

In vivo study on the effects of microcystin extracts on the expression profiles of proto-oncogenes (*c-fos*, *c-jun* and *c-myc*) in liver, kidney and testis of male Wistar rats injected i.v. with toxins

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ABSTRACT

Microcystins (MCs) are a potent liver tumor promoter, possessing potent tumor-promoting activity and weak initiating activity. Proto-oncogenes are known to be involved in the tumor-promoting mechanisms of microcystin-LR. However, few data are available on the effects of MCs on proto-oncogenes in the whole animal. To investigate the effects of MCs on the expression profile of the proto-oncogenes in different organs, male Wistar rats were injected intravenously with microcystin extracts at a dose of 86.7 µg MC-LR eq/kg bw (MC-LR eq, MC-LR equivalents). mRNA levels of three proto-oncogenes *c-fos*, *c-jun* and *c-myc* in liver, kidney and testis were analyzed using quantitative real-time PCR at several time points post-injection. Significant induction of these genes at transcriptional level was observed in the three organs. In addition, the increase of mRNA expression of all three genes was much higher in liver than in kidney and testis. Meanwhile, the protein levels of c-Fos and c-Jun were investigated by western blotting. Both proteins were induced in the three organs. However, elevations of protein levels were much lower than those of mRNA levels. These findings suggest that the expression of *c-fos*, *c-jun* and *c-myc* might be one possible mechanism for the tumor-promoting activity and initiating activity of microcystins.

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1. Introduction

The hepatotoxic microcystins (MCs) are cyclic heptapeptides produced by several cyanobacterial genera. They are widely distributed throughout the world in freshwater bodies, and have been a serious threat to public health. More than 80 microcystin variants have been detected to date, among which MC-LR and MC-RR are the most common and abundant microcystins (Dietrich and Hoeger, 2005). MCs primarily acted as hepatotoxins (Dawson, 1998), for they were predominantly absorbed, transported and accumulated into liver (Dahlem et al., 1989). However,

accumulation of MCs has also been reported in intestine, kidney, brain, heart, gonad and muscles of fish or mammals (Kankaanpää et al., 2005; Cazenave et al., 2005; Adamovský et al., 2007; Kagalou et al., 2008). Moreover, there was evidence about neurological or renal toxicity of MCs in vertebrates (Dietrich and Hoeger, 2005). Recently, MCs were also found to be toxic to the reproductive systems of male mice (Ding et al., 2006).

MC-LR or cyanobacterial extracts in drinking water could induce skin tumors in rats and mice after initiation with 7,12-dimethylbenz(a)anthracene (Fujiki et al., 1989; Falconer, 1991). In addition, glutathione-S-transferase placental form positive foci were detected in liver of rats after intraperitoneal injection of MC-LR and initiation with diethylnitrosamine (Fujiki, 1992; Nishiwaki-Matsushima et al., 1992). Moreover, there was an indication that MCs

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might also act as tumor initiators (Ito et al., 1997). Therefore, chronic exposure to low concentration of MCs in drinking water may be a serious hazard to public health, contributing to promotion of cancer in humans. The epidemiological survey of Yu (1989) has indicated that high concentrations of MCs in the drinking water were correlated both with high-endemic areas of liver cancer and with the number of hepatocellular carcinoma patients. Recent studies in China demonstrated that populations drinking MC-contaminated surface and well water had a higher risk for rectal and colon cancers than those drinking municipal groundwater (Ueno et al., 1996; Zhou et al., 2002). However, the molecular mechanisms by which MCs cause the susceptibility to carcinogenesis are not well understood.

