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Role of nitric oxide in the genotoxic response to chronic microcystin-LR exposure in human–hamster hybrid cells

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ABSTRACT

Microcystin-LR (MC-LR) is the most abundant and toxic microcystin congener and has been classified as a potential human carcinogen (Group 2B) by the International Agency for Research on Cancer. However, the mechanisms underlying the genotoxic effects of MC-LR during chronic exposure are still poorly understood. In the present study, human–hamster hybrid (A_L) cells were exposed to MC-LR for varying lengths of time to investigate the role of nitrogen radicals in MC-LR-induced genotoxicity. The mutagenic potential at the *CD59* locus was more than 2-fold higher ($p < 0.01$) in A_L cells exposed to a cytotoxic concentration (1 μmol/L) of MC-LR for 30 days than in untreated control cells, which was consistent with the formation of micronucleus. MC-LR caused a dose-dependent increase in nitric oxide (NO) production in treated cells. Moreover, this was blocked by concurrent treatment with the NO synthase inhibitor N^G-methyl-L-arginine (L-NMMA), which suppressed MC-LR-induced mutations as well. The survival of mitochondrial DNA-depleted (ρ^0) A_L cells was markedly decreased by MC-LR treatment compared to that in A_L cells, while the *CD59* mutant fraction was unaltered. These results provided clear evidence that the genotoxicity associated with chronic MC-LR exposure in mammalian cells was mediated by NO and might be considered as a basis for the development of therapeutics that prevent carcinogenesis.

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Introduction

The distribution of toxic cyanobacterial blooms in eutrophic rivers, lakes, reservoirs, and recreational waters worldwide has increased in recent years to become a global problem threatening human health and ecosystem safety. Cyanobacterial toxins are diverse in their chemical structure and are assigned to different substance classes such as hepatotoxins (microcystins and nodularins), neurotoxins (saxitoxins and anatoxins), cytotoxins (cylindrospermopsin), and irritant toxins (lipopolysaccharides) (Pearson et al., 2010). Of more than 100 variants, microcystin-LR is the most common and toxic congener, and has been classified as a potential human carcinogen (Group 2B) by the International Agency for Research on Cancer (Grosse et al., 2006). The general population is exposed to microcystin-LR (MC-LR) through natural water and food consumption and is vulnerable to the risks associated with long-term exposure (Chen et al., 2009). Experimental studies and epidemiologic data have linked MC-LR exposure to the development of liver

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and colorectal cancers (Yu, 1995; Zhou et al., 2002), possibly caused by an MC-LR-induced increase in the expression of tumor necrosis factor-α (Fujiki and Suganuma, 2011).

Various quantitative measurement of genotoxicity such as DNA strand breaks and mutations have been used to assess the carcinogenic potential of MC-LR, which remains controversial. MC-LR had no effect on the induction of micronuclei (MNs) in CHO-K1 cells (Lankoff et al., 2006b) and on the frequency of chromosomal aberration in lymphocytes (Lankoff et al., 2006a). Meanwhile, another study found that MN frequency was approximately 3.8-fold higher in human lymphoblastoid thymidine kinase (TK) 6 cells exposed to MC-LR at a concentration of 80 μg/mL for 24 hr than in control cells (Zhan et al., 2004). In human lymphocytes, the mitotic index was increased by MC-LR treatment in a dose- and time-dependent manner (Lankoff et al., 2004b). MC-LR-treated WRL-68 cells exhibited chromosomal instability and a high incidence of aneuploidy (Xu et al., 2012), and the potential carcinogenicity of MC-LR has also been suggested from *in vivo* studies (Ito et al., 1997). Mutagenic studies of MC-LR using the reverse Ames assay in tester strains TA98, TA100, and TA102 and the *Bacillus subtilis* multigene sporulation test using the 168 and hcr-9 strains have shown that MC-LR is inactive or only weakly active (Repavich et al., 1990). However, in another study, MC-LR induced a base substitution in codon 12 of *K-Ras* and ouabain resistance mutations in human RSa cells at concentrations greater than 15 μg/mL (Suzuki et al., 1998), as well as the loss of heterozygosity at the *TK* locus in human lymphoblastoid TK6 cells at greater than 20 μg/mL (Zhan et al., 2004). It should be noted that the concentration of MC-LR in hypereutrophic water bodies is less than 50 ng/mL (Nasri et al., 2007), but is nearly 1 μg/g in freshwater mussels due to bioaccumulation (Kann et al., 2010). Although alterations in microRNA expression in human WRL-68 cells were shown to play a critical role in MC-LR-induced carcinogenesis during chronic exposure (Xu et al., 2012), the underlying mechanisms are not well understood.

