Original Article

***In vivo* study on the effects of microcystin–LR on the apoptosis, proliferation and differentiation of rat testicular spermatogenic cells of male rats injected i.p. with toxins**

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ABSTRACT — Microcystin-leucine arginine (MC-LR), a cyclic heptapeptide produced by cyanobacteria, has strong reproductive toxicity. The present study was designed to elucidate the mechanism of declines in sperm quality as a result of exposure to MC-LR by using spermatogenic cells as a model system. MC-LR was intraperitoneally administered to male rats daily at 0, 50 and 100 µg/kg body weight for one week. Results showed that changes occurred in the structural of testis, the tubular diameter and the relative weight of the testes was significantly decreased following treatment with 100 µg/kg. Major differences in apoptosis and proliferation of testicular cells were observed at the 100 µg/kg MC-LR. The gene expression levels of testis-specific histone 2B (TH2B) and transition protein 2 (TP2) were both significantly decreased. Meanwhile, the stem cell factor receptor (c-kit) was increased after exposure to 50 or 100 µg/kg MC-LR. This study demonstrated that MC-LR can alter the apoptosis, proliferation and differentiation of spermatogenic cells *in vivo*.

Key words: Microcystin-LR, Spermatogenic cells, Seminiferous tubular diameter, Apoptosis, Proliferation, Differentiation

INTRODUCTION

Microcystins (MCs) are a class of cyclic heptapeptide compounds produced by several genera of freshwater cyanobacteria, and have received worldwide concern in recent decades (Carmichael *et al.*, 2001). MCs can be found in many freshwater bodies, which are sources for drinking water, due to the frequent occurrence of cyanobacterial blooms (Harada, 1999). The concentrations of total MCs may reach up to 1,300 µg/l in surface waters (Hermann Fromme, 2000). One study revealed that the concentrations of MCs in the blooming lakes in the United States ranged from 10 µg/l to 500 µg/l (Backer *et al.*, 2010). Thus, MCs may pose a health hazard to animals and humans higher up in the food chain because of the accumulation of MCs in aquatic creatures (Chen and Xie,

2005; Chen *et al.*, 2005; Xie *et al.*, 2004). MCs were identified for the first time in the serum of a chronically exposed human population together with indication of hepatocellular damage in a recent study (Chen *et al.*, 2009). The World Health Organization (WHO) set a provisional guideline which allows maximally 1 µg/l MCs in drinking water (WHO, 2004). So far, more than 100 different structural analogs of MCs have been identified, with microcystin-leucine arginine (MC-LR) being the most toxic and the most commonly encountered variant (Dietrich and Hoeger, 2005).

MCs have been well characterized as strong inhibitors of cellular serine/threonine protein phosphatases (PP) 1 and 2A in hepatocytes (Craig *et al.*, 1996). The inhibition is achieved through interaction with diverse molecular targets resulting in hyperphosphorylation of key proteins

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that regulate cell function. Current studies have confirmed that microcystins may generate nephrotoxicity and especially hepatotoxicity, as they are predominantly absorbed, transported and accumulated into liver (Svircev *et al.*, 2010; Wang *et al.*, 2010). Recently, some studies indicate that the gonads might be another important target organ of MCs (Chen and Xie, 2005; Chen *et al.*, 2005, 2011; Li *et al.*, 2008). A certain amount of MCs is still present in the gonads at 24 hr after intraperitoneal injection of MC extracts at a dose of MC-LR 80.5 mg/kg body weight, suggesting its resistance to elimination from the gonad. Our previous *in vivo* studies have demonstrated that treatment with MC-LR results in substantial toxicity to male reproduction with declines in sperm quality, decreased levels of serum testosterone, and injury to the testis (Chen *et al.*, 2011; Li *et al.*, 2008), which suggests that MCs are toxic to the male mammalian reproductive system. However, regardless of recent achievements, the mechanisms underlying MC reproductive toxicity remain elusive and little is known about the mechanisms of declines in sperm quality.

