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## Microcystin-LR-induced cytotoxicity and apoptosis in human embryonic kidney and human kidney adenocarcinoma cell lines

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Microcystin-LR (MC-LR) is a potent hepatotoxin, and increasing evidence suggests that it might also induce kidney injury. The aim of the present work was to evaluate the cytotoxicity and possible apoptotic effects of MC-LR on a human embryonic kidney cell line (HEK-293) and human kidney adenocarcinoma cell line (ACHN). Cells were exposed for 24 h to pure MC-LR (1.0–200  $\mu$ M) and the cytotoxic effects were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sulphorhodamine B (SRB) cell viability assays. Cell viability in both cell lines was significantly decreased after treatment with MC-LR at 50  $\mu$ M for 24 h ( $P < 0.001$ ). Moreover, MC-LR-treated ACHN and HEK-293 cells exhibited a marked dose-dependent loss of confluence as judged by phase-contrast microscopy. Similarly, fluorescence microscopic observations following acridine orange–ethidium bromide (AO/EB) staining confirmed that both cell types were undergoing apoptosis after treatment with MC-LR for 24 h. Expression of three apoptosis-related genes, *Bax*, *Survivin* and *p53*, was analysed by quantitative reverse transcriptase PCR analysis. Both *Bax* and *p53* functioned as promoters of MC-LR-mediated apoptosis in ACHN and HEK-293 cells. The *Survivin* gene acted as a suppressor of apoptosis at lower MC-LR concentration (1  $\mu$ M) and the gene was upregulated at higher MC-LR concentration (10  $\mu$ M) ( $P < 0.001$ ). Significant increases of caspase 3 ( $P < 0.0001$ ) and caspase 9 ( $P < 0.0001$ ) activity were detected in both cell lines after exposure to MC-LR for 24 h, indicating the MC-LR induces cytotoxicity and a marked apoptosis in both ACHN and HEK-293 kidney cell lines.

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### INTRODUCTION

Contamination of natural water bodies by cyanotoxins produced by cyanobacterial blooms is a worldwide problem, causing serious water pollution and health hazards to humans and livestock (Feurstein *et al.*, 2009a). Thus far, more than 46 cyanobacterial species have been reported to produce potent hepatotoxins and/or neurotoxins. Approximately 75% of water samples containing cyanobacteria also contain toxic cyanobacterial metabolites (Ernst *et al.*, 2005). Microcystins (MCs) comprise one of the major cyanobacterial toxin classes with a family of hepatotoxic heptapeptides

(Fischer & Dietrich, 2000). This group represents a toxin group of more than 100 structural congeners produced by freshwater cyanobacteria (Feurstein *et al.*, 2011). Severe hepatotoxicity of this assemblage of toxins has been confirmed both from acute human incidents and from animal poisoning incidents in different parts of the world (Chorus *et al.*, 2000; Duy *et al.*, 2000) and from *in vivo* and *in vitro* experimental studies (Gupta *et al.*, 2003; Žegura *et al.*, 2003).

Among the known microcystins, microcystin-LR (MC-LR) is the most commonly encountered variant of the family as it is the most potent toxic member of the group (Lankoff *et al.*, 2004). Although MC toxicity is primarily associated with liver disruption, possible damage to other vital organs such as the intestines, kidneys, thymus of quail and male reproductive organs has been suggested (Towner *et al.*, 2002; Zeller *et al.*, 2011; Zhang *et al.*, 2011). Furthermore, MCs are reported to have various other effects, including

Abbreviations: ACHN, human kidney adenocarcinoma cell line; AO/EB, acridine orange–ethidium bromide; CKD, chronic kidney disease; CKDu, CKD of unknown aetiology; HEK-293, human embryonic kidney cell line; MC, microcystin; MC-LR, microcystin-LR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OATP, organic anion-transporting polypeptide; RT-PCR, reverse transcriptase PCR; SRB, sulphorhodamine B.

neurotoxicity, genotoxicity and embryotoxicity (Chen *et al.*, 2005). Less severe health effects such as contact dermatitis, asthma-like symptoms and symptoms resembling hay fever have been attributed to MCs (Lankoff *et al.*, 2004; Torokne *et al.*, 2001). The World Health Organization (WHO) defined a drinking water guideline value of  $1.0 \mu\text{g l}^{-1}$  for MC-LR, revealing the importance of this toxin as a potential public health hazard (WHO, 1998). Moreover, Health Canada calculated a tolerable daily intake of  $0.013 \mu\text{g MC-LR}$  per kilogram of body weight per day (defined as a 60 kg adult consuming 1.5 litres of water per day, with an MC-LR content of  $0.5 \text{ ng ml}^{-1}$ ; Fischer *et al.*, 2001).

The toxicity of MCs is mainly due to their ability to inhibit serine/threonine protein phosphatases 1 and 2A (Fischer & Dietrich, 2000; Fischer *et al.*, 2000). This ability is due to the interaction of MCs with the catalytic subunits of these enzymes, demonstrating the association of these enzymes with MC-induced acute toxicity (Honkanen *et al.*, 1990; Yoshizawa *et al.*, 1990). MCs could damage to the cells by causing disorganization of cytoplasmic microtubules, cytokeratin intermediate filaments and actin microfilaments, thereby disrupting the cytoskeleton of the targeted cells (Falconer & Yeung, 1992). It has also been documented that prolonged, sublethal exposure to MCs can result in an increase in hepatic oxidative stress, apoptosis/necrosis, and even hepatic neoplasia in both *in vivo* and *in vitro* studies (Clark *et al.*, 2008; Gehring, 2004). MCs could be potent tumour promoters and tumour initiators (Zegura *et al.*, 2003). Increases in oxidative stress could lead to chemical carcinogenesis (Elrick *et al.*, 2005) and thus International Agency for Research on Cancer classified MC-LR as a possible human carcinogen (IARC, 2006).

