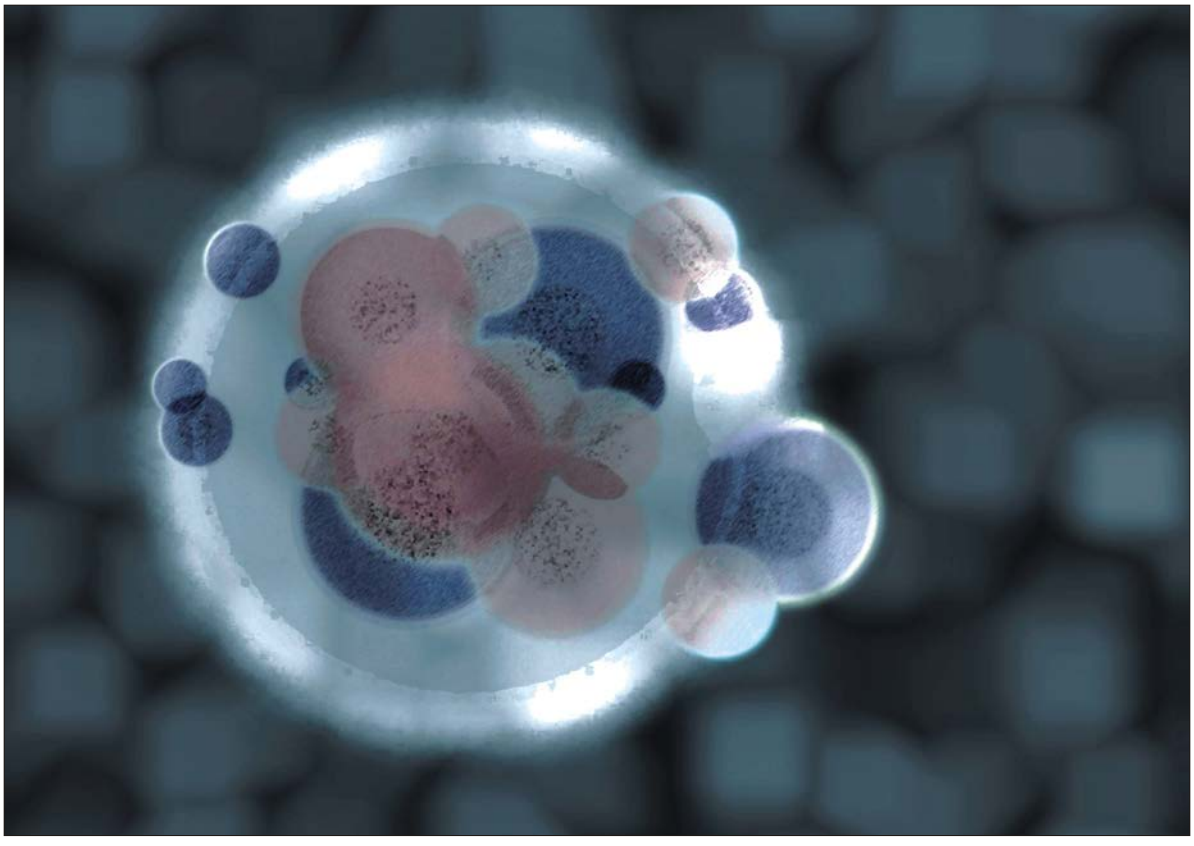

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Effect of Microcystin from Jordan on Ion Regulation and Antioxidant System on the Hepatocytes of Mice

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Abstract: The objective of this study was to evaluate mechanisms of microcystin (from King Talal Reservoir, Jordan) toxicity on the hepatocytes of mice. Five to seven weeks old male albino Balb/C mice (about 30 g wt.) were injected i.p. with normal saline (as control) or *Microcystis aeruginosa* aqueous extract (30 mg L^{-1}) at 24 h intervals for 48 h. After the exposure period of toxin-treated and control mice, Na^+, K^+-ATPase and glutathione-S-transferase (GST) activities were measured in hepatocytes extracts. Total oxyradical scavenging capacity (TOSC) and lipid peroxides (LPO) content were also determined. Na^+, K^+-ATPase and GST in the hepatocytes of mice was significantly lower in toxin treated mice than in control ones. Mice exposed to microcystin also showed a significant increase in the TOSC value against peroxyl radicals. Lipid peroxides level did not change in the hepatocytes of mice after exposure to the toxin. The increased levels of TOSC suggest on the occurrence of mice response against oxidative stress induced by toxin injection, which prevents lipid peroxidation.

