystin Plate Kit (Beacon Analytical Systems Inc.). Spectrophotometric analysis was carried out at 450 nm. The results were expressed as µg L⁻¹ total microcystin. The analytical procedure used was that described by Chu *et al.* (1990).

Histological analysis

After overnight fixing, kidney samples were dehydrated through a graded ethanol series, paraffin wax-embedded and cut in sections (7 µm) and mounted on slides. After deparaffinization in xylene, sections were rehydrated through a graded ethanol series, stained with haematoxylin and eosin (HE) and dehydrated through a graded again and cleared in xylene and mounted with Canada balsam for histological assessment under the light microscopy.

Statistical analysis

In order to identify the significant differences between body weights of the mice, the Kruskal-Wallis (KW) non-parametric test (at level of significance, $p = 0.05$) was employed.

RESULTS

The results of the phytoplankton analyses showed that community of Monjolinho reservoir was dominated by *Anabaena circinalis* and *Anabaena spiroides* (Cyanophyceae). This group represented more than 60% of the total algae present (Figure 2). Cyanobacterial cell density in the reservoir in the

sampling day was 0.35×10^6 for *A. circinalis* and 0.32×10^6 for *A. spiroides*, respectively. Other species belonging to the following taxonomic classes also occurred: Chlorophyceae, Bacillariophyceae, Xanthophyceae and Conjugatophyceae, although at low numbers.

After the oral intake of cyanobacterial extract the results evidenced that no mortality occurred during 7-d exposure to water with toxins. The results from histological analyses were shown in the Figure 3: a-h. No changes in the kidney histology were observed by light microscopy. The results of final body weight and weight gain of the mice were shown in the Table 1. The treatments did not differ among them and the control (Table 1).

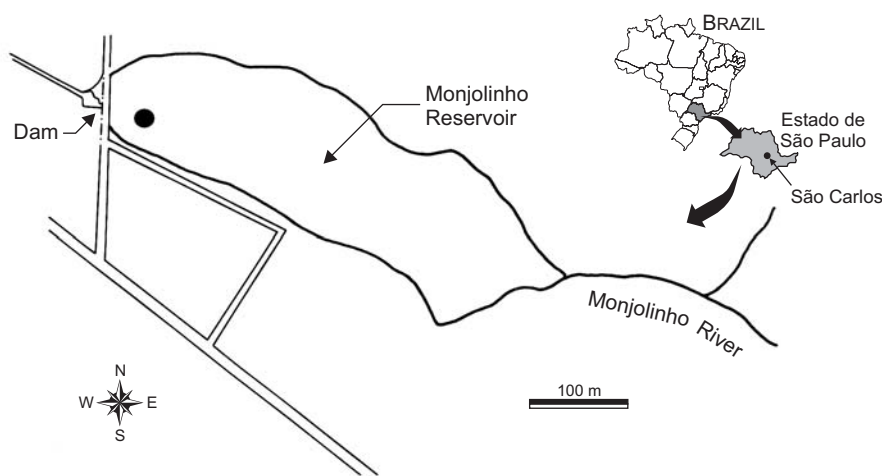


Figure 1 – Geographical location of studied reservoir in São Carlos city (São Paulo State, Brazil). The dot shows the location of the sampling site. Scale: 1:100,000.

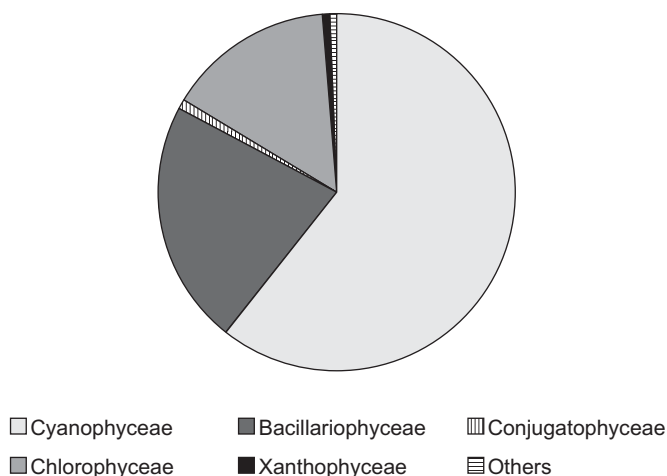


Figure 2 – Relative abundance of dominant groups in the phytoplankton community of Monjolinho Reservoir, on October 25th, 2004.