As a result of their complex structure and amino acid composition, MCs are somewhat hydrophilic and have large molecular masses ( $\sim 1 \text{ kDa}$ ; Carmichael *et al.*, 1988). Hence, passive diffusion is insufficient for passage through cell membranes, and MCs require active transport via specific transporters (Fischer *et al.*, 2010). Thus, MC uptake is mediated by various organic anion transporting polypeptides (e.g. rodent Oatps/human OATPs; Fischer *et al.*, 2005). Fischer *et al.* (2005) identified Oatp1b2, OATP1A2, OATP1B1 and OATP1B3 as MC-LR transporters in rat and human. The organ specificity of MCs is due to selective uptake of the toxin by the OATPs (Fischer *et al.*, 2005). The tissue distribution of the identified MC-LR transporting Oatps/OATPs explains the preferential organ toxicity of MCs in liver and brain. Oatp-mediated and MC-congener-dependent transport is responsible for MC-induced neuronal toxicity (Feurstein *et al.*, 2009b). MCs are mainly excreted by the hepatocytes, but up to 9% can be eliminated by urine (Bischoff, 2001) which makes the kidneys a potential target for MC-LR toxicity (Menezes *et al.*, 2013). Furthermore, OATP-A, one of the known MC active transporters, has been identified recently at mRNA level in the human kidney (Hagenbuch & Meier, 2003). Thus, the kidney might also be an important target organ for MCs (Dias *et al.*, 2009).

Chronic kidney disease (CKD) has become a burning health problem in Sri Lanka, and has been observed in many parts of the country, especially in North Central, North Western, Uva and Eastern provinces (WHO, 2010). CKD is a global health problem typically associated with diabetes, hypertension, infections and environmental nephrotoxins (Eckardt *et al.*, 2009; James *et al.*, 2010). Cases of CKD in Sri Lanka with unknown aetiology (CKDu) have increased alarmingly in recent decades, leading to national concern and an urgent need to identify the causes and for action. This is highlighted by admissions at the Anuradhapura General Hospital, the main hospital in the North Central region, which increased by 27% between 1992 and 2006 (Chandrajith *et al.*, 2011). Currently in excess of 8000 patients are receiving treatment. Progression of CKDu is generally symptomless until the advanced stages of disease, when the kidneys are damaged irreversibly resulting in mortality except where dialysis and/or transplantation are available. Since 2010 there has been extensive research funded by the National Science Foundation, Sri Lanka, and WHO to determine population prevalence, risk factors, treatment strategies and socioeconomic impact (Wanigasuriya, 2012). Findings to date indicate multiple risk factors, in particular chronic exposure to low levels of cadmium via the food chain. Other research has indicated that some cases are linked to ingestion of water, i.e. a higher incidence of disease in areas with surface water compared with areas where the population uses springs (Jayasekara *et al.*, 2013). However, despite extensive research in the North Central province, potential risk factors in the water supply (fluoride, heavy metals, pesticides, aluminium ochratoxin) were eliminated.

Given that the risk factors responsible for the occurrence of CKDu in Sri Lanka have not been clearly identified, further investigations are a priority. The role of cyanotoxins, some of the most potent natural toxins, in CKDu has been considered but not investigated. Thus, in 2013 a WHO report highlighted cyanotoxin-contaminated water as a possible explanation for CKDu in the affected area. Therefore, the present study was carried out to elucidate the possible cytotoxic effect of pure MC-LR on normal human embryonic kidney (HEK-293) and human kidney adenocarcinoma (ACHN) cell lines. Furthermore, the expression of some selected apoptosis-related genes in response to MC-LR was also studied.

## METHODS

**Cell lines and reagents.** ACHN (human renal adenocarcinoma; Catalogue number CRL-1611) and HEK-293 (human embryonic kidney; Catalogue number CRL-1573) cell lines, and Eagle's minimum essential medium (EMEM) were purchased from American type Culture Collection. TRIzol reagent (15596-018) was purchased from Invitrogen Life Technologies. FBS, trypsin-EDTA, Strep-penicillin and all other chemicals were purchased from Sigma-Aldrich.

Pure MC-LR standard was provided by the Cyanosole group in IDEAS, Robert Gordon University, Aberdeen, UK. Tissue-culture-treated cell culture flasks and plates were purchased from Sterilin.

**Cell culture.** Complete growth medium for both cell cultures was prepared by adding FBS and Strep-penicillin to give a final concentration in the medium of 10 and 0.1 %, respectively. Cell cultures were maintained at 37 °C with 5 % CO<sub>2</sub>/air and 90 ± 5 % humidity. Culture medium was changed every 2–3 days.

**Cytotoxicity assays.** Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sulphorhodamine B (SRB) assays as described previously with slight modifications (Samarakoon *et al.*, 2010). Briefly, for both assays cells were seeded at  $5 \times 10^3$  cells per well into a 96-well plate and incubated for 24 h for attachment. After the incubation period, cells in different wells were exposed to different concentrations of MC-LR (0.1, 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 and 200.0 µM) and subjected to MTT and SRB assays 24 h after incubation.

In the MTT assay, medium in each well was aspirated and the cells were washed with 1 × PBS. MTT solution (20 µl) was added to the 200 µl medium in each well and the plate was incubated at 37 °C for 4 h. The medium was aspirated carefully and then 100 µl 2-propanol/HCl was added per well. Finally, the plate was shaken for 30 min and the absorbance at 620 nm was measured using a microplate reader (Synergy HT micro plate reader, BIO-TEK instruments); results are expressed as a percentage of controls.