Key words: Microcystin, hepatocytes, total scavenging capacity, GST, lipid peroxidation, Na^+, K^+-ATPase

INTRODUCTION

Microcystins are group of monocyclic heptapeptide hepatotoxins produced by various freshwater cyanobacteria such as *Microcystis*, *Anabaena*, *Nostoc* and *Oscillatoria* species[1]. The general structure of microcystin is cyclo [-D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha-]. Apart from two variable L-amino acids, X and Z, microcystin consists of three D-amino acids: alanine (Ala), methylaspartic acid (MeAsp) and glutamic acid (Glu) and two unusual amino acids: N-methyldehydroalanine (Mdha) and 3-amino-9-methoxy-2, 6, 8,-trimethyl-10-phenyldeca-4, 6-dienoic acid (Adda)[2]. The Adda amino acid is responsible for the toxin biological activity[3].

So far more than 50 types of microcystins have been identified and among them microcystin-LR (MLR) is the most commonly encountered. Using tritiated microcystin, it has been demonstrated that the liver is the prime target organ affected[4], characterized by fulminant intrahepatic hemorrhage and death of animals[5]. Microcystin cannot penetrate the cell membrane through simple diffusion, but through the bile acid transport system[6]. This mechanism of cell entry could thus explain the cell specificity and organotropism of microcystin. In addition to its potent hepatotoxicity, microcystin also appears to have tumor promotion activity[7]. The exact mechanisms of microcystin-induced hepatotoxicity and tumor promotion activity have not been fully elucidated although changes in liver functions and programmed cell death have been considered as the main events. One of the most extensively studied mechanisms is that microcystins are potent inhibitors of protein phosphatases 1 (PP1) and 2A (PP2A), leading to increased protein phosphorylation[8].

Recently, others[5,7,9] have reported that microcystins are capable of initiating apoptosis in hepatocytes as evidenced by characteristic apoptotic morphological changes including membrane blabbing, cell shrinkage, externalization of membrane phosphatidylserine and chromatin condensation[10]. Ding and Ong[5] found that oxidative stress and mitochondria play a critical role in microcystin-induced apoptosis.

The activity of several enzymes are affected by reduced membrane fluidity, including the electrogenic sodium pump, Na^+, K^+ ATPase, a membrane protein known to be related to ion regulation in organisms[11-13]. It is known that oxidative stress can inhibit Na^+, K^+-ATPase and other ATPases through two different mechanisms[14,15]. The indirect mechanism promotes the generation of lipid peroxides, altering the membrane fluidity. A direct mechanism is considered to act through the direct oxidizing action of ROS on sulfhydryl groups of ATPases[13,16]. Previous studies by others[13,17] showed that microcystin inhibits *in vitro* Na^+, K^+-ATPase activity

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in fish gills through inhibition of phosphatase activity that this enzyme presents. This fact led this author to hypothesize that disruption of ionic regulation should be responsible for massive animal killing after *Microcystis aerugenosa* bloom, also, it led us to consider the possibility that the enzyme inhibition could be related to other processes such as oxidative stress generation. To test this hypothesis, the effects of extracts from the cyanobacteria *Microcystis aeruginosa* on the mice hepatocyte ion regulation (Na^+, K^+-ATPase) was analyzed.

MATERIALS AND METHODS

For the present study, cells of *Microcystis aeruginosa* (strain RST 9501)[18] were isolated from other types of cyanobacteria using light microscope according to Sakars method[19] identified according to Mattiensen *et al.*[18] and were cultured in BG 11 medium (containing 3.5 mM $NaNO_3$) according to Lehtimaki[20] and used as toxin source. Toxins were extracted according to the method recommended by Vinagre *et al.*[13] and the microcystin content of the extract was determined using a commercial enzyme-linked immunoassay (ELISA) with polyclonal antibodies (Cayman; USA).

Fifty μL of microcystin extract (30 mg toxin L^{-1}) diluted with 0.85% normal saline[21] were injected intraperitoneally (in toxin-exposed mice). Control mice were injected in the same way with equal volume of normal saline. Both control and toxin-exposed mice were injected twice (0 and 24 h) during the two days of experiment, then scarified.

Livers were removed immediately and processed as in the following : each liver was divided into two sections (part a and part b). Homogenates of the first group of liver sections (part a)were prepared according to Castilho *et al.*[12] modification of Vinagre *et al.*[13]. Briefly, liver sections were homogenized (10% w/v) in cold buffer containing sucrose (250 mM), PMSF (1 mM) and EDTA (5 mM), with pH adjusted to 7.6. After centrifugation at 10 000 xg (4°C) for 10 min, the supernatant was centrifuged at 10 000 xg for 20 min (4°C). The supernatant obtained was collected and used as GST source. The pellet was resuspended in the same buffer and centrifuged again (10000 x g at 4°C for 30 min). The last supernatant was then used as a source of Na^+, K^+-ATPase.

