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Involvement of Fas/FasL system in apoptotic signaling in testicular germ cells of male Wistar rats injected i.v. with microcystins

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ABSTRACT

Previous studies have shown that gonads were the second target organ of microcystins (MCs), and that MCs exposure exerted obvious toxic effects on male reproductive system of mammals. However, relevant molecular evidences are still lacking. Fas-signaling pathway plays a key role in toxicant-induced germ cell apoptosis. This study was to evaluate the responses of Fas/FasL system related genes and proteins in testes of rats injected intravenously with MCs. Enhanced apoptosis of germ cells in the testes of MCs-treated rats was detected by the terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL) associated with up-regulation of the Fas/FasL system. Both Fas and FasL protein expression were induced evidently from 1 h post-injection, and this high expression level maintained throughout the experiment. In addition, the activation of caspase-8 and caspase-3 protein was also observed, which were indicators of apoptosis. These results suggested the likely involvement of Fas/FasL system in the MCs-induced germ cell apoptosis. It is also suggested that MCs can cause damage to Sertoli cells directly.

1. Introduction

Microcystins (MCs), a group of cyclic heptapeptide compounds with specific hepatotoxins produced by cyanobacterial species, have received worldwide concern in the past decades (Cohen, 1989; Carmichael et al., 1997) and in a recent study by Chen et al. (2009), MCs were identified for the first time in the serum of a chronically exposed human population (fishermen at Lake Chaohu, China) together with indication of hepatocellular damage. So far, more than 80 different structural analogues of MCs have been identified (Fastner et al., 2002), with microcystin-LR being the most toxic (Mirura et al., 1989). MCs have been already well characterized as strong inhibitors of protein phosphatases 1 (PP1) and phosphatases 2A (PP2A) (Yoshizawa et al., 1990; Nishiwaki-Matsushima et al., 1992). This inhibition could lead to hyperphosphorylation of key proteins that regulate apoptosis (Fu et al., 2005). Previous studies confirmed that various cell types could be induced to undergo apoptosis by MCs, mainly characterized by cell membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, DNA fragmentation and formation of apoptotic bodies (McDermott et al., 1998; Ding et al., 2000).

Recently, some studies indicated that gonads might be the second target organ of MCs (Chen and Xie, 2005; Chen et al., 2005). Wang et al. (2008) report that contents of MCs (RR + LR) remained relatively stable (means 21–37 ng/g) in the rat gonad within 24 h after i.v. injection of MCs' extracts at a dose of 80.5 μg MC-$LR_{equivalent}$/kg body weight, and that even at 24 h, a certain amount of MCs was still present in the gonad, suggesting the difficulty of MCs' elimination from gonad. Ding et al. (2006) revealed various toxic effects of MC-exposure on the reproductive system of male mice through histological examination: damaged testes

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structure, increased space between the seminiferous tubules, decreased numbers of interstitial cells, Sertoli cells and mature sperm. Li et al. (2008) also found MC-LR can exert a generally chronic toxicity to male rat reproductive system and specific damage to the testes, including increased sperm abnormality, decreased sperm motility and concentration. As is known, the Fas-signaling system is implicated in the elimination of germ cells after testicular injury. When exposed to specific testicular toxicants, germ cell and Sertoli cell injuries were observed, followed by up-regulation of Fas and FasL in injured cells. This up-regulation of Fas–FasL resulted in an elevation of germ cell apoptosis (Lee et al., 1997, 1999; Boekelheide et al., 2000). Fas (APO-1, CD95) is a transmembrane receptor protein that belongs to the tumor necrosis factor/nerve growth factor receptor family (Watanabe-Fukunaga et al., 1992; Nagata and Golstein, 1995). FasL is a tumor necrosis factor-related type II transmembrane protein (Suda et al., 1993). Binding of FasL to Fas can induce trimerization of Fas receptors, which recruit Fas-associated death domain (FADD) through shared death domains (DD). FADD also contains a death effector domain (DED) at N-terminal region. Fas/FADD complex then binds to procaspase-8 through interactions between DED of the FADD and these caspase molecules. Aggregation of procaspase-8 causes self-activation, and generates active caspase-8, the initiator. At this point, there are two possible pathways through which caspase-8 can enter into: one is to directly activate procaspase-3 by proteolytic cleavage, and the other is to cleave Bid, a proapoptotic member of the Bcl-2 family. The cleaved Bid induces the release of cytochrome c from mitochondria, which interacts with apoptotic protease activating factor-1 (Apaf-1) and dATP to form the apoptosom. Apoptosom is a large oligomeric protein complex which can activate procaspase-9. Caspase-9 then activates procaspase-3. This pathway is called mitochondria-dependent apoptotic pathway (Budihardjo et al., 1999; Cohen, 1997). Both pathways converge on caspase-3 and other executioner caspases and then nucleases that drive the terminal events of programmed cell death. In the whole process of apoptosis regulation, the expression of Fas, FasL, FADD, caspase-8, Apaf-1, caspase-9 and caspase-3 is of vital importance.