For the SRB assay, 24 h after incubation, cells were briefly washed with 1 × PBS and fresh medium was placed in each well. Ice-cold 50 % trichloroacetic acid (25 µl) was layered on top of the fresh medium overlying the cells and incubated at 4 °C for 1 h to ensure cell fixation. The cells were then washed five times with tap water. The plate was air-dried and the fixed cells were stained with 0.4 % (w/v) SRB dissolved in 1 % acetic acid for 15 min at room temperature. The plate was then quickly washed five times with 1 % acetic acid and air-dried. Finally, 200 µl of unbuffered Tris-base solution (pH 7.5) was added to each well and the plate was placed on a plate shaker for 30 min at room temperature. The plate was then read (OD<sub>540</sub>) using the Synergy HT microplate reader and the results were expressed as a percentage of control values.

All the assays were performed in triplicate in three different experiments. IC<sub>50</sub> values of MC-LR on ACHN and HEK-293 cells were determined by analysing the percentage of control values in each cytotoxicity assay with sigmoid dose–response inhibition curves using Prism software version 5 (GraphPad Prism).

**Morphological observations using phase-contrast and fluorescence microscopy.** ACHN and HEK-293 cells were seeded separately in 24-well plates at  $5 \times 10^4$  cells ml<sup>-1</sup> on coverslips for 24 h in a humidified atmosphere at 37 °C in 5 % CO<sub>2</sub> to allow cell adherence. After incubation, cells were treated with different concentrations (1.0, 10.0, 50.0, 100.0 and 200.0 µM) of MC-LR. Treated cells were incubated for 24 h and at the end of the incubation cells were rinsed twice with ice-cold PBS. The cells on the coverslips were observed under a phase-contrast microscope (CKX41SF, Olympus) for morphological changes. Cells were then fixed with 4 % formaldehyde in PBS at room temperature for 15 min. Acridine orange-ethidium bromide (AO/EB) dye cocktail in PBS, containing 50 µg each dye ml<sup>-1</sup> was added to the fixed cells on coverslips and incubated for 10 min at room temperature in the dark. Cells were then visualized under a fluorescence microscope (BX51TRF, Olympus) using blue filters. Both assays were performed in triplicate in three different experiments.

**Gene expression analysis by reverse transcriptase PCR (RT-PCR).** ACHN and HEK-293 cells were cultured separately in T<sub>25</sub> flasks ( $5 \times 10^5$  cells per flask) for 24 h in complete EMEM culture medium. Cells were exposed to different concentrations of MC-LR (1.0 and 10.0 µM) for 24 h. Triplicate experiments were carried out for each concentration. Total RNA from cells in each flask was

extracted using TRIzol reagent according to the manufacturer's instructions with minor modifications.

Briefly, cells were lysed by adding 1 ml of TRIzol reagent followed by passing the lysate several times through a pipette. Cell lysate was transferred to a 1.5 ml microcentrifuge tube, mixed gently and incubated at room temperature for 5 min. The microcentrifuge tube was then centrifuged (D-78532, HETTICH) at 12 000 r.p.m. at 4 °C for 10 min. The supernatant was decanted into a fresh tube containing 200 µl of ice-cold chloroform. The mixture was shaken vigorously by hand for 30 s and incubated at room temperature for 2–3 min. Following centrifugation at 12 000 r.p.m. at 4 °C for 15 min, 150 µl of colourless upper aqueous phase containing RNA was collected to a new microcentrifuge tube. Ice-cold isopropyl alcohol (500 µl) was added and kept on ice for 15 min to precipitate RNA. Following centrifugation at 12 000 r.p.m. at 4 °C for 10 min, the supernatant was discarded and the RNA pellet was washed twice with 1 ml of ice-cold 75 % ethanol. The RNA pellet was then air-dried and resuspended in 15 µl RNase-free water. RNA quantification was done by measuring absorbance at 260 and 280 nm wavelengths using a BioSpec nanospectrophotometer (Shimadzu).

Synthesis of cDNA was carried out as follows. Each sample of isolated RNA was reverse-transcribed using an M-MLV reverse transcriptase system (A3500; Promega) to synthesize single-stranded cDNA. Extracted RNA (2 µg) was mixed with 50 ng of random primers. Subsequently, RNA-random primer mix was brought to total volume of 13.5 µl with water and incubated for 5 min at 70 °C. Reverse transcription reaction mix was prepared by adding 5 µl M-MLV 5 × reaction buffer, 5 µl 10 mM dNTP mix, 25 units of RNasin and 200 units of M-MLV RT enzyme. Total volume in the PCR mixture was adjusted to 25 µl using PCR water. This was then incubated at 37 °C for 60 min by using a thermal cycler.

Each PCR was carried out in a master mix MESA FAST quantitative PCR MasterMix Plus for SYBR Assay kit (*Taq* DNA polymerase, reaction buffer: MgCl<sub>2</sub> (4 mM), dNTP mix and SYBR Green I dye) with 0.2 mM of respective forward and reverse primers in 50 µl reaction mix. The PCR amplification was carried out in a Max 3000p real-time PCR system (Stratagene). *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) amplification was performed as an internal control. PCR conditions for *GAPDH* were 30 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. PCR conditions for *Bax* were 35 cycles at 94 °C for 1.5 min, 55 °C for 30 s and 72 °C for 1 min. PCR conditions for *Bcl-2* were 30 cycles at 94 °C for 1.5 min, 56 °C for 30 s and 72 °C for 1 min. PCR conditions for *survivin* were 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 50 s. The primers used for RT-PCR are detailed in Table 1.