In addition to the well-documented interaction of protein phosphatases PP1 and PP2A, oxidative stress induced by MC-LR is considered as a key determinant of MC-LR-mediated genotoxicity (Zegura et al., 2008). The involvement of reactive oxygen species (ROS) and the protective effects of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione-*S*-transferase have been investigated in various cell lines and in the animal liver, heart, and reproductive system (Li et al., 2008; Xiong et al., 2010). ROS, especially the hydroxyl radical ($OH^·$), directly or indirectly damage neighboring biomolecules such as DNA and membrane lipids (Ziech et al., 2010). There is also evidence that MC-LR induces oxidative DNA damage in HepG2 cells (Zegura et al., 2003), as well as the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in primary cultured rat hepatocytes and liver (Maatouk et al., 2004). $OH^·$ is a intracellular oxidant likely induced by MC-LR, but is so highly reactive that it can only diffuse a short distance from the site of formation; others may include reactive nitrogen species (RNS). MC-LR stimulates nitric oxide (NO) production in a dose-dependent manner in rat insulinoma (INS-1) cells (Ji et al., 2011). In the presence of superoxide anion (O_2^{-}), NO may form the more reactive and toxic peroxynitrite, which contributes to the nitration of proteins, hydroxylation or nitration of DNA, and mutations (Juedes and Wogan, 1996; Ducrocq et al., 1999). Although it has been postulated that NO and ROS cooperatively mediate the toxic effects of MC-LR, the origin of these species and the pathways involved in the generation of other secondary radicals remain to be elucidated.

Human–hamster hybrid (A_L) cells, which contain a full set of hamster chromosomes and a single copy of human chromosome 11, are sensitive to mutagens, such as ionizing radiation and certain chemical agents, that induce large multilocus deletions (Waldren et al., 1986; Hei et al., 1998). Since only a small part of 11p15.5 is required for the viability of A_L cells, chromosomal deletions up to 140 Mb can be detected in these cells. In the present study, A_L cells were exposed to a range of doses of MC-LR for varying time periods and then assayed for genotoxicity-related endpoints. Mitochondrial DNA encodes a nitric oxide synthase (NOS) that is a major source of NO (Giulivi et al., 1998), the role of mitochondria in MC-LR-induced mutagenesis was assessed in mitochondrial DNA-depleted (ρ^0) A_L cells.

1. Materials and methods

1.1. Cell culture

A_L hybrid cells, which contain a set of CHO-K1 chromosomes and a single copy of human chromosome 11, were used in the experiments. Chromosome 11 encodes cell surface markers that confer A_L cells with sensitivity to the cytotoxic effects of specific monoclonal antibodies in the presence of rabbit serum complement (Merck, Darmstadt, Germany) (Hei et al., 1998). Antibody E7.1 specific to the *CD59* antigen was produced from hybridoma cultures. A_L cells were cultured in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum (FBS), 25 μg/mL gentamicin, and 2×10^{-4} mol/L glycine. ρ^0 A_L cells were generated by treating A_L cells with the chemotherapeutic drug ditercalinium over a period of 3–4 months to deplete mitochondrial DNA by 95% (Liu et al., 2005). ρ^0 A_L cells were cultured in Dulbecco's Modified Eagle's Medium/F12 (1:1) supplemented with 15% heat-inactivated FBS, 2.7 g/L glucose, 584 mg/L glutamine, 50 μg/mL uridine, 25 μg/mL gentamicin, and 2×10^{-4} mol/L glycine. All cells were maintained at 37°C in a humidified 5% CO_2 incubator and passaged every 3 days.

1.2. MC-LR preparation and chemical treatment

A stock solution of MC-LR (Alexis Biochemicals, Lausanne, Switzerland) was prepared at a concentration of 50 μmol/L with doubled-distilled water and sterilized by passage through a 0.22-μm pore size syringe filter. Working solutions were diluted from the stock solution with complete F-12 medium. A_L and ρ^0 A_L cells in the exponential growth phase were incubated with 0.01–1 μmol/L MC-LR in 60-mm diameter petri dishes for varying time periods, with untreated time-matched control cells grown concurrently. Treated cells were subcultured every 3 days for up to 30 days with fresh medium containing MC-LR. After treatment, cells were washed twice with balanced salt solution and processed for various endpoints. In experiments involving nitric oxide synthase (NOS) inhibition, N^G-methyl-L-arginine (L-NMMA, Molecular Probes, Inc., Eugene, OR, USA) dissolved in doubled-distilled water and filter-sterilized was added to cultures concurrently with MC-LR for 1 or 3 days; during the 30-day treatment, confluent cells were subcultured with fresh medium containing MC-LR and L-NMMA every 3 days. After treatment, cells were collected for further analysis.