In carcinogenesis studies, the induction of the expression of proto-oncogenes has been shown to be involved in promoting the tumor activity (Defelipe and Hunt, 1994; Brecht et al., 1995; Hayashi et al., 2000). *c-Fos* and *c-Jun* are two important members of proto-oncogenes, and they are the major components of the transcriptional activator complex AP-1, which is composed of homodimers and heterodimers of the *Jun* and *Fos* proteins. Numerous studies have intensively investigated the induction mechanism of *c-fos* and *c-jun* by tumor promoters (Herschman, 1991). Previous studies had also provided the evidences that MC-LR induced expression of proto-oncogenes in primary cultured rat hepatocytes and zebra fish (Sueoka et al., 1997; Wei et al., 2008). The tumor-promoting activity of MCs was suggested to arise from its inhibition of PP2A (Nishiwaki-Matsushima et al., 1992; Gehringer, 2004), which negatively regulated several mitogen-activated protein kinases (MAPK). Thereby, MCs could activate MAPK by inhibition of PP2A, and the activated MAPK would activate proto-oncogene such as *c-jun* and *c-fos*, which initiate transcription of genes necessary for growth and differentiation (Gehringer, 2004). However, more detailed studies are necessary on both mRNA and protein level *in vivo* to examine whether it is the case.

In order to further fully understand the potent tumor promoter or carcinogenic mechanism of MCs, we intravenously injected rat with microcystin extracts (which are more close to the constitution of MCs in natural environments) and investigated the expressions of three proto-oncogenes (*c-jun*, *c-fos* and *c-myc*) in liver, kidney and testis. The result showed the significant raise of mRNA synthesis of *c-jun*, *c-fos* and *c-myc* in the three organs. The liver got more induction than other two tissues. But the protein level of *c-Jun* and *c-Fos* was only moderately elevated.

2. Materials and methods

2.1. Toxin

MCs (purity >85%) were isolated and purified from the surface blooms collected from Lake Dianchi, Yunnan, China. Freeze-dried algal cells were extracted three times with 75% methanol (V/V). The extract was centrifuged and the supernatant was applied to a C18 reversed phase cartridge, which had been firstly washed with 100% methanol and

then washed with distilled water. The cartridge was then washed with water and eluted with methanol. The elution was evaporated to dryness and the residue was dissolved in distilled water for the toxic experiment. MCs content in the extracts was determined by high performance liquid chromatography (HPLC, LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). The microcystin content was 80.5 μg MC-LR eq/ml, among which MC-RR and -LR were 167.7 $\mu\text{g}/\text{ml}$ and 47.0 $\mu\text{g}/\text{ml}$, respectively. Crude microcystin extracts were finally suspended in salt solution water (0.9% NaCl).

2.2. Animals

Male Wistar rats (8 weeks of age, weighing 200 ± 20 g) were obtained from the Center for Disease Control of Hubei Province, China. The rats were kept in stainless steel cages under 20 °C with a 12 h light–dark cycle, and given free access to standard rodent pellet diet and water. All procedures carried out on animals were approved by the Institutional Animal Care and Use Committee, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory.

2.3. LD₅₀ of cyanobacterial crude extracts containing MCs

The intravenous (i.v.) lethal dose 50 (LD₅₀) of cyanobacterial crude extract containing MCs in rat was determined according to the up–down method of Fawell et al. (1999).

2.4. MCs exposure and sample collection

Sixty healthy male Wistar rats weighing 200 ± 20 g were divided into two groups randomly. One group received intravenous injection of 1 mL microcystin extracts at LD₅₀ of 86.7 μg MC-LR eq/kg body weight. As a control, the other group was injected with the same volume of 0.9% saline solution. Five rats in both groups were sacrificed at 1, 2, 4, 6, 12, 24 h post-injection, respectively. The liver, kidney and testis samples were excised and immediately frozen at -80 °C for later analysis.

2.5. Total RNA extraction and cDNA synthesis

Total RNA from each sample (less than 100 mg) was isolated using Trizol reagent (Invitrogen, America) and treated by DNaseI to remove the contaminant DNA. The purified total RNA (2 μg) was then reversely transcribed using first strand cDNA synthesis kit (TOYOBO, Japan). The resultant cDNA was then diluted 20-fold and kept at -20 °C for later use.