As is known, the spermatogenic cells, Sertoli cells and Leydig cells are three main kinds of important cells in the testis. Specifically, Sertoli cells provide necessary nutrition for spermatogonia, while Leydig cells synthesize and secrete testosterone into seminiferous tubules to promote spermatogenesis (Bellve *et al.*, 1977; Svechnikov *et al.*, 2010). MCs can cause damage to Sertoli cells directly and Leydig cells indirectly (Li and Han, 2012; Wang *et al.*, 2012; Xiong *et al.*, 2009). Nevertheless, there is no *in vivo* evidence that MC-LR can affect spermatogenic cells, the most important cells in the testis. Toxicity studies demonstrate that MCs may compromise sperm quality affecting spermatogenic cells directly during spermatogenesis (Sohoni *et al.*, 2001). Therefore, in view of the adverse effects of MC-LR on sperm concentration and quality our concern was focused on its toxic effects on spermatogenic cells in the process of spermatogenesis. During spermatogenesis, diploid spermatogonia divide mitotically several times to provide a population of spermatocytes that proceed through meiosis to give birth to haploid spermatids which undergo morphological alterations leading to spermatozoa (White-Cooper and Bausek, 2010). The apoptosis, proliferation and differentiation of spermatogenic cells are involved in the entire process of spermatogenesis. Previous research suggests that these events occur spontaneously in rodents as well as in response to endocrine disruption and exposure to various environmental agents and chemicals (Billig *et al.*, 1995; Brinkworth *et al.*, 1995; Tapanainen *et al.*, 1993). To date, there is no report clarifying whether MCs can affect apop-

tosis, proliferation and differentiation of spermatogenic cells.

Given these findings, it is interesting to find out whether MC-LR has toxicity on spermatogenic cells directly *in vivo*. To this end, we undertook to detect cell apoptosis, proliferation and differentiation by means of immunohistochemistry and quantitative real-time PCR. The primary aim of this study was to investigate the mechanisms of decreased sperm quality following exposure to MC-LR and provide a better understanding of the mechanisms of MC-LR-mediated male reproductive toxicity.

MATERIALS AND METHODS

Main chemicals

MC-LR with a purity of > 96% was purchased from Alexis Biochemicals (Lausen, Switzerland). The *in Situ* Cell Death Detection Kit (POD) was purchased from Roche Co. (Berlin, Germany). Column animal RNAout kits were purchased from Tiandz, Inc. (Beijing, China). Easyscript first-strand cDNA synthesis super mix was obtained from Beijing TransGen Biotech Co. Ltd. (Beijing, China). Primer pairs were purchased from Nanjing Genscript Biotechnology (Nanjing, China). DNase and Proteinase K were purchased from Sigma-Aldrich (Munich, Germany).

Rabbit anti Rat c-kit antibody and Alexa Fluor 594 goat anti rabbit IgG were purchased from Santa Cruz (LA, CA, USA). Mouse anti proliferating cell nuclear antigen (PCNA) antibody, Cy3 goat anti mouse IgG and mouse anti β -actin were bought from Abcam (Cambridge, United Kingdom). 4', 6-diamidino-2-phenylindole (DAPI), HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were obtained from Boster Biotechnology Co. Ltd. (Wuhan, China). All the reagents were of analytical grade.

Animals and treatment

Healthy male Sprague-Dawley rats ($n = 30$) weighting from 40-50 g and aged 17 to 18 days were obtained from Sino-British Sippr-BK Lab. Animal Ltd., Co. (Shanghai, China), and were housed in the Nanjing University animal facility. Rats were fed with commercial rat chow and water *ad libitum* and kept on a 12 hr: 12 hr light-dark cycle. After one week of acclimation, the 24-25-day rats were randomly divided into three groups (group A, B and C) with 10 animals in each group.