1.3. Cell survival assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of MC-LR (Mosmann, 1983). A_L and ρ^0 A_L cells in the exponential

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growth phase were trypsinized and replated in 60-mm dishes at 1×10^5 cells/dish for 48 hr. Cultures were treated with 0.01–1 μmol/L MC-LR for 1, 3, or 30 days. After treatment, the growth medium was replaced with MTT working solution (1 g/L in medium) and cells were further incubated for 4 hr at 37°C. The medium was removed, and 1 mL dimethyl sulfoxide was added to dissolve the formazan crystals. The optical density was measured at 490 nm using a Spectra Max M2 fluorescence reader (Molecular Devices, Sunnyvale, CA, USA).

1.4. Quantification of mutations at the CD59 locus

After treatment with MC-LR and L-NMMA, the medium was removed and cells were washed three times with phosphate-buffered saline (PBS). A_L and ρ^0 A_L cells were then replated in 60-mm dishes and cultured in fresh complete medium for 7 days. This was necessary for surviving cells to recover from the temporary growth lag caused by MC-LR treatment and multiply sufficiently so that the progeny of mutated cells ceased to express lethal amounts of the *CD59* surface antigen. A total of 5×10^4 cells/dish were plated in six 60-mm dishes in 2 mL growth medium as described (Hei et al., 1992) and incubated for 2 hr to allow cell attachment, after which 0.2% antiserum and 1.5% (*V*/*V*) freshly thawed complement were added to each dish. Cultures were incubated for 8 more days; then, they were fixed and stained, and the number of *CD59* mutant colonies was scored. Controls included identical sets of dishes containing antiserum or complement alone, or neither. The mutant fraction (MF) at each dose was calculated as the number of surviving colonies divided by the total number of cells plated after correction for any nonspecific cytotoxicity due to complement alone.

1.5. MN assay

The frequency of MN formation was measured with the cytokinesis-block technique (Fenech, 2000). After treatment with MC-LR, 3×10^4 A_L cells were seeded in 35-mm dishes and incubated for 2 hr. The growth medium was replaced with fresh complete medium containing 2.5 μg/mL cytochalasin B (Sigma, St. Louis, MO, USA), followed by incubation for 30 hr. As a positive control, 3 μmol/L mitomycin C was instead added for 24 hr. Cells were rinsed in PBS, fixed in a solution of methanol:acetic acid (*V*/*V* = 9:1), and stained with 0.01% (*W*/*V*) acridine orange for 5 min before MNs in binucleated cells were visualized using a fluorescence microscope (Olympus, Tokyo, Japan) and morphologically evaluated (Fenech and Morley, 1986). At least 1000 binucleated cells were scored in each experiment for each data point to measure the frequency of MN induction. To assess cell proliferation, the cytokinesis-block proliferation index (CBPI) was calculated from 500 cells per culture as the following equation:

$$\mathrm{CBPI} = N_{\mathrm{MNCs}} + 2N_{\mathrm{BNCs}} + 3N_m/N_t$$

where, N_{MNCs} is the number of mononucleated cells (MNCs); N_{BNCs} is the number of binucleated cells (BNCs); N_{m} is the number of multinucleated cells, and N_{t} is the total number of cells.

1.6. Nitric oxide assay

The generation of NO was monitored with the fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate (Molecular Probes, Inc., Eugene, OR, USA), which shows a stable and intense fluorescence signal across a wide range of pH values after reacting with NO (Kojima et al., 1998). A_L cells were seeded at the same density of 8×10^4 cells/well in 96-well microtiter plates after treatment for the specified durations. Confluent cultures were washed with Tyrode solution at 37°C for 2 min and then stained with 5 μmol/L DAF-FM diacetate prepared at 10 mmol/L in saline buffer containing 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $MgCl_2$, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethanes-ulphonic acid, 10 mmol/L glucose, 6 mmol/L sucrose, and 2 mmol/L $CaCl_2$ for 30 min at 37°C, followed by washing with cold tyrode solution twice. After the excess probe was removed, cells were washed three times with cold Tyrode solution. Fluorescence was measured with a Spectra Max M2 fluorescence reader (Molecular Devices, Sunnyvale, CA, USA) at excitation/emission wavelengths of 495/515 nm.

1.7. Statistical analysis

Data were pooled from at least three independent experiments and were presented as mean ± S.D. Differences in the percentage of micronucleated cells measured by MN test in the test groups *versus* the vehicle control groups were analyzed statistically using Chi-square test. Multiple comparisons between the groups were performed using Bonferroni correction, which sets the significance cut-off at 0.05/4 (α/n, the significance level for each test). Differences for the measurement data in other tests were assessed by one-way analysis of variance with a post hoc least significant difference test. The $p < 0.05$ was considered statistically significant.

