Original Article

Cardiotoxic Injury Caused by Chronic Administration of Microcystin-YR

(microcystin-YR / chronic intoxication / cardiomyocytes / rat)

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Abstract. Microcystins are cyclic peptide toxins. Chronic intoxication with well-known members of the microcystin family – microcystins-LR – induces liver tumour formation, injury of kidney and heart. Despite worldwide distribution in the environment, the effects of microcystins-YR have not been studied extensively. The aim of the study was to evaluate whether microcystins-YR, in relatively low doses, have a toxic effect on cardiomyocytes of chronically treated rats. Male adult Wistar rats were treated every second day for 8 months with microcystins-YR (10 μg/kg i.p., N = 5). Control groups were treated either with vehicle (ethanol and methanol 4 : 1 v/v; N = 5) or with physiologic saline (N = 4). The heart sections of microcystin-YR-treated rats revealed decreased volume density of cardiac muscle tissue (microcystins-YR = 0.485 mm3/mm3 ± 0.003; vehicle = 0.493 mm3/mm3 ± 0.002; saline = 0.492 mm3/mm3 ± 0.002) due to fibrous proliferation. A few lymphocyte infiltrates were observed. Most of cardiomyocytes were enlarged (microcystins-YR = 20.19 μm ± 1.34, vehicle = 17.45 μm ± 0.52, saline = 16.00 μm ± 1.43), with enlarged and often bizarre-shaped nuclei and decreased myofibril volume fraction (microcystins-YR = 0.416 mm3/mm3 ± 0.009; vehicle = 0.472 mm3/mm3 ± 0.009; saline = 0.479 mm3/mm3 ± 0.010). No TUNEL-positive cells were found in the heart sections of rats in all groups. The results allow the conclusion that chronic exposure to low doses of microcystins-YR may cause atrophy and fibrosis of the heart muscle.

Introduction

Bloom-forming cyanobacterial genera produce numerous cyclic cyanopeptides possessing various biological activities (Sedmak et al., 2009). The most extensively studied among them is the microcystin (MC) family of more than 70 structurally cyclic peptide toxins frequently synthesized in considerable amounts (Fastner et al., 2002; Cazenave et al., 2005; Sedmak et al., 2008). MCs were reported to cause mortality in animals and illness in animals and humans (Carmichael and Falconer, 1993; Moreno et al., 2003). These threats have led the World Health Organization (WHO) to establish a provisional guideline value for microcystin as 1 μg/l of drinking water (WHO, 1998).

Following absorption, MCs are taken up through the multispecific transport system for bile acids characteristic of liver, ileum, kidney and brain (Runnegar et al., 1981; Eriksson et al., 1990; Dawson, 1998; Fischer et al., 2005). MCs generally inhibit both protein serine/threonine phosphatases-1 and 2A (MacKintosh et al., 1990; Meriluoto et al., 1990), increase formation of reactive oxygen species (ROS) (Ding et al., 2001; Zegura et al., 2003, 2004) and interact with mitochondrial ATP synthase (Mikhailov et al., 2003), aldehyde dehydrogenase (Chen et al., 2006) and also with mitochondrial oxidative phosphorylation (La-Salete et al., 2008).

A well-known and the most investigated member of MC family is MC-LR. High doses of MC-LR cause necrosis/apoptosis of hepatocytes and pooling of blood into the liver (Dawson, 1998; Hooser, 2000), but in chronic intoxication it promotes liver tumour formation (Ito et al., 1997; Sekijima et al., 1999) and induces kidney injury (Milutinovic et al., 2002, 2003; La-Salete 2008). MC-LR also induces myocardial damage in acute

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Abbreviations: ANOVA – analysis of variance, CMvd – volume density of cardiac muscle, HE – haematoxylin and eosin, i.p. – intraperitoneally, MC – microcystin, MC-LR – microcystin-LR, MC-YR – microcystin-YR, MFvf - myofibril volume fraction, ROS – reactive oxygen species, TUNEL – terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

(Zhang et al., 2002; Qiu et al., 2009) and chronic intoxication (Milutinovic et al., 2006). Despite the wide distribution and abundance in the environment, the effects of MC-YR have been studied less extensively, and there is very little information on the toxicity profile of MC-YR. The aim of the study was to find out whether chronic application of relatively low doses of MC-YR is toxic to the heart muscle.