The Intraperitoneal (i.p.) lethal dose 50 (LD_{50}) of MC-LR in rat was determined by intraperitoneal injections according to the up-down method of small animals (Dawson, 1998). Rats in groups B and C received an

intraperitoneal injection of MC-LR at doses of 50 µg/kg (1/3LD₅₀) and 100 (2/3 LD₅₀) µg/kg body weight every day for 7 days according to the LD₅₀ and the previous studies (Miura *et al.*, 1991). The rats in the control group (group A) were injected daily with 7.5 ml/kg body weight 0.9% saline solution. MC-LR was dissolved in a minimum amount of methanol (0.1%) and diluted to the required concentration in normal saline. The final volume was equal to the saline volume used in the control group. The animals were handled in accordance with guidelines approved by the Experimentation Ethics Review Committee of Nanjing University (ethics approval no. A9089).

Histopathological evaluation

The testes were fixed over night at 4°C in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.4). They were dehydrated successively using 30%, 50%, 75%, 80%, 85%, 90%, 95% and 100% ethanol. Then they were embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin (H&E) for light microscopic examination.

Tubular diameter measurements

The method used for determining tubular diameter was based on previously published methods (Katerina Kovačević, 2006). Testes from 24-25-day rats (5 rats from each group) exposed to MC-LR for 7 days were cut on a microtome at 5 µm and stained with hematoxyline and eosin using standard procedures. Seminiferous tubular diameter was measured at 20x magnification using the Image-Pro plus Version 6.0 software. The diameters of 50 randomly chosen tubules were measured in each testis from each animal. Only tubules considered round by eye examination were used for the diameter measurement to avoid oblique cutting of tubules. The testis relative weights were calculated according to the observed net weight gain and testis absolute weights.

Measurement of testicular cell apoptosis via TUNEL staining

TUNEL was carried out following the producer's directions. The paraffin embedded testicular tissues were sectioned at 5 µm and placed on the glass slides at 37°C over night, followed by storage at 4°C until standard processing. Briefly, 5 µm sections of testicular tissues were stained by H&E followed by deparaffinization, rehydration, fixation in 4% paraformaldehyde in PBS, permeabilization with Proteinase K, pre-equilibration, and labeling of DNA strand breaks with reaction mixture for 1 hr. Samples were visualized after DAPI nuclear stain in the mounting medium using a confocal microscope

(Fluoview FV10i, Japan) to detect the localized green fluorescence of apoptotic cells.

Positive TUNEL POD labeling was prepared using DNase treatment. After pretreatment, histological sections were incubated with DNase at 37°C for 10 min to induce DNA strand breaks. Negative control was carried out by omitting terminal transferase from the labeling procedure (label solution only instead of TUNEL reaction mixture).

Detection of testicular cell proliferation via immunohistochemistry

Sections were mounted on slides. Before incubation with primary antibodies, sections were dewaxed, rehydrated in graded ethanol, washed in water and PBS followed by blocking endogenous peroxidase by incubating the section for 10 min in 3% H₂O₂. After disrupting cell membranes with 0.3% Triton X-100, the sections were incubated in blocking buffer containing 3% bovine serum albumin for 30 min. Mouse anti-PCNA antibody (1:200) was applied in a humidified atmosphere and incubated overnight. The fluorochrome-conjugated secondary antibody (1:200) was added and samples were incubated for 60 min at 37°C. Nuclei were counterstained with 5 µg/ml DAPI. Finally, slides were mounted with glycerin and visualized using a confocal microscope.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from each sample using Column Animal RNAout according to the manufacturer's instructions. 2 µl total RNA was eluted with 48 µl RNase-free water and purity was determined by the quotient of OD at 260/280 nm. The Easyscript first-strand cDNA synthesis super mix kit was used for reverse transcription polymerase chain reaction (RT-PCR). The resultant cDNA was stored at -20°C.