2. Results

2.1. Chronic exposure to MC-LR has lethal effects

The MTT assay was used to monitor the cytotoxic effects of MC-LR in A_L and ρ^0 A_L cells exposed to MC-LR for varying lengths of time. There was no change in the survival fraction of A_L cells at MC-LR concentrations ranging from 0.01 to 1 μmol/L after 1 day (Fig. 1a); however, after a 3-day exposure at 1 μmol/L, cell viability was significantly reduced to 89.2% ±1.39%, compared to the untreated cells ($p < 0.01$). After exposure to 0.1 and 1 μmol/L MC-LR for 30 days, the viability of A_L cells was decreased to 86.67% ± 5.59% ($p < 0.05$) and 73.27% ± 4.91% ($p < 0.01$), respectively, compared to the control cells. These data indicate that chronic exposure to low levels of MC-LR reduces viability in A_L cells in a time- and concentration-dependent manner. ρ^0 A_L cells exhibited even greater sensitivity to the cytotoxic effects of MC-LR (Fig. 1b): upon exposure to 1 μmol/L MC-LR for 1 day, cell viability was decreased to 83.77% ± 3.64% compared to the control cells ($p < 0.01$); When AL cells were exposed to 1 μmol/L MC-LR for 3 and 30 days, cell viability was further reduced to 80.3% ±2.55% and 59.1% ± 7.97% ($p < 0.01$).

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2.2. Mutations at the CD59 locus are induced by chronic MC-LR exposure

To assess the mutagenic potential of MC-LR, A_L cells were exposed to graded concentrations of MC-LR for specified durations. The normal plating efficiency of A_L cells was about 80% ± 5%, and the background mutation frequencies after 1, 3, and 30 days of exposure were 43 ± 22, 65 ± 28, and 109 ± 17 mutants per 10^5 survivors, respectively. The MF was increased slightly compared to controls upon exposure to 1 μmol/L MC-LR for 1 or 3 days (Fig. 2); after a 30-day exposure, the number of mutants induced by 0.1 μmol/L or 1 μmol/L MC-LR was increased to 230 ± 143 and 249 ± 24 mutants per 10^5 survivors, respectively. The mutation frequency at the *CD59* locus induced by a 30-day treatment of 1 μmol/L MC-LR was more than 2-fold higher than non-treated controls ($p < 0.01$). These data suggest that chronic exposure to MC-LR is mutagenic in mammalian cells.

2.3. MN formation is induced by chronic MC-LR exposure

The genotoxicity of MC-LR was evaluated by the MN assay. Fractions of binucleated cells with MNs and associated parameters are shown in Table 1. Binucleated cells were observed at frequencies of 71.23%, 65.12%, and 62.48% in control cells cultured for 1, 3, and 30 days, respectively. MN fractions of A_L cells remained unchanged when exposed to MC-LR for 1 or 3 days. However, for a 30-day exposure, MN fractions were increased from 1.21% ± 0.31% in the control group to 1.95% ± 0.58% for 0.1 μmol/L and 2.33% ± 0.56% for 1 μmol/L MC-LR. The increase in MN induction was more than 1.9-fold higher in A_L cells exposed to 1 μmol/L MC-LR for 30 days than in control cells ($p < 0.01$). In addition, a CBPI value of 1.0 indicates that all cells are mononucleated representing 100% cytotoxicity of the test agent (OECD, 2010). Our present study showed that the CBPI values induced by MC-LR did not change significantly with dose and time.

2.4. MC-LR-induced mutagenesis is caused by RNS

NOSs are hemoproteins with a cytochrome P450-like active site that catalyzes the oxidation of arginine to NO and citrulline (White and Marletta, 1992). To evaluate the contribution of NO to MC-LR-induced mutagenesis, A_L cells were exposed to MC-LR for 30 days with or without L-NMMA, which competitively blocks the activity of NOSs in cells (Hong et al., 2010). When A_L cells were concurrently treated with 1 μmol/L MC-LR and 0.2 mmol/L L-NMMA, the number of mutants was decreased to 134 ± 18 mutants per 10^5 survivors from 283 ± 75 mutants per 10^5 survivors with 1 μmol/L MC-LR alone. The mutation frequency was decreased by more than 2-fold ($p < 0.05$) in A_L cells (Fig. 3), while L-NMMA alone had little effect after a 30-day treatment ($p > 0.05$).