Based on these factors, it is of consequence to find out whether Fas/FasL system is involved in the mechanism of MCs-induced injury or apoptosis in testes. The primary aims of this study were to evaluate the time-dependent responses of the Fas/FasL system related genes and proteins in testes of rats exposed to MCs, and further to elucidate the underlying toxicological mechanisms.

2. Materials and methods

2.1. Toxin

The cyanobacterial material used in this experiment was collected from surface blooms (phytoplankton cells) of Lake Dianchi, Yunnan in China during May and June, 2006. According to microscopic examinations, the predominant species was *Microcystis aeruginosa*. Freeze-dried crude algae were extracted three times with 75% (V/V) methanol. The extract was centrifuged and the supernatant was applied to a C_{18} reversed phase cartridge, which had been preconditioned by washing with methanol and then distilled water. The cyanobacterial material was analyzed for MCs content via a reverse-phase high-performance liquid chromatography (HPLC, LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) equipped with an ODS column (Cosmosil 5C18-AR, 4.6×150 mm, Nacalai, Japan) and an SPA-10A UV–vis spectrophotometer set at 238 nm. Crude extract concentrations were determined by comparing the peak areas of the test samples with those of the standards available (MC-LR and MC-RR, Wako Pure Chemical Industries, Japan). Crude MCs' extracts were finally suspended in salt solution (0.9% NaCl).

2.2. Animals

Male Wistar rats weighing 180–200 g were supplied by Hubei Laboratory Animal research Center (Hubei, China). The rats were housed under controlled conditions of 12 h light/dark cycle, $50 \pm 5\%$ humidity and $23 \pm 1\,^{\circ}C$. The animals were allowed free access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory.

2.3. LD_{50} of cyanobacterial crude extracts

Male Wistar rats ($n = 40$) were divided into five groups, and rats in each group were administered by an i.v. injection of MCs at different dose of MC-$LR_{equivalent}$/kg body weight. Calculating the death number and mortality of rats in each group during 24 h, we obtained the LD_{50} level for 24 h by using the formula $LD_{50} = \log^{-1}[Xm - I(\Sigma p - 0.5)]$ (Xm: the log of max dose; p: mortality; Σp: the sum of mortality in each group; I: the difference between the log of adjacent two group dose). The LD_{50} studies were approved by the IACUC.

2.4. MCs exposure

Healthy male Wistar rats weighing 200 ± 20 g were divided in equal numbers into two groups randomly. One group received intravenous injection (i.v.) of 1 mL MCs extracts at LD_{50} of 80.5 μg MC-$LR_{equivalent}$/kg body weight. An equivalent volume of 0.9% saline solution was applied to control ones. Six sampling points were set during a period of 24 h in the experiment (1, 2, 4, 6, 12 and 24 h). Five rats from each group were killed at each time points and the testes were quickly removed, minced and stored frozen at liquid nitrogen for later analysis.

2.5. Terminal deoxynucleotide transferase-mediated deoxy-UTP nick end labeling (TUNEL)

For TUNEL staining (Gavrieli et al., 1992), the standard protocol for frozen sections was followed (ApopTag, Oncor, Gaithersburg, MD). Frozen cross-sections (8 μm) from testes were prepared, fixed in 10% neutral buffered Formalin for 10 min at room temperature, rinsed in PBS,

postfixed in acetone for 5 min at −20 °C, and then incubated in 2% H_2O_2 for 15 min to quench endogenous peroxidases. Five slides were prepared per testis and the number of TUNEL-positive cells was counted in each seminiferous tubule (ST). To quantitate the incidence of apoptosis at each time point, the TUNEL-positive cells within a ST cross-section were counted. All TUNEL-positive cells within the seminiferous epithelium were considered as germ cells. The percentage of apoptotic germ cells in both the control and MCs-treated rat testes was determined by counting a total of 1000 germ cells (including both apoptotic and non-apoptotic cells) from STs with cross-section.