Liver Na^+, K^+-ATPase activity was measured according to the method described by Castilho *et al.*[12]. Incubation medium A contained ATP (4 mM; from Sigma), NaCl (100 mM), KCl (25 mM) and $MgCl_2$ (7.8 mM) with pH adjusted to 7.6. In the incubation medium B, KCl was replaced by NaCl (25 mM) and ouabain (1 mM; from Sigma) was added. Aliquot of liver homogenate (0.762 mg protein) was incubated at 30°C in both medium A and B, for 1 h. Enzymatic reaction was stopped by addition of trichloroacetic acid 50%. The inorganic phosphorus released was determined using a commercial kit (Cayman; USA) in a microplate reader (Cayman; USA) at 660 nm. Difference in phosphorus concentration between medium A and B was attributed to Na^+, K^+-ATPase activity.

GST activity was measured to evaluating the conjugation of 1 mM of glutathione with 1-chloro-2, 4-dinitro-benzene (CDNB) at 340 nm during 1 min[13,22]. Aliquot of liver homogenate was incubated at 25°C in 100 mM phosphate buffer (pH 7.0). An extention coefficient of 9.6/mM/cm was employed in order to calculate the moles of CDNB conjugated.

Na^+, K^+-ATPase and GST measurements were normalized considering the total protein content in the homogenates, which was determined using a commercial kit (Cayman; USA) based on the Biuret reagent. Determinations were done at 550 nm.

Total oxyradical capacity (TOSC) was determined using part b of liver sections[13,23]. Liver sections were homogenized (1:10) in phosphate buffer 50 mM, pH 7.50, plus NaCl (2.5%) and protease inhibitors (aprotinin: 1 μg mL^{-1}; leupeptin: 1 μg mL^{-1} and pepstatin: 0.5 μg mL^{-1}). Homogenates were centrifuged at 54000 xg at 4°C for 1 h. The supernatant was used for the measurement.

Alpha-keto-y-methiolbutyric acid (KMBA; 0.2 mM; from Sigma) was employed as substrate. KMBA reacts with ROS, producing ethylene[13] and TOSC values reflect the sample capability to scavenge the oxyradicals produced, thus inhibiting ethylene formation. TOSC values were calculated according to Winston *et al.*[24] and referred to the total protein content in the homogenates. Lipid peroxidation was measured according to the method recommended[25], modification of Vinagre *et al.*[13]. Liver sections were homogenized in chloroform:ethanol (2:1) (10% w/v) and centrifuged at 1000Xg, for 10 min. The supernatant obtained was collected, dried and resuspended (50% w/v) in cold methanol 100%. Lipid peroxides were determined using 500 μL of $FeSO_4$ (1 mM) prepared immediately before use, 200 μL of H_2SO_4 (250 mM), 200 μL of xylenol orange (1 mM; Sigma), 900 μL dH_2O and 40 μL of the methanolic extract. Samples absorbance (580 nm) was spectrophotometrically (Unicam; UK.) recorded after 3 h of incubation at room temperature and quantified in terms of cumene hydroperoxide (CHP; from Sigma) equivalents, which was used as standard (5 nmol mL^{-1}).

Significant changes in Na^+, K^+-ATPase and GST activity, TOSC values and lipid peroxidation level were

analyzed by means of analysis of variance and student t-test.

RESULTS

In this study, it was observed that the activity of Na^+, K^+-ATPase from hepatocytes of toxin injected mice was 6.08±0.11 μmole pi h^{-1} mg^{-1} protein. This value was significantly lower than the activity of Na^+, K^+-ATPase (8.36±0.21 μmole pi $h^{-1}mg^{-1}$ protein) from hepatocytes of control mice as shown in Fig. 1.