The fluorescent probe DAF-FM diacetate was used to quantify the production of NO in A_L cells exposed to MC-LR. The increase in NO level upon MC-LR treatment was dose-dependent and independent of exposure time (Fig. 4a), and was observed at ≥0.1 μmol/L MC-LR ($p < 0.05$); this was suppressed in cells treated simultaneously with 1 μmol/L MC-LR and 0.2 mmol/L L-NMMA (Fig. 4b). These results suggest that the mutagenic effects of chronic MC-LR exposure are linked to the generation of RNS, especially NO.

2.5. MC-LR-induced mutagenesis results from mitochondrial NO production

The role of mitochondria in MC-LR-induced mutagenesis was investigated in ρ^0 A_L cells exposed to 1 μmol/L MC-LR for 30 days. The background MF of ρ^0 A_L cells was 1562 ± 143 mutants per 10^5 survivors; treatment with 1 μmol/L MC-LR had little effect (Fig. 5). In contrast, the MF was increased by more than 2-fold in A_L cells treated with 1 μmol/L MC-LR relative to non-treated controls ($p < 0.01$). These results suggest that the mutagenic effects of MC-LR are exerted by NO produced by mitochondrial NOSs.

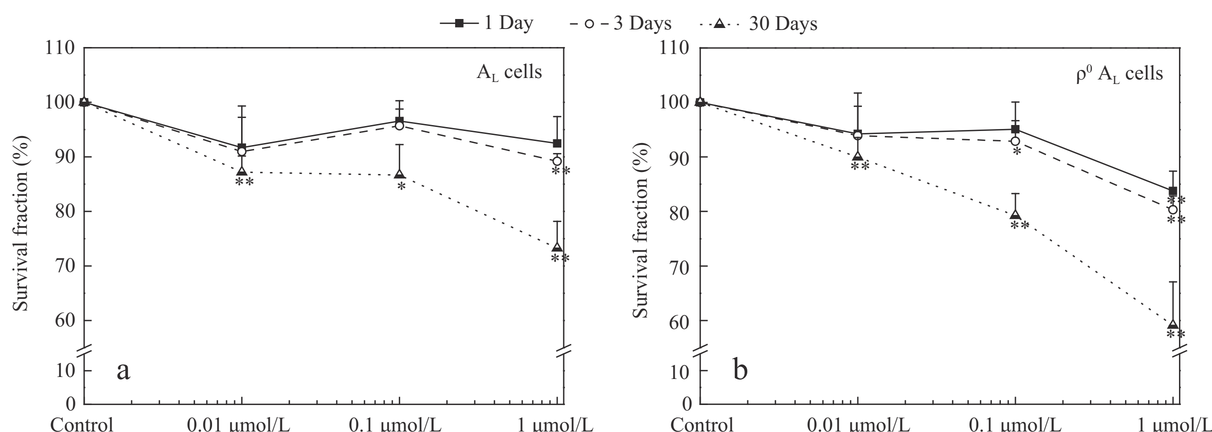

Fig. 1 – Effect of microcystin-LR (MC-LR) on cell survival. A_L (a) and ρ^0 A_L (b) cells in the exponential growth phase were treated with the indicated concentrations of MC-LR for 1, 3, or 30 days, and the survival fraction was calculated. Data were pooled from three independent experiments and represented mean ± S.D. *$p < 0.05$, and **$p < 0.01$.

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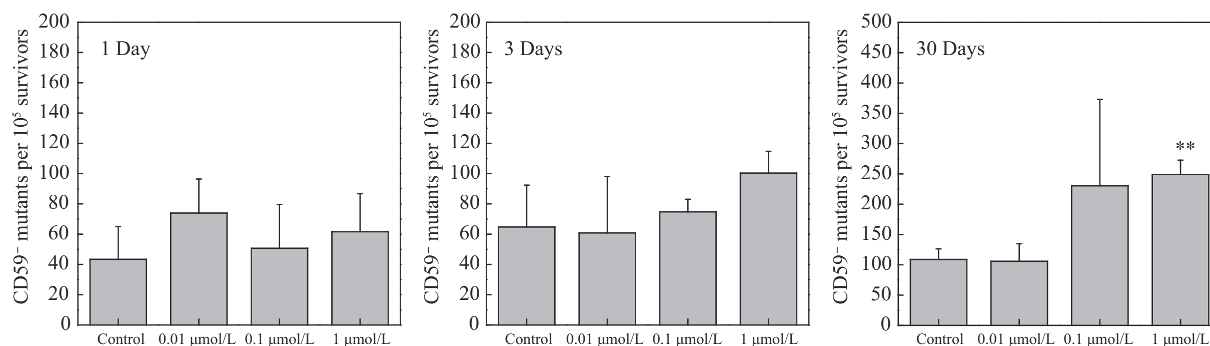

Fig. 2 – Frequency of *CD59⁻* mutants per 10^5 survivors in A_L cells exposed to MC-LR. Cells in the exponential growth phase were treated with the indicated concentrations of MC-LR for 1, 3, or 30 days. Data were pooled from six independent experiments and represented mean ± S.D. $^*p < 0.05$, $^{**}p < 0.01$.