2.6. Quantitative real-time PCR (Q-PCR)

The primers were designed based on the gene sequence of *Rattus norvegicus* present on the NCBI (<http://www.ncbi.nlm.nih.gov>) so as to amplify a 160–210-bp section. The sequences of the *c-fos* (NM_022197) primers were 5'-ACC ATGATGT TCTCGGTT TCAAC-3' (sense) and 5'-GAGAT GGCTGTACCGTGGGGAT-3' (antisense), for *c-jun* (X17163)

5'-GACCGACGAGCAGGAGGGCTT-3' (sense) and 5'-AGCCCTTCCACGATGCCAAAGTTGT-3' (antisense), for *c-myc* (AY294970) 5'-CCACAC AGCCACTGGTCCT-3' (sense) and 5'-GGCTGGAGC ATTTGCGG TTGT-3' (antisense), for and *GAPDH* (NM_017008) 5'-ATGGAGA AGGCTGGGGCTCA CCT-3' (sense) and 5'-GCCACAGCGGGAGCGACCAT-3' (antisense). Specification of each pair of primers was tested by randomly sequencing three clones, and further confirmed by the melting curve analysis using Q-PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid. Only primers with similar amplification efficiency were used in this experiment. Q-PCR was conducted by amplifying 1.0 μ l of diluted cDNA with the SYBR Green Q-PCR kit (Finnzymes Company, Finland) on a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA). Cycling conditions were as follows: 4 min at 94 °C, 40 cycles of 20 s at 94 °C, 20 s at 58 °C or 60 °C, and 20 s at 72 °C. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in triplet repeat, and PCR reactions without the addition of the template were used as blank controls. The relative quantification of the expression of the target genes was measured using Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA as an internal control, which had been reported to be not affected by MCs (Chen et al., 2005; Hudder et al., 2007). After completion of the PCR amplification, data were analyzed with the Option Monitor software 2.03 version (MJ Research, Cambridge, MA).

2.7. Western blot analysis

Tissues were homogenized for extract proteins in ice-cold protein extraction buffer (Wuhan Boster Biological Technology Company, China). The homogenates were centrifuged at 12,000g for 10 min to collect the supernatants. Bradford method was used to determine the concentration of proteins. About 20 μ g of protein from each sample was separated on SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad). Membranes were blocked in 5% nonfat dry milk in PBS buffer for 1.5 h at room temperature. Blocked membranes were incubated in rabbit polyclonal antibodies specific for rat c-Fos, c-Jun (Santa Cruz, Santa Cruz, CA) or *GAPDH* (Wuhan Boster Biological Technology Company, China), in PBS (containing 1% nonfat dry milk) overnight at 4 °C. Antirabbit secondary antibody (Wuhan Boster Biological Technology Company, China), at a concentration of 1:1000 in PBS (containing 1% nonfat dry milk), was added to membranes and incubated for 1.5 h at room temperature. The protein signal was developed using NBT/BCIP system. The results of Western blotting were quantified with Gene Snap software (Syngene, America).

2.8. Statistic analyses

Data were expressed as the mean \pm standard deviation. One-way ANOVA was used to elucidate if there were significant differences between treatments groups and the control groups ($p < 0.05$ was considered as a statistically significant difference).

3. Result

3.1. Effects of MCs on proto-oncogenes expression in liver

Using Q-PCR, gene-specific mRNA abundance was quantified in liver from rats exposed to microcystin extracts for 1, 2, 4, 6, 12 and 24 h. As shown in Fig. 1, the mRNA levels of the three genes were all increased immediately after 1 h of exposure, and reached a peak at 4 h post-exposure, and then returned to a relatively lower level after 24 h of exposure. Among these genes, the highest increase was observed in *c-fos*, followed by *c-jun*. The expression of *c-fos* was remarkably up-regulated to as high as one thousand times of the control level at 4 h post-exposure, meanwhile expression of *c-jun* was increased to above 70-fold (Fig. 1).