Material and Methods

Animals and treatment

Male Wistar rats weighing from 444 g to 599 g at the beginning of the experiment were used in all the experiments. The animals were handled following the guidelines in the Slovenian Law for Animal Health Protection and Instructions for Granting Permit for Animal Experimentation for Scientific Purposes. Rats were treated every second day for 8 months with MC-YR (N = 5) in relatively low doses (10 µg MC-YR/kg intraperitoneally, i.p). The control group was treated with vehicle (N = 5) (0.8% ethanol and 0.2% methanol dissolved in 0.9% saline) in a volume of 3.7 ml/kg or pure saline (N = 4). At the end of the experiment the animals were sacrificed in CO_2 anaesthesia.

Microcystin YR

Microcystin YR (MC-YR) was isolated from a toxic bloom at the artificial Koseze pond used for recreational activities as described before (Sedmak and Kosi, 1997, 1998).

Staining with haematoxylin and eosin and sirius red

Hearts were quickly removed, fixed in buffered 10% formalin for 24 hours and embedded in paraffin. Thin sections (4 µm) were then cut on microtome, and stained with haematoxylin and eosin (HE) and sirius red.

Evaluation of the volume density of cardiac muscle tissue and the myofibril volume fraction

Morphometric analysis (Weibel, 1979) was performed under the light microscope using Weibel's test system. Volume density of cardiac muscle tissue (CMvd) and myofibril volume fraction (MFvf) were estimated as described previously (Zorc et al., 2003). The average value (CMvd and MFvf) of each treatment group was then expressed as the average value ± SD. The statistical significance of the differences of the measured parameter between the treatment groups was evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's post hoc analysis ($P < 0.05$).

Measurement of the size of the cardiomyocytes

Three histological sections of the heart left ventricles of each animal were performed under the light microscope at an objective magnification of 40x. The diameter of 50 cardiomyocytes was measured by using Zeiss Axioscope software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). The average diameter of the cardiomyocytes of each treatment group was then expressed as the average level ± SD. The statistical significance of the differences of the measured parameter between the treatment groups was evaluated by one-way ANOVA followed by Scheffe's post hoc analysis ($P < 0.05$).