**Table 1.** Primer sequences for *GAPDH*, *Bax*, *Survivin* and *p53* genes

| Gene            |         | Sequence                      |
|-----------------|---------|-------------------------------|
| <i>GAPDH</i>    | Forward | 5'-GAAGGTGAAGGTCGGAGTC-3'     |
|                 | Reverse | 5'-GAAGATGGTGATGGGATTTTC-3'   |
| <i>Bax</i>      | Forward | 5'-GGACGAAGTGGACAGTAACATGG-3' |
|                 | Reverse | 5'-GCAAAGTAGAAAAGGGCGACAAC-3' |
| <i>Survivin</i> | Forward | 5'-TGGCCGCTCCTCCCTCAGAAAA-3'  |
|                 | Reverse | 5'-GCTGCTGCCTCAAAGAAAGCG-3'   |
| <i>p53</i>      | Forward | 5'-GTTCCGAGAGCTGAATGAGG-3'    |
|                 | Reverse | 5'-TCTGAGTCAGGCCCTTCTGT-3'    |

Relative quantification for expression of each gene was determined using the method described by Livak & Schmittgen (2001). Expression of each gene was compared with *GAPDH*, the internal control gene. Relative gene expression was given by  $2^{(-\Delta\Delta C_t)}$ , where  $C_t$  is the cycle at which the threshold is crossed,  $\Delta C_t = C_t$  (target gene) –  $C_t$  (*GAPDH* gene) and  $\Delta\Delta C_t = \text{average } \Delta C_t$  (treated sample) – average  $\Delta C_t$  (untreated sample).

#### Measurement of caspase-3 and caspase-9 enzyme activity.

Caspase-3 and caspase-9 activity was measured using GenScript colorimetric assay kits (Cat. Nos. L00289 and L00304) according to the method described by Samarakoon *et al.* (2012). Briefly,  $5 \times 10^6$  cells of either cell line per one T<sub>75</sub> flask were cultured for 24 h prior to the experiment. Cells were then exposed to different concentrations of the toxin (1.0 and 10.0  $\mu\text{M}$  MC-LR) for 24 h to induce apoptosis. Triplicate experiments were carried out for each concentration. Cells were harvested by trypsinization and centrifuged at 150 r.p.m. at 4 °C for 7 min. The resultant cell pellet was washed with PBS twice by centrifugation at 2000 r.p.m. at 4 °C for 5 min. The clean cell pellet was lysed for 1 h by adding 50  $\mu\text{l}$  of cold lysis buffer. The cell lysate was centrifuged at 10 000 r.p.m. at 4 °C for 1 min. The supernatant was transferred to a new tube and total protein in the extract was quantified using the Bradford method.

An aliquot containing 200  $\mu\text{g}$  of protein was separated from the extract and 50  $\mu\text{l}$  of  $2 \times$  reaction buffer was added. Caspase substrate (5  $\mu\text{l}$  of caspase-3 or caspase-9) was added and incubated in the dark for 4 h at 37 °C. An extinction value was obtained at 400 nm using a BioSpec nanospectrophotometer (Shimadzu). Finally, caspase activity values in the cells exposed to the toxin were obtained by calculating  $\text{OD}_{\text{induced}}/\text{OD}_{\text{negative control}}$ .

**Statistical analysis.** All assays were carried out in triplicate in three different experiments. Therefore, the total number of replicates was nine for each concentration of MC or control for each experiment. All data are represented as mean  $\pm$  SD. Statistical differences were analysed with a *t*-test or with an ANOVA followed by Dunnett's post test using GraphPad Prism software as appropriate. Values of  $P < 0.001$  were considered statistically significant.

## RESULTS

### Cytotoxicity and cell viability assays

The cytotoxic effect of MC-LR on HEK-293 and ACHN cells as assessed by SRB and MTT assays is shown in Fig. 1. Exposure to MC-LR caused a significant ( $P < 0.001$ , d.f. = 3, one-way ANOVA) dose-dependent cytotoxicity in both cell lines (mean absorbance values for control wells with HEK-293 and ACHN cells, respective to 100 % cell survival, were 0.3416 and 0.3648 for the MTT assay and 0.3025 and 0.3284 for the SRB assay, respectively).

When  $\text{IC}_{50}$  values were compared using the results of the SRB assay, a comparatively lower  $\text{IC}_{50}$  value was observed in HEK-293 [16.32  $\mu\text{M}$  (16.24  $\mu\text{g ml}^{-1}$ )] than in ACHN cells [90.85  $\mu\text{M}$  (90.41  $\mu\text{g ml}^{-1}$ );  $P < 0.01$ , d.f. = 3, paired *t*-test]. The MTT assay also showed a significantly ( $P < 0.001$ , d.f. = 3) lower  $\text{IC}_{50}$  in the HEK-293 cells than in the ACHN cells, but the value for HEK-293 cells was much higher than the value determined by the SRB assay. The  $\text{IC}_{50}$  values of the MTT assay for HEK 293 cells and ACHN cells were 72.62  $\mu\text{M}$  (72.27  $\mu\text{g ml}^{-1}$ ) and 97.09  $\mu\text{M}$  (96.62  $\mu\text{g ml}^{-1}$ ), respectively.

### Morphological observations using phase-contrast and fluorescence microscopy

Characteristic morphological changes of apoptosis in MC-LR-treated and control HEK-293 and ACHN cells after 24 h observed under phase-contrast microscopy are shown in Figs 2 and 3, respectively. Control cells and cells exposed to lower doses of MC-LR (up to 1  $\mu\text{M}$  in HEK-293 cells and 10  $\mu\text{M}$  in ACHN cells) displayed similar higher confluence (Figs 2a, b and 3a–c). Higher concentrations of the toxin caused a lower cell confluence and a high proportion of rounded cells resulting from a higher percentage of detached cells from the culture monolayer. These morphological changes increased dose dependently and were more pronounced in cells exposed to concentrations higher than 10  $\mu\text{M}$  in HEK-293 cells (Fig. 2c–f) and 50  $\mu\text{M}$  in ACHN cells (Fig. 3d–f).

