

## In vitro genotoxicity of microcystin-RR on primary cultured rat hepatocytes and Hep G2 cell line detected by Comet assay

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### Introduction

Microcystins are hepatotoxic cyclic heptapeptides produced by different species of bloom forming cyanobacteria (*Microcystis*, *Anabaena*, *Nostoc Oscillatoria*).<sup>1</sup> The primary target of the toxin is the liver. The uptake of microcystins into the hepatocytes occurs via carrier-mediated transport system. Microcystins cause cytoskeletal damage, necrosis and pooling of blood in the liver, with a consequent increase in the liver weight. The cause of death is a massive hepatic haemorrhage.<sup>2</sup>

Microcystins are inhibitors of serine/threonine protein phosphatases 1 and 2A and act as tumor-promoters.<sup>3</sup> Ito and coworkers<sup>4</sup> demonstrated that microcystins induced neoplastic nodules in the liver after repeated injections without an initiator, which indicates that they might act also as tumor initiators.

The aim of our studies was to elucidate possible genotoxic effects of microcystin-RR (MCYST-RR) at molecular level using Comet assay. The Comet assay is a sensitive method for detection of DNA strand breaks at the level of a single cell.<sup>5</sup> DNA single-strand breaks can lead to mutations, which are the first step in carcinogenesis.

### Materials and methods

#### *Primary cultured rat hepatocytes and the cell line Hep G2*

Primary rat hepatocytes were isolated from rat liver by a three-step collagenase perfusion method described by Doyle.<sup>6</sup> The isolated hepatocytes and Hep G2 (human hepatoma cell line) were cultured in Williams Medium containing 10% FBS for four h at 37 °C in humidified atmosphere with 5% CO<sub>2</sub>.

#### *Cell treatment*

The primary cultured rat hepatocytes were rinsed with phosphate buffer saline (PBS) (Mg<sup>2+</sup> and Ca<sup>2+</sup> free) and then incubated with different concentrations (0.01, 0.1 and 1 µg/ml) of microcystin-RR (MCYST-RR) in Williams Medium containing 10% FBS for 3 and 13 h. Control cultures were treated with solvent (methanol) only. After the treatment hepatocytes were rinsed with PBS, trypsinised and centrifuged at 1000 rpm for 10 min. Similarly, the Hep G2 cells were incubated with different concentrations of MCYST-RR in Williams Medium containing 10% FBS for 13 h, rinsed with PBS, trypsinised and centrifuged at 1000 rpm for 10 min.

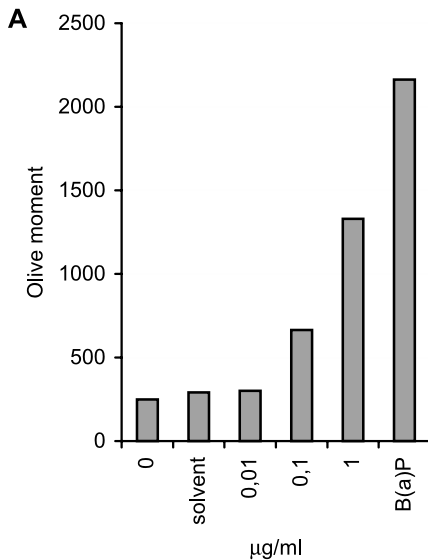
#### *Comet assay*

30 µl of a freshly prepared suspension of cells (3,5x10<sup>5</sup>) and 70 µl low melting point agarose were added to a fully frosted microscope slide,

precoated with 80  $\mu$ l of 1% normal melting point agarose, covered with the top slide. The top slide was removed after solidifying and the cells were lysed at 4 °C for 1 h (2.5M NaCl, 100mM EDTA, 10 mM Tris, 1% Triton X-100, pH10). The slides were then placed in the electrophoresis solution (300mM NaOH, 1mM EDTANa<sub>2</sub>, pH13) for about 20 min at 4 °C to allow DNA unwinding before electrophoresis. Samples were electrophoresed for 20 min at 25 V (300 mA). After electrophoresis the slides were placed in 0.4 M Tris buffer (pH7.5) for 15 minutes in order to neutralise and then stained with ethidium bromide (5  $\mu$ g/ml). The 100 cell nuclei were examined at 400 magnification using a fluorescence microscope (Olympus) and analyzed with the software VisCOMET. MTT cytotoxicity test was performed as described previously by Imamura.<sup>7</sup>

## Results

Significant increase in DNA damage of the primary cultured rat hepatocytes treated with

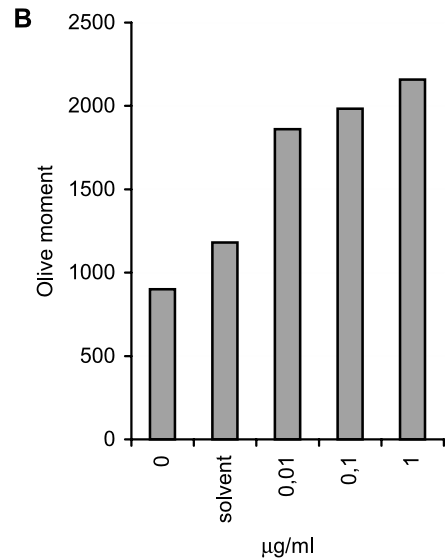


MCYST-RR when compared to the (solvent) control was observed only after prolonged treatment for 13 h. The MTT test showed that MCYST-RR was not toxic at the tested concentrations (results not shown).

Hep G2 cells were treated with the same concentrations of MC-RR and for the same time as primary cultured rat hepatocytes. The results showed a dose-response similar to that of isolated cells. MC-RR was not toxic to Hep G2 cells at the tested concentrations.

## Discussion

The genotoxic potential of microcystin RR was evaluated since it is the most frequent and abundant hepatotoxin in Slovene surface water bodies.<sup>8</sup> The results demonstrated that MCYST-RR induced dose dependent DNA damage in both, primary cultured rat hepatocytes and in Hep G2 cell line at the nontoxic concentrations. With the comet assay DNA damage has already been demonstrated in primary cultured rat hepatocytes after the



**Figure 1.** Olive tail moment of primary cultured rat hepatocytes (A) and the cell line Hep G2 (B) treated with different concentrations of microcystin-RR. The cells were treated for 13 h. Mean values are presented. Methanol (solvent) was used as negative and B(a)P was used as a positive control.

treatment with MCYST-LR (1 µg/ml).<sup>9</sup> In their experiments the DNA damage was induced after 4 h treatment, while in our experiment MCYST-RR was effective only after treatment for 13 h. MCYST-RR is known to be 10 times less biologically active in comparison to MCYST-LR.<sup>10</sup> This could explain the difference in exposure time, needed to induce DNA damage. The fact, that the effects are time dependent corroborates the importance of chronic *in vivo* experiments for the extrapolation of health risks.

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