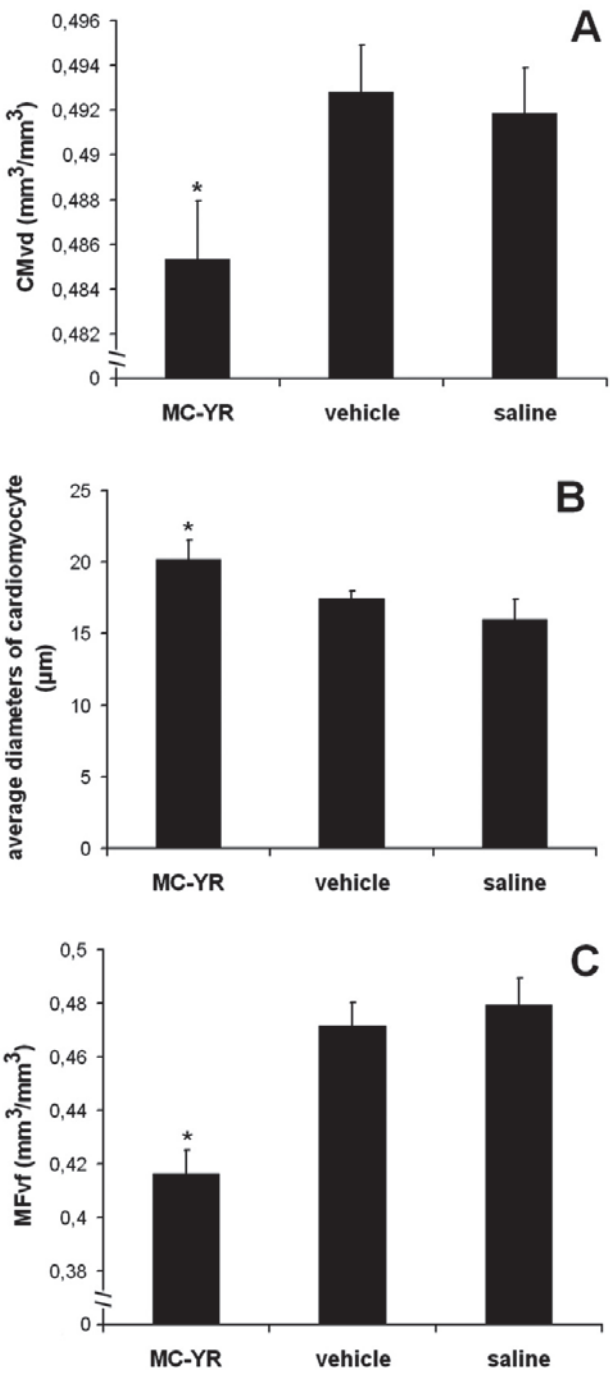

Fig. 1. Evaluation of the volume density of cardiac muscle tissue (**CMvd** ± S.D.) (**A**), average diameter of cardiomyocytes (± S.D.) (**B**) and the myofibril volume fraction of cardiomyocytes, (**MFvf** ± S.D.) (**C**) of heart sections of rats that were treated with **MC-YR, vehicle** and **saline**

TUNEL assay

Detection of apoptosis was performed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) method (Apo Taq plus Peroxidase Kit ONCOR, Gaithersburg, MD) following the manufacturer's instructions as described previously (Zorc et al., 2003).

Results and Discussion

Our results showed that chronic intoxication of experimental animals with relatively low doses of MC-YR causes degeneration of heart muscle. One of the MC-YR-treated rats died before the end of the experiment and was excluded from the study. The heart sections of MC-YR-treated rats revealed decreased volume density of cardiac muscle tissue compared to both control groups (MC-YR = 0.485 $mm^3/mm^3 \pm 0.003$; vehicle = 0.493 $mm^3/mm^3 \pm 0.002$; saline = 0.492 $mm^3/mm^3 \pm 0.002$) (Fig. 1A) due to fibrous proliferation in the MC-YR-treated rats. Most of the cardiomyocytes of the MC-YR-treated animals were enlarged (Fig. 2A, C) compared to the cardiomyocytes of the control animals (MC-YR = 20.19 μm ± 1.34, vehicle = 17.45 μm ± 0.52, saline = 16.00 μm ± 1.43) (Fig. 1B; Fig. 2A, B, C, D).

Some of the cardiomyocytes had enlarged and often bizarre-shaped nuclei as shown in Fig. 2A. The short runs of myocardial fibre were interrupted by connective tissue, degenerative muscle fibres with myocytolysis, and a few lymphocyte and macrophage infiltrates were observed (Fig. 2A). Morphometrical analysis of cardiomyocytes showed decreased myofibril volume fraction in the MC-YR group when compared to the control groups (MC-YR = 0.416 $mm^3/mm^3 \pm 0.009$; vehicle = 0.472 $mm^3/mm^3 \pm 0.009$; saline = 0.479 $mm^3/mm^3 \pm 0.010$) as shown in Fig. 1C and Fig. 2A, B. Treatment of rats with the vehicle had no effect on the morphology of the heart tissue. The TUNEL test showed no apoptotic cells in the sections of control and MC-YR-treated animals.

The morphological changes in myocardium of the MC-YR-treated rats were similar to the changes observed in rats intoxicated with MC-LR (Milutinovic et