3. Discussion

As MC-LR is the most widespread cyanotoxin in brackish and freshwater blooms, chronic MC-LR exposure may be more hazardous to human and animal health than other isomers produced by cyanobacteria. MC-LR can act as a potent tumorigenic agent (Ito et al., 1997) or genotoxic carcinogen (Zegura et al., 2008). Epidemiological studies have suggested that MC-LR is a risk factor for primary liver cancer (Ueno et al., 1996), which has a high incidence in certain areas of China. However, there is little information on the genotoxic risk to humans who may be chronically exposed to MC-LR in the drinking water.

Studies on MC-LR cytotoxicity have mostly focused on mammalian liver cells and cultured hepatocytes (Svircev et al., 2010); one study reported that MC-LR at concentrations greater than 1 μmol/L activated apoptosis-related signaling pathways in rat hepatocytes (Ding et al., 2002). However, liver is not the only target of MC-LR *in vivo*. The viability of CaCo-2 cells was also significantly reduced when exposed to MC-LR at the concentrations of 10, 50, or 100 μmol/L for 24 hr (Huguet et al., 2013), while proliferation was inhibited in T-cells at 25 μg/mL and in B-cells at 1, 10, and 25 μg/mL (Lankoff et al., 2004a). A 24-h treatment with MC-LR had concentration-dependent cytotoxic effects starting at 20 μg/mL in human lymphoblastoid TK6 cells, affecting relative survival and relative suspension growth (Zhan et al., 2004). Treatment with 10 and 20 μg/mL MC-LR enhanced early apoptosis in CHO-K1 cells in a dose-dependent manner (Lankoff et al., 2006b). In contrast, 0.01–1 μg/mL MC-LR was not cytotoxic to the HepG2 human hepatoma cell line, as determined by the

Table 1 – Effects of MC-LR on binucleated cells with micronuclei (BNMN), cytokinesis-block proliferation index (CBPI) in A_L cells.

Treatment group		Parameters					
MC-LR dose (μmol/L)	Treatment time (day)	Mononucleated cells	Binucleated cells	Trinucleated cells	Quadrinucleated cells	BNMN	CBPI
Control	1	144	364	2	1	9.9 ± 0.65	1.758 ± 0.05
	3	176	336	3	1	10.17 ± 0.91	1.721 ± 0.066
	30	189	318	1	1	12.07 ± 3.07	1.663 ± 0.039
0.01 μmol/L	1	156	351	3	1	10.6 ± 0.4	1.741 ± 0.032
	3	187	327	4	1	9.17 ± 1.17	1.71 ± 0.045
	30	182	327	2	0	12.93 ± 1.45	1.683 ± 0.064
0.1 μmol/L	1	154	350	3	1	10.93 ± 1.61	1.726 ± 0.032
	3	175	338	4	1	9 ± 1.32	1.73 ± 0.036
	30	188	322	1	0	19.5 ± 5.79	1.667 ± 0.033
1 μmol/L	1	145	359	3	1	11 ± 1.3	1.751 ± 0.046
	3	188	325	1	0	11.1 ± 0.7	1.683 ± 0.034
	30	200	308	3	0	23.33 ± 5.55 *	1.652 ± 0.033
3 μmol/L MMC	1	252	260	1	0	53.7 ± 6.67 **	1.553 ± 0.063 *

Cells in the exponential growth phase were exposed to the indicated concentrations of MC-LR for 1, 3, or 30 days. BNMN and CBPI were determined by the occurrence of micronucleated cells and cytokinesis block in proliferating cells, respectively. MMC: mitomycin C. Data were pooled from three independent experiments and represented mean ± S.D.

* $p < 0.05$

** $p < 0.01$

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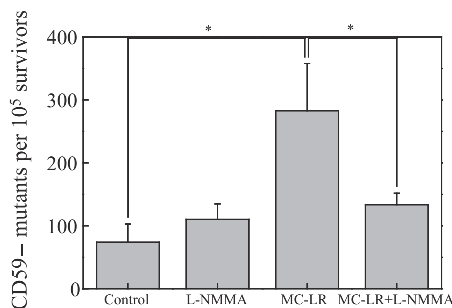

Fig. 3 – Mutagenic effect of nitric oxide synthase (NOS) inhibition in A_L cells. Cells were exposed to 1 μg/mL MC-LR with or without 0.2 mmol/L N^G-methyl-L-arginine (L-NMMA) for 30 days. Data were pooled from three independent experiments and represented mean ± S.D. *$p < 0.05$.