2.6. Total RNA isolation

Total RNA was isolated from 50 to 100 mg sections of testis using Trizol reagent (Invitrogen, America) and quantified by determination at OD_{260}. RNA was extracted according to the manufacturer's protocol, resuspended in 50 μl RNase-free water, and stored at −80 °C. Quantification was done using Eppendorf Biophotometer (Germany). The purified total RNA (2 μg) was then reverse transcribed. Reverse transcription was performed with oligo (dT) 18 primer using first strand cDNA synthesis kit (TOYOBO, Japan). The resultant cDNA was then diluted 20 fold and kept at −20 °C.

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

All the primers used in Q-PCR were listed in Table 1. The primers were designed based on the gene sequences of *Rattus norvegicus* present on the NCBI homepage (http://www.ncbi.nlm.nih.gov). The specification of each pair of primers was confirmed by randomly sequencing six 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. GAPDH was used as the internal control gene for Q-PCR assay. Q-PCR was conducted with the SYBR Green qPCR kit (Finnzymes, Finland) on a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA). The reactions were performed in a 20 μl volume mix containing 10 μl SYBR Green I mixture, 1 μl primers, 1 μl cDNA and 1 μl sterile, distilled–deionized water. Cycling conditions were as follows: 3 min at 95 °C, 44 cycles of 15 s at 95 °C, 20 s at 62 °C or 64 °C, and 15 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 three tubes, and PCR reactions without the addition of the template used as blanks. After completion of the PCR amplification, data were analyzed with the Option Monitor software 2.03 version (MJ Research, Cambridge, MA).

2.8. Western blot analysis

For Western blotting sample preparation, small tissue sections were homogenized in ice-cold protein extraction buffer (Wuhan Boster Biological Technology Company, China). After centrifugation at 12,000 *g* (4 °C) for 10 min to remove debris, the supernatant was carefully recovered. Protein concentrations were determined using the Bradford assay (Bradford, 1976). All samples were stored at −80 °C prior to electrophoresis.

Aliquots from supernatant containing 20 μg of proteins were mixed with equal volume of 2× sample buffer. The sample was boiled for 5 min and subjected to 12% SDS-PAGE. After electrophoresis, the resolved proteins were transferred to nitrocellulose membrane using an electro blotting apparatus (Bio-Rad, America). Membranes were blocked at room temperature for 2 h in blocking buffer containing 5% nonfat dry milk to prevent non-specific binding of reagents, and then incubated with anti-Fas (sc-716; Santa Cruz Biotechnology; 1:200), anti-FasL (sc-834; Santa Cruz Biotechnology; 1:200), anti-caspase-8 (Wuhan Boster Biological Technology Company, China; 1:100), anti-caspase-3 (H-277; Santa Cruz Biotechnology; 1:200) or GAPDH (Wuhan Boster Biological Technology Company, China) at 4 °C overnight. The membranes were washed in TBST (50 nmol/L Tris-Cl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 30 min and incubated with IgG (H + L) conjugated secondary antibody (Wuhan Boster Biological Technology Company, China; 1:1000) for 1 h at room temperature. The protein signal was developed using NBT/BCIP system. The results of Western blots were quantified with Gene Snap software (Syngene, America).

2.9. Statistical analysis

Significance of differences between the treated and control groups was analyzed by Student's *t* test. Statistical significance was concluded at $P < 0.05$ and 0.01.

Table 1
Q-PCR primers used in this experiment.

Target gene	Primer sequence (5′–3′)		Size (bp)
	Forward	Reverse	
Fas	GCTGTCCTGCCTCTGGTGCTTGCT	GCAGTTGTTGTCGGTTTCACGAACG	117
FasL	TCCACCACCACCTCCATCACCACT	CCATTCCAACCAGAGCCACCAGCAC	143
Fadd	CCTCGCTGGGCAGACACGACCTACT	CACAATGTCAAATGCCACCCGCAGA	110
Caspase-8	TAAGACCTTTAAGGAGCTTCATTTTGA	TCCGTTCCGTAGACGATGCCCTT	168
Apaf-1	GGTCCTAAGTATGTTATCCCTGTGGAG	TGAGGTAGTATGCCCAGCGATTGG	205
Caspase-9	TGAGCCAGATGCTGTCCCATACCAG	CCTGGGAAGGTGGAGTAGGACAC	114
Caspase-3	GAACGAACGGACCTGTGGACCT	GCCTCCACTGGTATCTTCTGGCAT	187
GAPDH	ATGGAGAAGGCTGGGGCTCACCT	AGCCCTTCCACGATGCCAAAGTTGT	209