Fluorescence microscopic observations of ACHN and HEK-293 cells treated with different concentrations of MC-LR stained with AO/EB dyes after 24 h of incubation (Figs 4 and 5, respectively) also demonstrated that MC-LR can mediate pro-apoptotic effects when compared with the untreated cells.

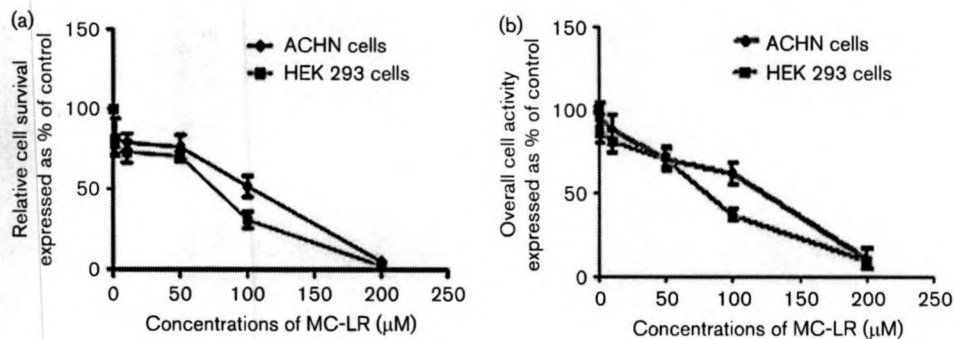
As is evident from Figs 4(a) and 5(a), respectively, nuclei of viable ACHN and HEK-293 cells in control groups are stained uniformly bright green by AO. Early apoptotic cells in both cell lines have greenish yellow nuclei (Figs 4b, c and 5b) while late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin (Figs 4d, e and 5c). Necrotic cells stained uniformly orange to red (Figs 4f and 5d–f). Thus, the degree of apoptosis appeared to be dose dependent in the ACHN and HEK-293 cells treated with different concentrations of MC-LR.

### Gene expression analysis by RT-PCR

Expression of the *Bax*, *Survivin* and *p53* genes was evaluated relative to the *GAPDH* gene using quantitative RT-PCR and the results are shown in Fig. 6. Exposure to MC-LR resulted in a dose-dependent significant upregulation of *Bax* ( $P < 0.01$ , d.f. = 3,  $R^2 = 0.8879$ , one-way ANOVA) and *p53* ( $P < 0.001$ , d.f. = 3,  $R^2 = 0.8802$ , one-way ANOVA) gene expression in both ACHN and HEK-293 cells compared with the control. In contrast, *Survivin* gene expression was significantly ( $P < 0.001$ , d.f. = 3,  $R^2 = 0.8937$ , one-way ANOVA) downregulated at the lower concentration of MC-LR (1  $\mu\text{M}$ ) while it was upregulated at the higher concentration of MC-LR (10  $\mu\text{M}$ ).

### Measurement of caspase-3 and caspase-9 enzyme activity

The effect of MC-LR on activation of caspase-3 and caspase-9 in ACHN and HEK-293 cells is shown in Fig. 7. Caspase-3 activity was significantly increased by MC-LR at concentrations of 1 and 10  $\mu\text{M}$  in both cell lines compared with the untreated control cells ( $P < 0.001$ , one-way ANOVA).

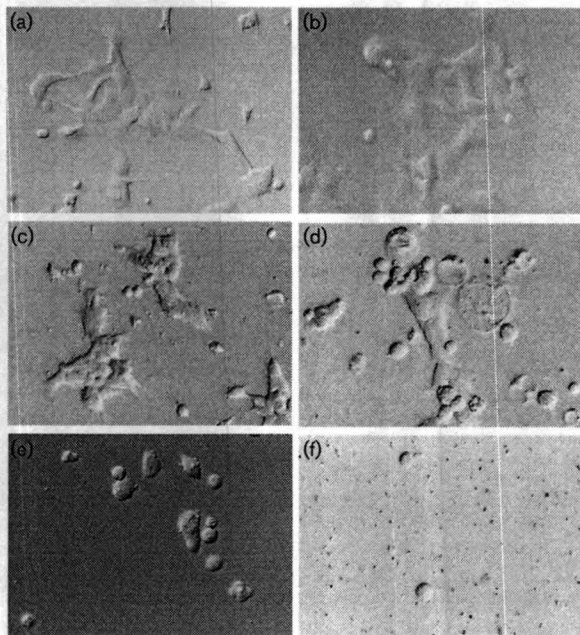


**Fig. 1.** Viability of ACHN and HEK-293 cells exposed to different concentrations of MC-LR for 24 h assessed by SRB assay (a) and MTT assay (b). Results are expressed as the mean ( $\pm$  SD) percentage of three replicates relative to control cells treated only with the medium ( $P < 0.001$ ).

with Dunnett's post test). Caspase-9 activity in both cell lines significantly ( $P < 0.001$ ) increased when exposed to 10  $\mu$ M MC-LR whereas 1  $\mu$ M MC-LR had no effect when compared with controls.

## DISCUSSION

Although the toxic effects of MC-LR on the human liver is well documented (Žegura *et al.*, 2003), an increasing number of recent publications have emphasized the need for a thorough evaluation of their effects on other organs.