MCs caused statistically significant increase of c-Jun protein level from 2 h of exposure analyzed by western blot ($p < 0.05$). In liver, the protein expression profile of c-Fos was similar to that of c-Jun, except for 6 h post-exposure when the increase of protein was not significant (Fig. 2A and B).

3.2. Effects of MCs on proto-oncogenes expression in kidney

In kidney, *c-fos* mRNA synthesis was induced to the highest level immediately after injection, and around 45-fold of the control at 1 h time point. Interestingly, it kept this high level to 2 h time point, and then dramatically went down to 5 times of control at 4 h. Until 24 h, it maintained the relatively low level. For *c-jun* and *c-myc* mRNA synthesis, both began to rise after injection and reached the highest level at 4 h, around 25-fold for *c-jun* and 10-fold for *c-myc*. Similarly to *c-fos*, their abundances were reduced to the relatively low level (less than 3-fold of control) after 6 h time point until 24 h (Fig. 1).

Unlike mRNA changes, in kidney the protein level of c-Fos was not significantly elevated until 6 h post-exposure. Moreover, only small significant increase was detected from 6 to 24 h compared to the remarkable change of mRNA level. In addition, in the whole experiment, no dramatic change was found for protein c-Jun in kidney, compared to that of the control (Fig. 2A and B).

3.3. Effects of MCs on proto-oncogenes expression in testis

Similarly to that in kidney, *c-fos* mRNA was significantly up-regulated to 10 times level of control within 1 h post-exposure in testis. But with further progress, its mRNA abundance declined quickly to the control level. The level of *c-jun* mRNA was relatively stable from 1 h to 6 h, but was significantly induced to be approximate 4-fold of control at 12 h and then went back to the lower level at 24 h time point. In testis, *c-myc* mRNA got the similar change pattern during the whole exposure process. However, overall there was a relatively moderate change of these three proto-oncogenes' mRNA abundance in testis compared to liver and kidney (Fig. 1).

Western blot analysis showed that c-Fos significantly increased but only about 1.2-fold increase at 2 h post-exposure. Furthermore, at the other time points, there was no significant change observed between the treated groups