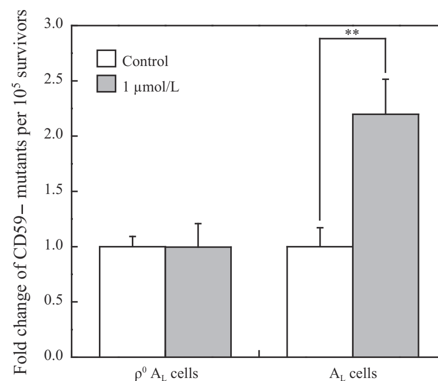

Fig. 5 – Frequency of *CD59*⁻ mutants per 10^5 survivors in A_L and $ρ^0$ A_L cells exposed to MC-LR. Cells in the exponential growth phase were treated with 1 μg/mL MC-LR for 30 days. Data were pooled from three independent experiments and represented mean ± S.D. **$p < 0.01$.

MTT assay (Zegura et al., 2003). The data presented here showed that mammalian cell viability was decreased in the presence of MC-LR, with the effects slightly less in A_L than in $ρ^0$ A_L cells (Fig. 1).

MC-LR induces ROS production, which can result in oxidative DNA damage (Zegura et al., 2003). Acute exposure to high concentrations of MC-LR can lead to large deletions, homologous recombination, and rearrangements (Maatouk et al., 2004; Zhan et al., 2004), and results in chromosomal breakage in bacterial systems (Repavich et al., 1990). Large deletions caused by mutagens such as radiation and arsenite have been readily identified by examination of the *CD59* locus of A_L cells (Hei et al., 1988, 1992). *CD59* mutants were detected at a higher frequency in A_L cells chronically exposed to 1 μmol/L MC-LR than in controls (Fig. 2), which was associated with the occurrence of MNs (Table 1), demonstrating the genotoxicity of MC-LR during chronic exposure. The number of MNs in CHO-K1 cells treated with 1, 10, and 20 μmol/L MC-LR for 6 hr and control cells was previously shown to be similar (Lankoff et al., 2006b). However, in the present study, the number of MNs was markedly increased after treatment with 1 μmol/L MC-LR for 30 days (Table 1), suggesting that MN occurrence is associated with prolonged rather than short-term treatment. A molecular analysis indicated that most TK mutations induced by MC-LR resulted from the loss of heterozygosity (Zhan et al., 2004), and MC-LR also suppressed radiation-induced DNA damage repair (Lankoff et al., 2004b). These observations implicated that chromosome loss might be the direct cause of DNA mutagenesis induced by MC-LR.

NO is an inter/intracellular signaling molecule that regulates various physiological processes such as neural transmission (Ignarro, 1990) and immune response (Wink et al., 2011). NO is mutagenic in TK6 cells (Nguyen et al., 1992),