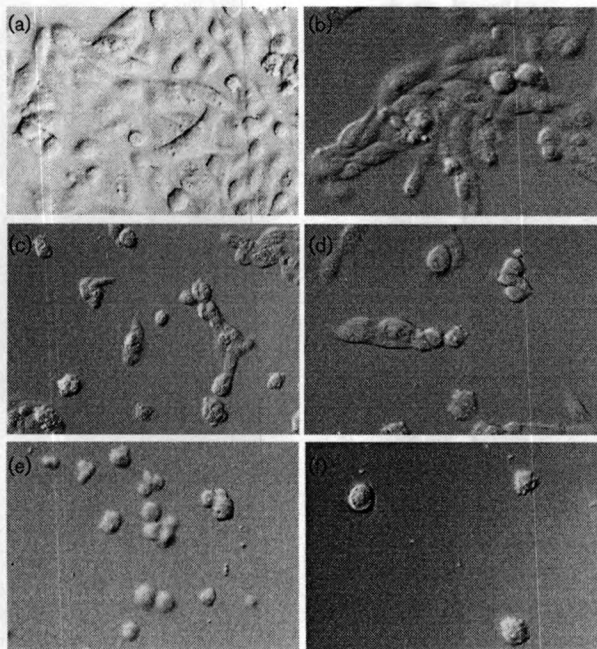


**Fig. 2.** Morphological characteristics of HEK-293 cells observed by phase-contrast microscopy exposed to different concentrations of MC-LR for 24 h: (a) control (0  $\mu$ M), (b) 1  $\mu$ M, (c) 10  $\mu$ M, (d) 50  $\mu$ M, (e) 100  $\mu$ M and (f) 200  $\mu$ M (magnification  $\times 1000$ ).

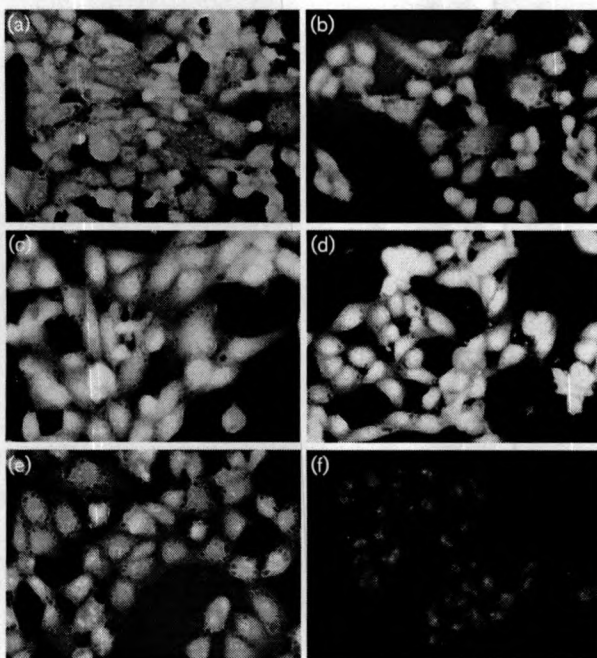
The adverse effects of MC-LR in distinct organs is an important issue for risk assessment, because the guideline value for MC in drinking water (1 nM) is still a provisional one, based on limited toxicological data (WHO, 2008). Exposure to low doses of MC-LR is to the most reasonable kidney intoxication scenario, given that it is not the main target organ for MC-LR. However, the role of the kidney in toxin elimination might expose kidney cells to a low internal dose that can be biologically effective in inducing nephrotoxic effects (Menezes *et al.*, 2013).

Therefore, the present study was carried out to investigate the cytotoxic and apoptotic effects of MC-LR on HEK-293 and ACHN cell lines. The major aim was to evaluate the potential damage of MC-LR on normal kidney cells using a disease-free embryonic kidney HEK-293 cell line and to detect whether this cyanotoxin is a potential cancer promoter using a kidney adenocarcinoma ACHN cell line as that is a proven liver cancer promoter. Cytotoxicity and morphological studies were carried out to elucidate whether MC-LR is equally cytotoxic to the both cell lines or is only toxic to normal kidney cells. Morphological evaluation was also used to demonstrate whether this toxin could promote the proliferation of ACHN cells, as this would provide evidence for its cancer-promoting action. Gene expression analysis and caspase studies were carried out to evaluate the pathways of apoptosis used by the toxicity of MC-LR in both cell lines, as cytotoxicity and morphological studies showed that MC-LR is toxic to both cell lines.

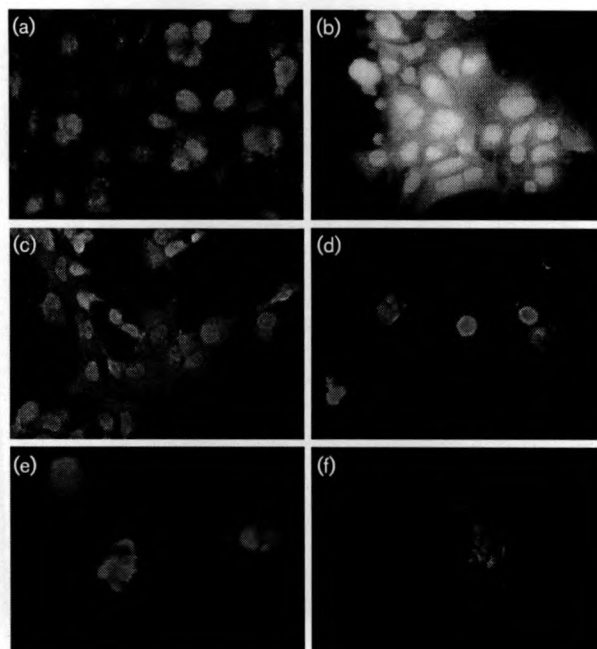
Cytotoxicity evaluation demonstrated that MC-LR significantly reduced relative cell survival of both ACHN and HEK-293 cell lines. When the  $IC_{50}$  values of MC-LR in the two cell lines were compared, HEK-293 had lower values than the ACHN cells at 24 h post-incubation in both MTT and SRB assays, suggesting that MC-LR is more cytotoxic to normal embryonic kidney cells than to kidney adenocarcinoma cells. Dias *et al.* (2009) reported that exposure to MC-LR at 22  $\mu$ M for 24 h could induce a marked cytotoxic response (cell viability  $\leq 50\%$ ) in a monkey kidney cell line (Vero E6). Alverca *et al.* (2009) also reported a



**Fig. 3.** Morphological characteristics of ACHN cells observed by phase-contrast microscopy exposed to different concentrations of MC-LR for 24 h: (a) control (0  $\mu\text{M}$ ), (b) 1  $\mu\text{M}$ , (c) 10  $\mu\text{M}$ , (d) 50  $\mu\text{M}$ , (e) 100  $\mu\text{M}$  and (f) 200  $\mu\text{M}$  (magnification  $\times 1000$ ).