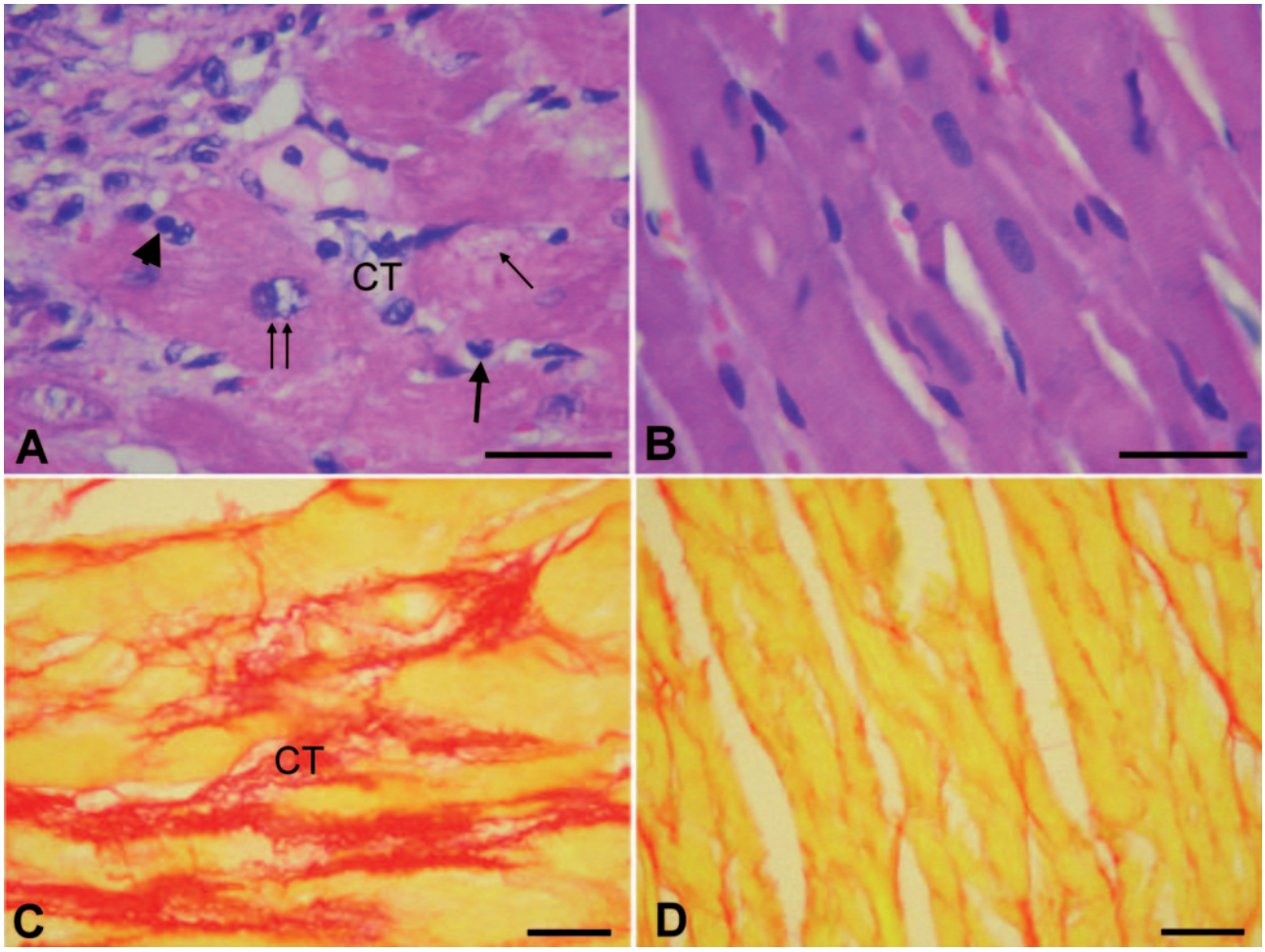

Fig. 2. Heart section of a rat treated with **MC-YR** (A, C) and **vehicle** (B, D) and stained by the HE (A, B) and sirius red (C, D) method. MC-YR section shows a short run of enlarged myocardial fibre interrupted by connective tissue (**CT**) (A, C), a sparse lymphocyte (arrow-head, A) and sparse macrophage (arrow, A) infiltrates, enlarged cardiomyocyte nucleus (two thin arrows, A) and fragmentation of cardiomyofibrils (thin arrow, A). Bar (A, B, C, D) = 30 μm

al., 2006), but the effects were less prominent. Our findings are in agreement with the report of Gupta et al. (2003), who showed that the acute LD_{50} determination of the MC-LR and MC-YR variants showed differences in toxicity, namely that MC-LR was more toxic than MC-YR. Nevertheless, MC-YR is also highly toxic and its presence in potable water poses significant environmental health hazard to livestock and humans (Gupta et al., 2003). In conclusion, chronic exposure of experimental animals to low doses of MC-YR causes atrophy and fibrosis of the heart muscle.

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