3. Results

3.1. TUNEL staining

As shown in Fig. 1(A), only a few apoptotic germ cells were observed in the basal compartment of STs in the control rats. In contrast, a remarkable increase in the number of apoptotic germ cells (spermatogonia, spermatocytes and round spermatids) was observed in the STs of MCs-treated rats (Fig. 1(B)). The percentage of apoptotic germ cells in MCs-treated rats was significantly higher than that in the control group (Fig. 2). The level of apoptosis peaked at 6 h after treatment and the elevation of the incidence of apoptosis was evident from 1 h onward.

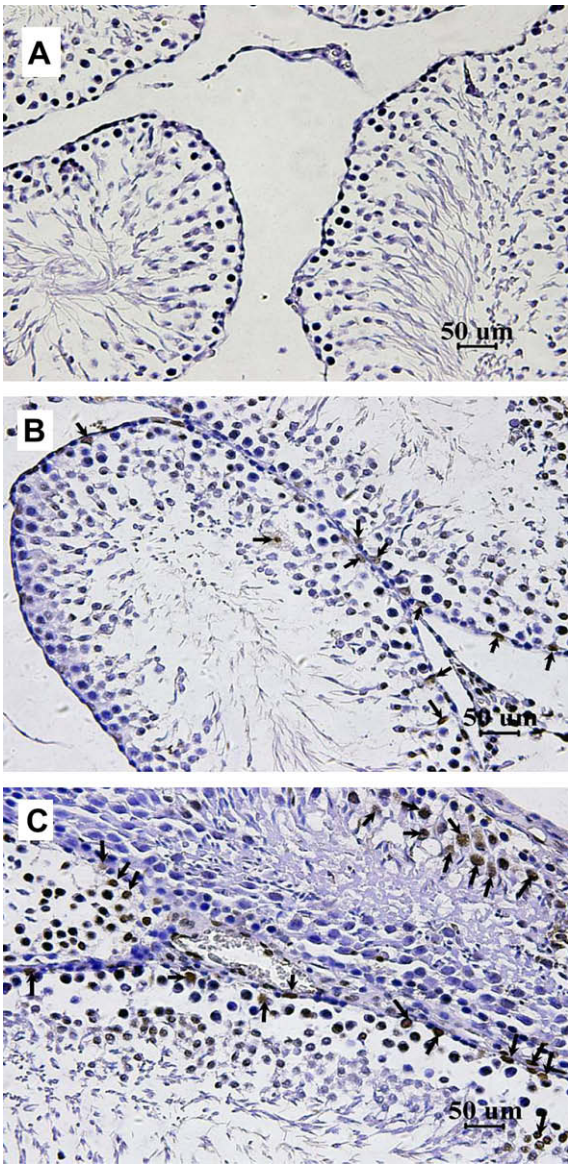

Fig. 1. TUNEL labeling of sections from treated and control rats. (A) Control section showing very few cells positive for TUNEL (brown deposits). (B) Increased number of apoptotic germ cells (arrows) is observed in the STs of MCs-treated rats 4 h after exposure. (C) A marked increase in the number of apoptotic germ cells (arrows) is observed in the STs of MCs-treated rats 6 h after exposure.

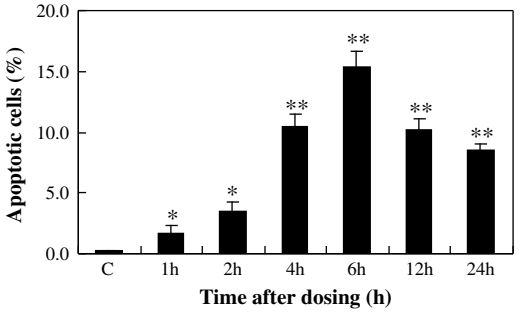

Fig. 2. Time course of changes in percentages of apoptotic cells in testes of control and MCs-treated rats. (*indicates significant change at $P < 0.05$, **indicates significant change at $P < 0.01$).