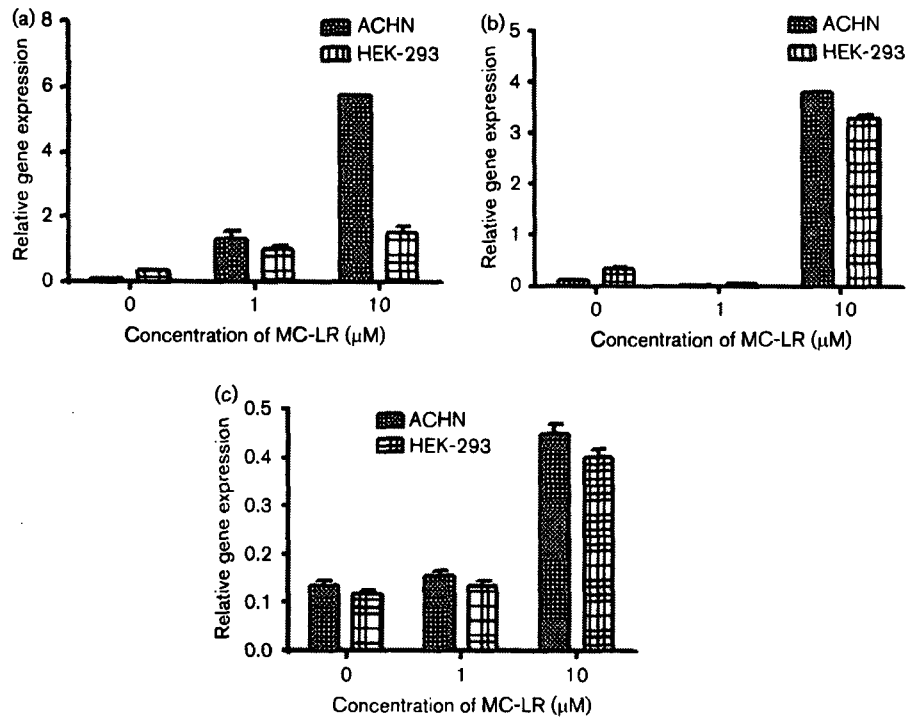
**Fig. 4.** Fluorescence microscopy observations of MC-LR-treated and non-treated ACHN cells stained with AO/EB at 24 h post-incubation: (a) control (0  $\mu\text{M}$ ), (b) 1  $\mu\text{M}$ , (c) 10  $\mu\text{M}$ , (d) 50  $\mu\text{M}$ , (e) 100  $\mu\text{M}$  and (f) 200  $\mu\text{M}$  (magnification  $\times 1000$ ).



**Fig. 5.** Fluorescence microscopy observations of MC-LR-treated and non-treated HEK-293 cells stained with AO/EB at 24 h post-incubation: (a) Control (0  $\mu\text{M}$ ), (b) 1  $\mu\text{M}$ , (c) 10  $\mu\text{M}$ , (d) 50  $\mu\text{M}$ , (e) 100  $\mu\text{M}$  and (f) 200  $\mu\text{M}$  (magnification  $\times 1000$ ).

significant reduction of cell viability when the same cell line was exposed to 30  $\mu\text{M}$  MC-LR for the same incubation period. Other literature has reported a significant reduction in the viability of rat Sertoli cells upon exposure to MC-LR at 50 and 500 nM for 24 h and at 10.05–20.10  $\mu\text{M}$  (10–20  $\mu\text{g ml}^{-1}$ ; Li & Han, 2012; Zhang *et al.*, 2011).  $\text{IC}_{50}$  values of MC-LR in kidney cells in the present study were different from those of MC-LR reported for other cell types, perhaps due to different cell types in the human body having different responses to MC-LR (Yi *et al.*, 2011).

Morphological analysis of MC-LR-treated ACHN and HEK-293 cells allowed us to clarify the cellular damage underlying MC-LR-induced cytotoxicity. According to our phase-contrast microscopy observations, the confluence of both normal ACHN and HEK-293 cells decreased dose dependently after 24 h of exposure, when compared with the control experiments. This loss of higher confluence was observed at MC-LR doses of 10  $\mu\text{M}$  or higher in HEK-293 cells and at 50  $\mu\text{M}$  or higher in ACHN cells, confirming a greater toxicity of MC-LR in HEK-293 cells than in ACHN cells. Furthermore, fluorescence microscopy confirmed the apoptotic effects of MC-LR in both HEK-293 and ACHN cells. Several studies have reported toxic and apoptotic effects of MC-LR using light/electron microscopy and fluorescence microscopy on different types of human, monkey, rat and primary cell cultures (Alverca *et al.*, 2009; Dias *et al.*, 2009; Li & Han, 2012; McDermott *et al.*, 1998). It is hard to compare these data as the source of the toxin