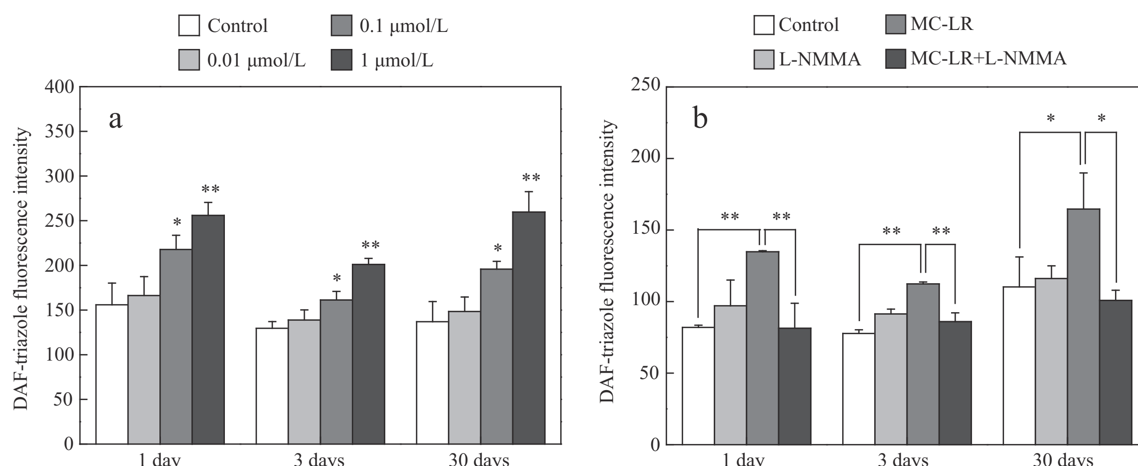

Fig. 4 – Effect of MC-LR on NO production in A_L cells. Fluorescence intensity was measured for 4-amino-5-methylamino-2′,7′-difluoro fluorescein (DAF-FM) diacetate-loaded cells in the exponential growth phase after exposure to indicated concentrations of MC-LR for 1, 3, or 30 days (a); and 1 μg/mL MC-LR and/or 0.2 mmol/L L-NMMA for 1, 3, or 30 days (b). Data were pooled from three independent experiments and represented mean ± S.D. *$p < 0.05$, **$p < 0.01$.

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causing A:T to G:C transversions in plasmids (Routledge et al., 1993) and C to T transitions in a bacterial system (Wink et al., 1991). Here, NO production was stimulated dose-dependently by MC-LR irrespective of treatment time (Fig. 4a), consistent with results from a previous study in which NO level was upregulated in INS-1 cells exposed to MC-LR (Ji et al., 2011).

MC-LR exerts immunosuppressive effects on peritoneal macrophages of BALB/c mice *via* inhibition of inducible *(i)NOS* mRNA expression (Chen et al., 2005). However, MC was also found to activate lipopolysaccharide (LPS)- and cytokine-mediated *iNOS* expression and NO production in rat primary astrocytes, while inhibiting these processes in resident macrophages (Pahan et al., 1998). These results demonstrate that inhibitors of protein phosphatases 1 and 2A differentially regulate *iNOS* expression depending on cell type. The generation of NO (Fig. 4b) and increase in the *CD59* MF (Fig. 3) induced by chronic MC-LR exposure were suppressed by L-NMMA, a competitive NOSs inhibitor, indicating that NO mediates MC-LR-induced genotoxicity in mammalian cells. While mutagenesis plays an important role in tumor development, the contribution of NO merits further investigation *in vivo*. It is also of interest that NO production was detected in A_L cells after exposure to MC-LR for 1 day, while mutations were detected only after 30 days (Fig. 2). This may be due to the fact that NO is induced soon after MC-LR treatment through the activities of organelle-specific NOSs isoforms (Giulivi et al., 1998; Chen et al., 2005; Ji et al., 2011). In contrast, mutations typically arise over a longer term and are dependent on multiple factors, including chronic oxidative stress and failure of the DNA damage response (Jena, 2012). Chronic exposure to mutagenic agents disrupts the balance between lesion formation and DNA repair processes, resulting in gene mutations (Nguyen et al., 1992).

Mitochondria are the major producers of cellular energy and intracellular sources of free radicals (Lee and Wei, 2000). Previous studies have shown that mitochondria are targeted by cellular responses to MC-LR such as apoptosis and oxidative stress (Ding and Nam Ong, 2003). Functional alterations in mitochondria are usually manifested in the mitochondrial membrane potential and mitochondrial permeability transition; changes in these parameters have been observed in MC-LR-induced toxicity, which result in cytochrome c release, calmodulin activation, and hepatocyte apoptosis (Ding et al., 2002). NO is also an important trigger for mitochondria to exert their physiological functions (Giulivi et al., 1998; Chen et al., 2008). In the present study, MC-LR-induced mutagenicity was blocked in mitochondrial DNA-depleted (ρ^0) A_L cells (Fig. 5). Although ditercalinium treatment to achieve DNA depletion increased the background MF, ρ^0 A_L cells had a clonogenic efficiency similar to that of wild-type cells and were not sensitive to the mutagenic effects of MC-LR. However, ρ^0 A_L were more sensitive than wild-type cells to MC-LR-induced cytotoxicity (Fig. 1). Although increases in intracellular oxidant levels are mediated by other cytosolic components including lysosomes, endoplasmic reticulum, and membrane-bound nicotinamide adenine dinucleotide phosphate oxidase (Shafique et al., 2013), the results from ρ^0 A_L cells demonstrate that mitochondrial function is involved in MC-LR-induced genotoxicity in mammalian cells.

4. Conclusions

Although MC-LR has been regarded as the most abundant and toxic microcystin congener, the genotoxic mechanisms of MC-LR during chronic exposure are not well understood. Chronic MC-LR exposure increased MF and MN formation in A_L cells. In addition, the genotoxic effects of MC-LR were mediated by NO and mitochondria. However, the mechanisms underlying these processes in MC-LR-induced genotoxicity needs to be further elucidated, which will provide a basis for better understanding the carcinogenic target of MC-LR and making interventional approach in prevention.

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