3.2. mRNA level of the related genes of Fas/FasL system

Fig. 3 showed the transcriptional changes of Fas, FasL and their downstream effectors-FADD, Caspase-8, Apaf-1, Caspase-9 and Caspase-3 in the rat testes within 24 h. The transcription of Fas was significantly induced since 2 h post-injection, and reached a peak value at 6 h and then returned to the original level at 24 h. FasL mRNA was significantly increased at 1 h post-injection and peaked at 4 h, and returned to the control level at 24 h. The expression of FADD was suppressed evidently in the first two hours and then significantly enhanced at 4 h post-injection. The transcription of caspase-8 was significantly elevated at all time points. Apaf-1 mRNA was induced at 6 h and showed a tendency to recover after 12 h. The transcription of caspase-9 was induced from 2 h to 6 h and returned to the original level since 12 h. The mRNA expression of the terminal executioner-caspase-3 was induced from 4 h to 6 h but was depressed at 24 h. The mRNA expressions of Fas, FasL, FADD, caspase-8, Apaf-1, caspase-9 and caspase-3 were all significantly increased at 6 h, among which Fas, Apaf-1 and caspase-3 reached to the maximum at this time point.

3.3. Western blot analysis

The protein expressions of Fas, FasL, Caspase-8 and Caspase-3 were detected by Western blot. Both Fas and FasL proteins were induced obviously at 1 h after MCs treatment, and the high expression level remained throughout the experiment. Quantification of the two proteins revealed that the expressions of both Fas and FasL were significantly increased from 1 h post-injection, and reached to the maximum at 6 h and 4 h, respectively. Associated with this increase was the activation of caspase-8, the initiator caspase in this apoptotic pathway. The terminal executioner, caspase-3 was also examined by Western bolt analysis. The results showed that caspase-8 and caspase-3 cleavage was detectable from 1 h onward using Western blots (Fig. 4). In