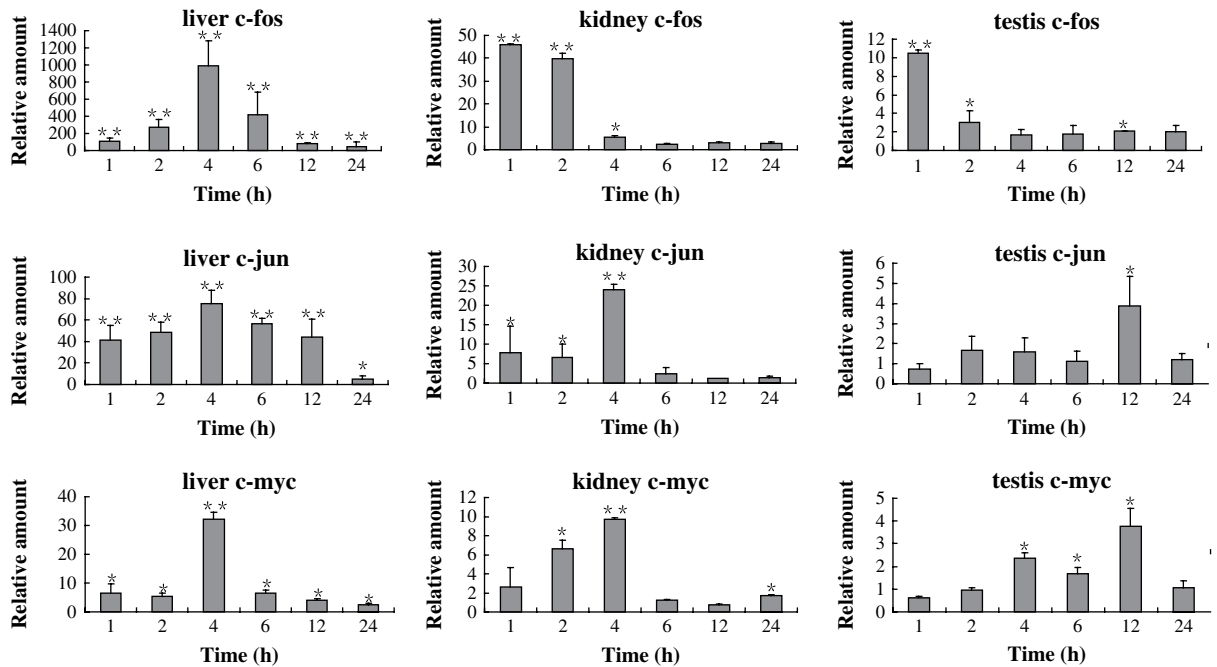


Fig. 1. The temporal changes of proto-oncogene transcript after microcystin extracts exposure compared with the controls. All expression values were normalized to the value of GAPDH gene. Relative amount was calibrated based on the transcript amount of the corresponding gene in untreated controls. Significance is indicated as: * $p < 0.05$; ** $p < 0.01$.

and the control. c-Jun protein did not increase significantly until 12 h post-exposure (Fig. 2A and B).

4. Discussion

c-fos and *c-jun* were two important members of the proto-oncogene family. Activation of these genes would result in the production of related proteins that could form the dimeric transcription factor AP-1. AP-1 has been speculated to be involved in the induction of tumorigenesis (Dosch and Kaina, 1996). At the same time, *c-myc* gene was the best-studied member of Myc oncogene family, which encoded a transcriptional regulator involved in carcinogenesis (Richardson and Johnson, 1993; Nasi et al., 2001). In the present study, we investigated the influence of MCs on the mRNA and protein levels of *c-jun*, *c-fos* and *c-myc* gene in the three organs from rats to evaluate their tumor-promoting potential.

In this study, the exposure to MCs led to great induction of *c-fos*, *c-jun* and *c-myc* in liver from rats on the transcriptional level. The result of GeneChip indicated the up-regulation of *c-fos* and *c-jun* in liver from zebra fish intraperitoneally injected with a dose of 400 μg MC-LR (Wei et al., 2008), which was in accordance with our results. In primary cultured rat hepatocytes, the induction of *c-fos* and *c-jun* gene was also observed (Sueoka et al., 1997). Based on the observation of higher mRNA level of *c-fos* and *c-jun* in liver, we explored the time course of protein expression of c-Fos and c-Jun. Parallel to the profile of mRNA changes, the expression of c-Fos and c-Jun proteins significantly increased in liver nearly almost throughout the experiment. But the change level and pattern of protein did not match

with that of mRNA. It was known that c-Jun protein was a positive regulator of proliferation and induced positive regulators of cell cycle progression (Szremska et al., 2003), while c-Fos had oncogenic activity with frequent over-expression in tumor cells (Verde et al., 2007). Therefore, the accumulation of these two proteins in liver might be one of the molecular events related to the tumor promotion activity of MCs.

Furthermore, we demonstrated in this study that changes in the expression on mRNA level did not often correlate with changes on protein level, especially not in the liver for the expression of *c-fos* gene at 4 h post-exposure. This unexpected discordance between mRNA and protein level changes could be reasoned by post-transcriptional control of *c-fos* expression. Moreover, both Fos and Jun protein might undergo extensive post-translational modifications to influence protein stability (Cohen and Curran, 1988). Therefore, MCs might have distinct influences on mRNA transcription and protein synthesis machines. Several recent reports also proved that alterations in mRNA and protein levels of proto-oncogenes were not always directly linked. Payson et al. (2008) found that although *c-fos* and *c-jun* gene showed a significant reduction in leiomyoma compared with myometrial samples, Western blot did not show significant differences in protein expression for these genes. In liver of male rats after 100 weeks post-exposure to phenobarbital, a tumor promoter, RNA and protein levels of *c-fos* gene did not correlate, too (Bitsch et al., 1999). As for other kinds of genes, similar phenomenon also existed. In squamous cell carcinoma samples, in contrast to the suppression of *Pdcd4* mRNA, *Pdcd4* protein level remained unchanged or even