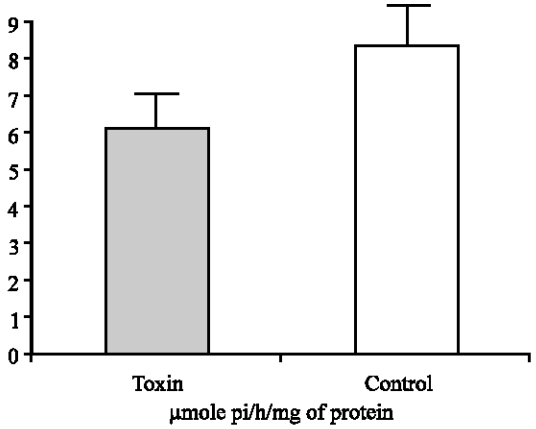

Fig. 1: Na^+, K^+-ATPase activity (μmole Pi/h/mg of total proteins) in mice hepatocytes; injected with aqueous extracts (30 mg of microcystin/l) of cultured cells of *Microcystis aeruginosa* (toxin) or with saline (control)

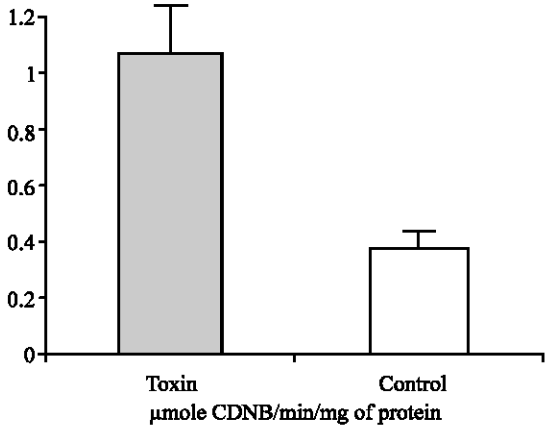

Fig. 2: Glutathione-S-transferase (GST) activity (μmoles CDNB/min/mg of total proteins) in mice hepatocytes. Mice were injected with aqueous extract (30 mg of microcystin/l) of cultured cells of *Microcystis aeruginosa* (toxin), or with 50 μL of saline (control). CDNB stands for 1-chloro-2, 4-dinitrobenzene, the substrate employed for GST determination

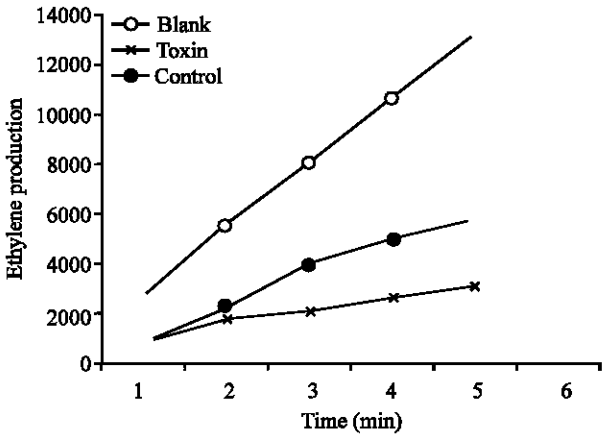

Fig. 3: Ethylene production over incubation time using a peroxyl radical generation system (ABAP, 20 mM) without sample extract (blank) or with extracts of hepatocytes from control or toxin-injected mice

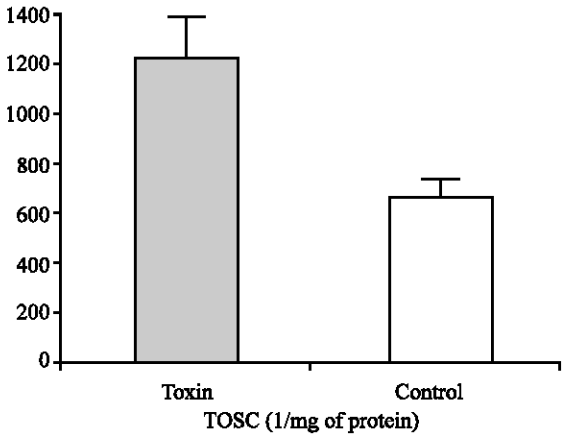

Fig. 4: Total oxyradical scavenging capacity (TOSC) (l/mg of total proteins) using a peroxyl radical generation system

GST activity from hepatocytes of toxin injected mice was significantly higher (1.07±0.03 μmole CDNB min^{-1} mg^{-1}) of total protein than GST activity value observed in hepatocytes of control mice (0.38±0.01 CDNB min^{-1} mg^{-1} of total proteins (Fig. 2).