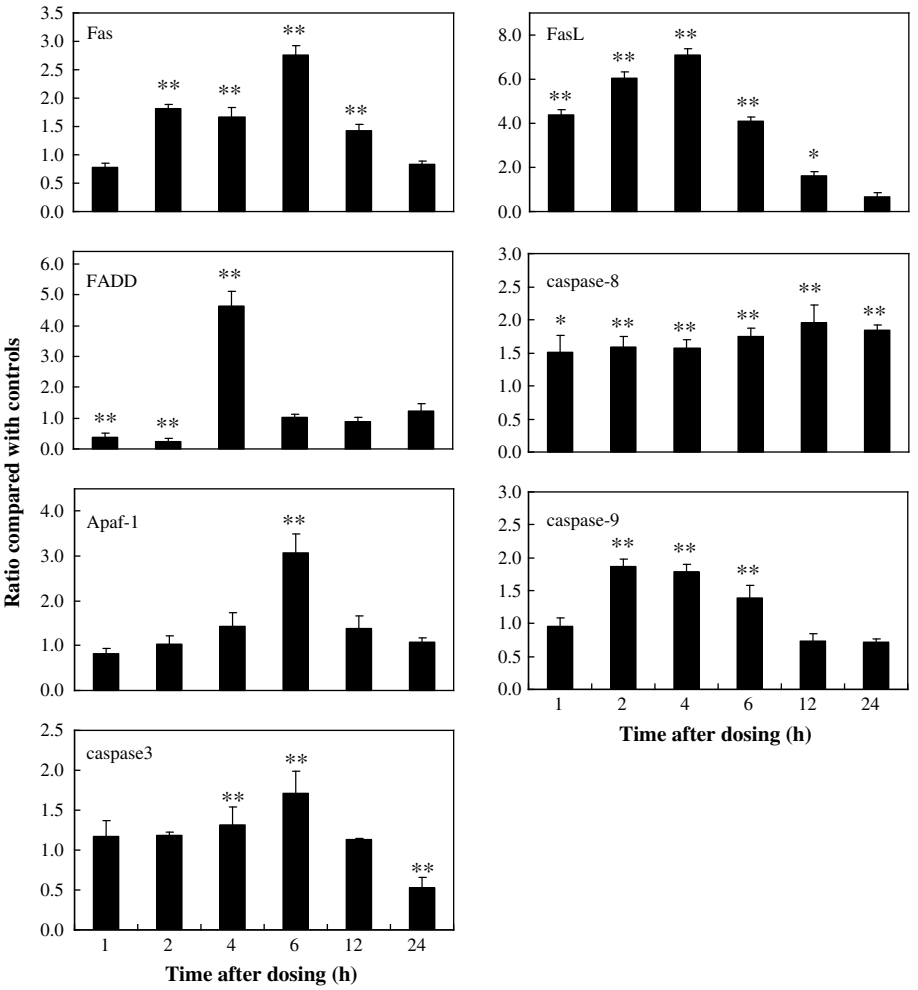

Fig. 3. Time course of Fas/FasL system related mRNA expression by Q-PCR analysis in rat testis after treatment with 80.5 μg MC-$LR_{equivalent}$/kg body weight. (*indicates significant change at $P < 0.05$, **indicates significant change at $P < 0.01$).

addition, procaspase-3 and procaspase-8 expression was significantly elevated. The procasepase-8 and procaspase-3 expression peaked at 4 h and 6 h, respectively.

4. Discussion

The principal finding of the present study was that enhanced apoptosis of germ cells in the testes of MCs-treated rats was associated with up-regulation of the Fas-signaling system. The TUNEL assay demonstrated the presence of a few apoptotic cells and apoptotic STs in the control testes, which was in agreement with previous reports (Lee et al., 1997, 1999; Richburg et al., 1999; Richburg, 2000). In contrast, a significant increase in the percentage of apoptotic germ cells was observed in the testes of MCs-treated rats. From these results, it could be deduced that MCs exposure induced germ cell apoptosis in testes of male Wistar rats. Monitoring of time course changes could facilitate analysis of toxicological mechanisms including cause and effect relationship (Kijima et al., 2004). In the present study, the mRNA expressions of Fas, FasL and their downstream effectors-FADD, caspase-8, Apaf-1, caspase-9 and caspase-3 were all significantly increased within 24 h post-injection as determined by Q-PCR. The simultaneous expression of these apoptosis-related genes might suggest a multiple wave like propagation of apoptosis signal from FasL, Fas, FADD down to caspase-3 via a pathway including caspase-8, Apaf-1, caspase-9. Moreover, we found that the expression profiles of Fas and FasL in protein level were correlated closely with that of mRNA level. Significant elevation of FasL and Fas protein with similar folds to that of mRNA was observed in testis from 1 h onward. Accompanied with this increase was the activation of caspase-8, the initiator caspase and caspase-3, the terminal executioner. As is known, activation of caspase-8 is required for Fas-induced apoptosis (Juo et al., 1998), while activation of caspase-3 is a central event upon which numerous signaling pathways converge and through which multiple downstream substances such as caspase-activated DNase are cleaved (Jacobson et al., 1997; Kim et al., 2000, 2001). The time-dependent up-regulation of mRNA and activation of proteins suggest that the