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>) to amplify a 150-300 bp section. The sequences of the primer pairs used in this assay are shown in Table 1. Specification of each pair of primers was tested by randomly sequencing four clones, and further confirmed by the melting curve analysis using Q-PCR. Only primers with similar amplification efficiency were used in this experiment. Q-PCR was conducted by amplifying 50 µl of diluted cDNA with the SYBR Green Q-PCR kit (Roche) with 7300 Real-Time PCR System (ABI, NY, NY, USA). Cycling conditions were as follows: 10 min at 95°C, 40 cycles of 20 sec at 94°C, 25 sec at 60°C, and 30 sec at 72°C. Melting curve analysis of the

Table 1. Primer used for Q-PCR

Target gene	Primer sequence (5'-3')		Size (bp)
	Forward	Reverse	
c-kit	GCATCACCATCAAAAACGTG	GATAGTCAGCGTCTCCTGGC	332
TH2B	TGAGACGTTGGAGTGGACAA	GTA ACTCTCTTCGCGGCATC	152
TP2	GGCCTCAAAGTCACACCAAT	TTCCCTTCCAAGGTCTTCCT	205
GAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA	118

Fig. 1. Effect of MC-LR on the testicular microstructures of male rats. Paraffin sections were prepared from testes of rats exposed to saline (A), MC-LR at 50 µg/kg (B) and 100 µg/kg (C) for 7 days. The sections were stained with H&E. Bar = 100 µm. In comparison with control, the spermatogenic epithelium became sparse following treatment with MC-LR at 100 µg/kg. The structure of the spermatogenic epithelium was lost, deranged, thinner and blocked after treatment in a dose-dependent manner. The black arrow shows blockage of the seminiferous tubules, while the red arrow shows sparse seminiferous tubules and loss of spermatogenic cells.

amplification products was carried out at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in triplicate, 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 is reported to be not affected by MCs (Chen *et al.*, 2005; Hudder *et al.*, 2007). After completion of the PCR amplification, data were analyzed by the 7300 Sequence Detection Software (ABI).

Statistical analysis

The data were expressed as the mean ± standard error (SE). All calculations and statistical analyses were performed using SPSS for windows version 11.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze the difference between groups, followed by the Dunnett’s t-test. *P* < 0.05 was regarded as statistically significant.

RESULTS

Disrupted histopathological structures of rat testicles

After exposure to MC-LR, rat testicular tissue structure was observed through paraffin sections by H&E staining (Fig. 1). A regular arrangement of spermatogenic epithelium in seminiferous tubules in control tissues was seen (Fig. 1A). The results demonstrated that the pathologic findings in the 50 µg/kg group changed slightly with no significant difference (Fig. 1B). The spermatogenic epithelium appeared loosened in its organization, and blockage of the seminiferous tubules and the spermatogenic cell arrangement disorder were evident in group C which was treated with 100 µg/kg MC-LR. In addition, this high dose treatment resulted in sparse seminiferous tubules and loss of spermatogenic cells (Fig. 1C).

The effect of MC-LR on tubular diameter

Compared to the control group we observed a significant (*P* < 0.05) decrease in the mean absolute weight of the testes in rats treated with MC-LR (Table 2). At the

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Table 2. Effect of MC-LR on body weight and testes weights of male rats in comparison to control rats

Groups	Net weight gain(g)	Testis absolute weights(g)	Testis relative weights (g/100g weight)
0 µg/kg MC-LR (n = 10)	98.03 ± 3.0	0.85 ± 0.02	0.88 ± 0.04
50 µg/kg MC-LR (n = 10)	91.75 ± 3.6	0.75 ± 0.03*	0.82 ± 0.03
100 µg/kg MC-LR (n = 10)	85.42 ± 3.0 *	0.56 ± 0.03**	0.65 ± 0.04*

Data are shown as mean ± S.E.

* Response that is significantly different from the control ($P < 0.05$).