Concerning the TOSC analysis, Figure 3 shows a typical measurement of ethylene production over time in samples without (blank) and with hepatocytes extracts. As it can be observed, gas production is highly reduced when homogenate aliquots are added in the system. TOSC values in hepatocyte extracts of toxin exposed animals were different to controls depending of the oxyradical generating system employed. Figure 4 shows higher values of TOSC in hepatocytes of toxin injected mice toward peroxyl radicals (1225±7.06 TOSC mg^{-1} of

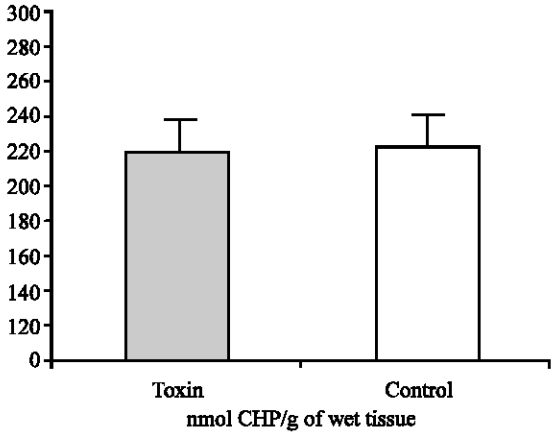

Fig. 5: Lipid peroxidation content (nmole CHP/g of tissue) in hepatocytes of mice injected with aqueous extract (30 mg of microcystin/l) of cultured cells of *Microcystis aeruginosa* (toxin) or with saline (control)

protein) when compared to those observed in control mice (682.2±2.02 TOSC mg^{-1} protein).

Finally, lipid peroxidation levels for hepatocytes were similar in both control (222.5±5.08 nmol CHP g^{-1} wt.) and toxin-injected mice (218±6.07 nmol CHP g^{-1} wt.) (Fig. 5).

DISCUSSION

Results obtained in the present study showed that microcystin inhibited Na^+, K^+-ATPase activity in the liver of mice. It was found that microcystin extracts from lysed cells of *Microcystis aeruginosa* are devoid of long chain fatty acids which are known to be Na^+, K^+-ATPase inhibitors[13,15,26]. Thus, the results obtained in the present study must be discussed in light of possible direct and indirect toxic effects of microcystin.

After toxin exposure, GST activity was significantly high in the liver extracts assuming that the potentiality of microcystin conjugation with glutathione can be estimated by GST activity, since a higher conjugation capability should lower the intracellular concentration of free microcystin[13].

Takenake[27] postulated that glutathione may play an important role in the detoxification and clearance of microcystin from vertebrate hepatocytes.

Liver extracts of treated mice showed augmented TOSC values against peroxyl radicals. Regoli and Winston[23] determined that depending on the oxyradical generating system employed, the scavenging efficiency of non-enzymatic antioxidants measured by TOSC could be quite different[28]. Ding *et al.*[10] found that glutathione gave so much higher TOSC values with a peroxyl radical generation than with a hydroxyl radical generation (iron/ascorbate).

Vinagre *et al.*[13] stated that other non-enzymatic antioxidants such as uric acid and trolox, also discriminated well between peroxyl and hydroxyl radicals. In this context, it should be considered that the higher GST activity in the liver extract of treated mice is not the only responsible for observed higher TOSC values. Glutathione is one of the more abundant cytosolic antioxidant and also involved in microcystin conjugation[27]. Ding *et al.*[10] found that primary cultured rat hepatocytes exposed to microcystin showed an augmented intracellular glutathione concentration after 6 h of exposure. They suggested that the higher tripeptide content should be a compensatory response via the activation of y-glutamylcysteine synthetase, one of the enzymes involved in glutathione synthesis[13]. Augmented levels of glutathione should also contribute to rise TOSC values and, as previously mentioned, this tripeptide is a better antioxidant against peroxyl radicals than hydroxyl radicals[10]. In this way, the observed higher TOSC values against peroxyl radicals should be at least partially related to an increased glutathione contents.

The fact that in the present study no difference in lipid peroxides content between control and toxin injected mice was found, suggests that this mechanism is not responsible for the observed Na^+, K^+-ATPase inhibition in hepatocytes. However, it must be considered that the augmented TOSC values against peroxyl radical could imply a compensatory response in mice against reactive oxygen species, which is consistent with previous results of Ding *et al.*[29] showing that microcystin exposure augments ROS generation and glutathione content. Several authors reported direct inhibition of Na^+, K^+-ATPase by ROS[13,30].

There are more evidences in agreement with the idea that microcystin inhibits Na^+, K^+-ATP ase activity through indirect mechanisms. Vinagre *et al.*[31] study strongly suggest the existence of indirect mechanisms of microcystins toxicity that remain to be investigated. Betorello and Katz[14] suggested that a-subunit phosphorylation of Na^+K^+-ATPase could induce a conformational change on its structure, modifying its affinity for one or more of its major substrates. In this context, toxins like microcystin can inhibit the dephosphorylation step catalyzed by phosphatase that restore catalytic activity of Na^+, K^+-ATPase.

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