The measured concentrations of microcystin from the test solutions are also shown in Table 1. Data obtained here showed clearly that as the extract concentration increased, the microcystin concentrations equally increased.

DISCUSSION

In the present experiment we aimed to explore some of the cellular mechanisms that could be responsible for the kidney injury induced by chronic treatment with cyanobacterial extract containing microcystins. Some studies have reported that chronic and acute cyanotoxins exposure can cause kidney damage (Nobre et al., 1999, 2003; Milutinovic et al., 2002; Milutinovic et al., 2003; Moreno et al., 2005). In contrast, our results showed that oral ingestion of MCs in the form of extract (ranging from 0.5, 1.0 and 2.0 mg mL⁻¹) of a lyophilized from *Anabaena* bloom during 7 days, apparently did not result in histological alterations of kidney, as observed by light microscopy.

The amount of microcystin present in the test solutions varied between 1.77 and 6.12 µg L⁻¹. The maximum concentration of MCs allowed in drinking water for human consumption is 1.0 µg L⁻¹ (WHO, 1998) consequently the concentration of total MC contained in the highest concentration tested here was up to 6 times the allowed value for human consumption. Moreover, the dominant species in this bloom were *Anabaena circinalis* and *A. spiroides*. It is known that these species from *Anabaena* genus also produce neurotoxins (Beltran & Neilan, 2000). Therefore, some histological effect in the kidney could be expected.

We can think of two possible explanations, hypothetically: although microcystin concentrations present in the solutions test were high, the period of exposure was short and not enough to cause histological effects. Another fact is that in the present work the microcystin concentration measured represented all variants present in the sample whereas in those studies purified microcystin of a toxic strain was used, possibly with higher toxicity than that in the extract we used.

Table 1 – Extract concentration in drinking test solutions, microcystin concentration, initial and final body weight and weight gain.

Extract concentration in drinking water	Microcystin concentration (µg L ⁻¹)	Animal	Initial body weight (g)	Final body weight (g)	Weight gain (g)
0.0 mg mL ⁻¹ (Control group)	–	1	31.3	35.8	4.5
		2	30.1	36.2	6.1
		3	36.9	42.7	5.8
		Mean	32.8 ± 2.1	38.2 ± 2.2	5.5 ± 0.5
0.5 mg mL ⁻¹	1.77	1	36.3	43.4	7.1
		2	32.6	36.5	3.9
		3	34.0	39.6	5.6
		Mean	34.3 ± 1.1	39.8 ± 2.0	5.5 ± 0.9
1.0 mg mL ⁻¹	2.32	1	33.7	37.4	3.7
		2	32.9	34.8	1.9
		3	29.7	38.1	8.4
		Mean	32.1 ± 1.2	36.8 ± 1.0	4.7 ± 1.9
2.0 mg mL ⁻¹	6.12	1	28.5	37.2	8.7
		2	29.4	36.4	7.0
		3	30.0	31.6	1.6
		Mean	29.3 ± 0.4	35.1 ± 1.7	5.8 ± 2.1
KW			3.0	0.49	
P			0.39	0.92	

KW = Kruskal-Wallis test; p = probability (significance level p < 0.05).

It is important also to observe that at the end of the tests all mice from the treatments with toxins had body weights and weight gain similar to control. Additionally no mortality and no other reactions or changes in behavior such as hunched posture or reduced motor activity were observed until the end of the experiment.

Fawell *et al.* (1999) testing the adverse effect of purified microcystin on mouse liver have found that microcystin-LR is 30-100 times less toxic via oral ingestion than via intraperitoneal injection. Therefore, in a next step it will be relevant to characterize the dominant type of microcystin present in Monjolinho Reservoir cyanobacterial blooms in order to better interpreting the results here obtained.

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