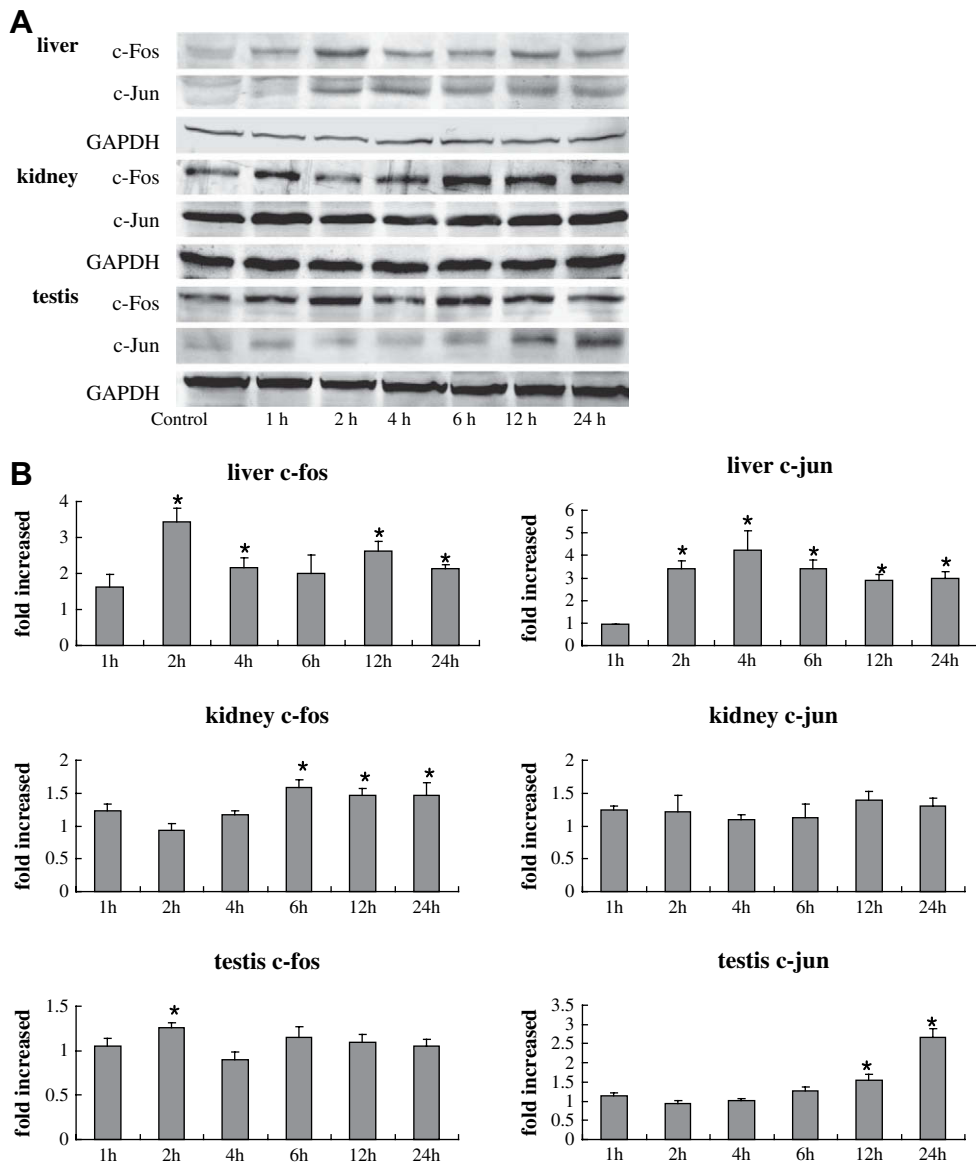


Fig. 2. Western blot analysis of proteins from differently induced rat liver, kidney and testis with antibodies to c-Fos, c-Jun, or GAPDH. (A) Western blot analysis was performed with antibody against c-Fos, c-Jun or GAPDH. Each figure corresponded to a representative experiment out of three experiments. (B) Each column and bar represented the mean \pm SD of three individual samples. Mean protein expression in each treated groups was shown as a fold increase compared to mean expression in control group which has been ascribed an arbitrary value of 1 (* indicates significant change at $p < 0.05$).

up-regulated (Kalinichenko et al., 2008). Bergmann et al. (2008) demonstrated that Ferroportin and DMT1 mRNA levels were significantly higher in cirrhotic human livers than in controls, whereas the levels of proteins were lower in cirrhotic livers than in controls. In another report, iron excess led to a significant increase of the mRNA level but not the protein level of Dcytb in Caco-2 cells (Balusikova et al., 2008).

Recently, MCs were found to be toxic to the reproductive systems of male mice and also to kidney (Ding et al., 2006; La-Salette et al., 2008). However, there had no reports on the possibility of tumor-promoting activity of MCs in these two organs yet. The current result showed that exposure to MCs

could lead to induction of the transcript of *c-fos*, *c-jun* and *c-myc* gene in kidney and testis. Meanwhile, the up-regulation of c-Fos and c-Jun protein was also observed in these two tissues. However, both Q-PCR and Western blotting analysis indicated that MCs had the slighter effects on the mRNA and protein synthesis of *c-fos*, *c-jun* and *c-myc* gene in kidney and testis than in liver. From these results, it seemed that there was also potential tumor-promoting activity in kidney and testis when exposed to MCs, although such potential was very weaker in kidney and testis than in liver of rat. It was reported that exposure of mice to MC-LR at 1 LD₅₀ intraperitoneally could significantly inhibit the expression of protein phosphatases 1 and