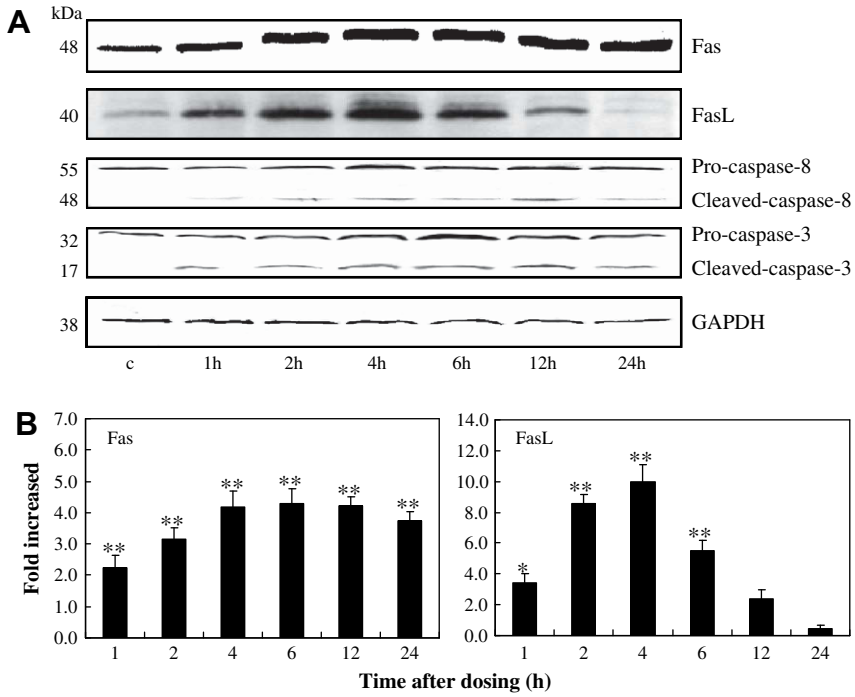

Fig. 4. Western blot analysis of proteins from rat testes with antibodies to Fas, FasL, caspase-8, caspase-3 and GAPDH. (A) Western blot analysis was performed with antibody against Fas, FasL, caspase-8, caspase-3 and GAPDH. Each figure corresponds to a representative experiment out of three experiments. (B) Each column and bar represent the mean ± SD of three individual samples. Mean protein expression in each treated groups is shown as a fold increased compared to mean expression in control groups which has been ascribed an arbitrary value of 1. (*indicates significant change at $P < 0.05$, **indicates significant change at $P < 0.01$).

apoptogenic effect of MCs might be mediated via the up-regulation of the Fas-signaling system and the activation of caspases.

It is generally believed that Sertoli cells are the main cells expressing FasL in STs (Xu et al., 1999; Hu et al., 2003; Koji and Hishikawa, 2003; D'Abrizio et al., 2004) and Fas is localized to specific germ cell subtypes (Xu et al., 1999). The Fas-signaling system is initiated differentially depending on the primary cellular site of toxicity. Exposure to toxicants, such as 2, 5-hexanedione or mono-2-ethylhexyl phthalate, which target the Sertoli cells and disrupt their function, will lead to up-regulation of FasL followed by Fas (Lee et al., 1997, 1999). Radiation-induced injury or testicular hyperthermia, which both directly affect germ cells failed to up-regulate FasL while up-regulating Fas mRNA expression (Lee et al., 1999). Our studies revealed an evident induction of both Fas and FasL mRNA in testis after MCs treatment, when compared to control groups. The highest expression of FasL mRNA was detected at 4 h after MCs treatment, while the expression of Fas mRNA reached to the maximum at 6 h when the level of apoptosis also peaked. This result was in accordance to that of Lee et al. (1999), who reported an early onset of FasL mRNA up-regulation after Sertoli cell injury. Actually, large vacuoles, distended endoplasmic reticulum and increased lipid droplets were found in the Sertoli cell through electron microscopy at 6 h post-exposure in this experiment (unpublished data). Therefore, we supposed that MCs damaged Sertoli cells in the testis of rats. This corroborated previous reports that MCs could damage Sertoli cells in the testes (Ding et al., 2006).

In normal state, Sertoli cells maintain the homeostasis by providing hormonal, nutritional and physical support to most healthy germ cells, and killing a few Fas-positive germ cells with FasL. Toxicants that injure or disrupt the functions of Sertoli cells can effectively reduce their supportive capacity and, as a result, germ cell cannot be supported adequately. The Fas-signaling pathway between Sertoli cells and germ cells plays a key role in mediating germ cell apoptosis in the testis after Sertoli cell injury (Richburg et al., 1999; Richburg, 2000; Giammona et al., 2002; Richburg and Nanez, 2003). In the present study, both mRNA and protein expression of Fas and FasL in the testis of male Wistar rats were significantly induced. According to the working model for the Fas-signaling system in the testis proposed by Lee et al. (1999), it is most probable that after injury, the disfunctional Sertoli cells increased the expression of FasL which translocates and diffuses towards the apical side of STs to facilitate the elimination of the inadequately supported germ cells that express Fas. In this way, the germ cells' population can be reduced to a level which the Sertoli cells can support. Thereby, a new equilibrium state can be achieved to match the reduced supportive capacity of the disfunctional Sertoli cells with fewer germ cells (Richburg et al., 1999; Richburg, 2000).

In summary, the present study indicated for the first time that MC-exposure could induce germ cell apoptosis in rat testes. And the enhanced apoptosis of germ cells was

associated with the increased expression of Fas/FasL system related genes at both mRNA and protein level, indicating the likely participation of the Fas/FasL system in MCs-induced germ cell apoptosis. Our study also suggested that MCs had Sertoli cell toxicity.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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