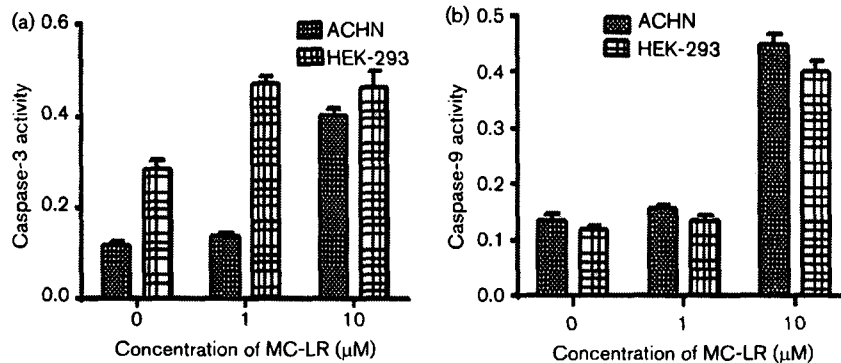


**Fig. 6.** Effects of MC-LR on mRNA expression of (a) *Bax*, (b) *Survivin* and (c) *p53* genes in ACHN and HEK-293 cells 24 h post-incubation.

(pure or crude extracts), dosages and end points used for evaluation differed between the studies.

Apoptosis is an active process of cellular self-destruction that requires the expression of specific genes, including *Bax*, *p53* and *caspase 3* (Zhang *et al.*, 2011). The mitochondria-mediated pathway of apoptosis is regulated by Bcl-2 family proteins including Bax, a pro-apoptotic protein (Hu *et al.*, 2010). In the present study, expression of three apoptosis-related genes *Bax*, *Survivin* and *p53*, was evaluated. Li *et al.* (2011) stated that MCs could contribute to apoptotic death

of rat testicular cells by leading to a persistent increase of transcriptional and protein levels of *p53* and *Bax* genes. When cells undergo apoptosis, the pro-apoptotic protein Bax can translocate to the outer membrane of mitochondria leading to the release of pro-apoptotic factors inducing apoptosis. The *p53* gene has been implicated in the induction of apoptosis. It is a tumour suppressor gene that affects the cell cycle through controlling the progression of the G1 stage (Zhang *et al.*, 2011). It appears to play an important role in programmed cell death. Overexpression of *p53* can



**Fig. 7.** Expression of caspase-3 (a) and caspase-9 (b) in ACHN cells and HEK-293 cells exposed to different concentrations of MC-LR for 24 h. Results are expressed as mean  $\pm$  SD of triplicate experiments.

induce apoptosis in a wide range of cell types (Zhang *et al.*, 2011). In the present study, MC-LR at higher concentrations increased expression of the pro-apoptotic gene *Bax* and tumour suppressor gene *p53* significantly. This scenario suggests that MC-LR induces apoptosis in ACHN and HEK-293 cells by inducing the expression of Bcl-2 family *Bax* and *p53* genes. *Survivin* is an inhibitor of apoptosis proteins (Chen *et al.*, 2005). In the present study, MC-LR down-regulated expression of the *Survivin* gene at a lower concentration of MC-LR (1  $\mu$ M), while upregulating at a higher (10  $\mu$ M) MC-LR concentration. Although the published literature on expression of the *Survivin* gene after exposure of human or animal cells to MC-LR is limited, Chen *et al.* (2005) reported the same expression pattern of the *Survivin* gene in mouse liver cells as in the present study.

Li & Han (2012) have demonstrated a change in oxidative stress upon MC-LR exposure of rat Sertoli cells and suggested that this would induce apoptosis. An increased peroxide concentration may trigger the dissociation of cytochrome *c*. Once cytochrome *c* is released from mitochondria, the cell will die by activation of the apoptotic caspase cascade (Li & Han, 2012). Furthermore, caspase-3 plays an important role in mediating nuclear apoptosis. For instance, caspase-3-dependent signalling transduction mediates the morphological changes in nuclei during apoptosis (Zhang *et al.*, 2011). Li & Han (2012) revealed increased expression of caspase-3 and -9 in rat Sertoli cells after exposure to MC-LR for 24 h. Similarly, in the present study caspase-3 and caspase-9 protein levels were increased in both cell lines after treatment with MC-LR for 24 h. Wang *et al.* (2013) and Zhang *et al.* (2011) also reported increased caspase-3 activity upon exposure to MC-LR.

The higher MC-LR concentrations used in the present study have rarely been reported in natural water bodies contaminated with cyanobacteria, although long-term accumulation *in vivo* might lead to the reported levels (Yu *et al.*, 2009).

Given the results obtained here, we established that MC-LR is more toxic to normal human kidney cells than to human kidney adenocarcinoma cells. Moreover, morphological studies also revealed that MC-LR is toxic to cancer cells, but not as highly toxic as to normal kidney cells. MC-LR does not promote cell division of human kidney adenocarcinoma (ACHN) cells, showing that it could not be a promoter of kidney cancer. Gene expression analysis and caspase studies revealed that apoptosis of both types of cells was induced by MC-LR through a caspase-dependent pathway. On the other hand, all the experiments showed that MC-LR is more toxic to normal kidney cells than to cancerous cells, explaining the different behaviour of the two cell lines. Further studies are needed to evaluate the toxic mechanisms underlying these differences.

In conclusion, the present study provides direct evidence that MC-LR exposure can induce apoptosis-related morphological changes, can upregulate expression of the *Bax* and *p53* genes, can modulate expression of the *Survivin*

gene, and increase activity of caspase-3 and caspase-9 in HEK-293 and ACHN cells. Hence, *Bax*, *p53*, *Survivin*, caspase-3 and caspase-9 are most likely to be involved in MC-LR-induced cellular damage. Moreover, this study contributes to elucidating the toxicological mechanism underlying the effects of MC-LR on the human kidney. Understanding the detailed toxicological role of MC-LR in apoptosis-related signalling pathways will require further investigations.

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