2A activity in liver after 30–120 min post-exposure but not in kidney (Jayaraj and Lakshmana Rao, 2006), which was in agreement with our results.

Even though the detailed mechanism of tumor-promoting activity of MCs has not been well elucidated, it has been suggested that activation of MAPK might be an underlying mechanism for the tumor-promoting activity of the Ser/Thr PP inhibitors resulting in increased cellular proliferation (Toivola and Eriksson, 1999). *c-jun* and *c-fos* were reported to be the targets of the MAPK pathway (Delaney et al., 2008), once activated MAPK is transferred to the nucleus, it could activate *c-jun* and *c-fos* gene (Gehring, 2004). In addition, *c-fos* and *c-jun* could be induced via oxidative mechanisms (Amstad et al., 1992). Recently, accumulating evidences indicated that oxidative stress is involved in cellular injury induced by MCs (Bouaicha and Maatouk, 2004; Žegura et al., 2004). The direct involvement of reactive oxygen species (ROS) in AP-1 activation had been demonstrated using well-defined ROS generating systems to challenge cultured cells (Ding et al., 1999). Therefore, oxidative stress induced by MCs might also be involved in the activation.

In summary, this study provided evidence that proto-oncogenes, especially *c-jun* and *c-fos*, were significantly up-regulated in the three organs from rats in response to MCs. These data suggested that the effects of MCs on the expressions of *c-fos* and *c-jun* could be a possible factor when trying to explain the tumor-promoting activity of MCs in liver. Furthermore, the elevated level of proto-oncogene might be correlated to the tumor-promoting activity in different organs induced by MCs.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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