** Response that is significantly different from the control ($P < 0.01$).

Fig. 2. Effect of MC-LR on the seminiferous tubular diameter of male rats. Samples were prepared as described in Fig. 1. Seminiferous tubular diameters were measured by Image-pro Plus. (A) A schematic diagram of diameter measurement shown in pixels. (B) Bars represent mean ± S.E. Asterisk denotes a response that is significantly different from the control (** $P < 0.01$). Tubular diameters showed no significant differences at 50 µg/kg but significantly decreased at 100 µg/kg MC-LR.

same time the relative weight of the testes and the net weight gain mean in rats treated with 100 µg/kg MC-LR were significantly lower than those in control group. The seminiferous tubular diameter was measured to determine whether MC-LR could affect spermatogenic cells (Fig. 2). Compared to the control, the tubular diameter remained unchanged at 50 µg/kg. But the tubular diameter was significantly decreased following treatment with 100 µg/kg compared with the control ($P < 0.01$).

The effect of MC-LR on testicular cell apoptosis

In comparison with the control, the TUNEL positive staining revealed no significant difference in the number of apoptotic cells (arrow) at 50 µg/kg MC-LR; however, there was a dramatic increase in the number of apoptotic testicular cells at the highest doses of MC-LR (100 µg/kg). Almost all apoptotic cells were located in spermatogenic cell niches (Fig. 3).

The effect of MC-LR on testicular cell proliferation

The effect of MC-LR on testicular cell proliferation was determined by immune fluorescence staining of PCNA (Fig. 4). No major difference was observed between control and MC-LR at 50 µg/kg. The number of proliferating cells (arrow) within the testis, which form the major spermatogenic cells, significantly decreased at the highest MC-LR concentration (100 µg/kg) (Fig. 4C).

The effect of MC-LR on the gene expression of spermatogenic cells

Gene-specific mRNA abundance in spermatogenic cells from rats exposed to various MC-LR concentrations was quantified by Q-PCR. As shown in Fig. 5, the mRNA levels of both TH2B and TP2 were decreased at 50 µg/kg MC-LR and the reduction was much more pronounced at 100 µg/kg exposure. The gene expression of c-kit was remarkably up-regulated to as high as 4 times of

Fig. 3. Effect of MC-LR on apoptosis of spermatogenic cells. Paraffin sections were prepared from testes of rats exposed to saline (A), MC-LR at 50 µg/kg (B) and 100 µg/kg (C) for 7 days. The sections were stained by TUNEL staining. At 100 µg/kg, the number of apoptotic spermatogenic cells (arrow) significantly increased. (Bar = 100 µm). (D) The percentage of TUNEL positive testicular cells is plotted. The TUNEL positive histogram represents the apoptosis rate in the testes of male rats after treatment with MC-LR for 7 days (mean ± S.E.). Asterisk denotes a response that is significantly different from the control (** $P < 0.01$, $n = 5$).

the control at 100 µg/kg. Meanwhile, expression of TP2 was decreased by 25 times (Fig. 5).

DISCUSSION

The reduced fertility of animals and humans as a result of environmental exposure has drawn the attention of WHO, the scientific community, and the public (Swan and Elkin, 1999). Chen and his colleagues found that large amounts of MCs are accumulated in the gonads and ovaries of aquatic fauna in the Chaohu Lake, China (Chen *et al.*, 2005). MCs have been demonstrated to be toxic to the male reproductive system through acute, sub-acute, and chronic low-dose exposures (Chen *et al.*, 2011;

Li *et al.*, 2008). At low concentrations of MC-LR, the increase of sperm abnormalities is accompanied by the decrease in sperm concentration, sperm motility and testicular weight. However, the mechanisms of MC-induced sperm quality decline remain unclear. Spermatogenesis is a highly organized process involving spermatogonial proliferation, spermatocyte meiosis, and morphological alterations in spermatids (White-Cooper and Bausek, 2010). Disruption in any one of such processes will compromise sperm quality. Spermatogenic cells are the core cells in the process of spermatogenesis. These findings prompted us to investigate whether sperm abnormality was associated with injury of spermatogenic cells in the early stage after exposure to MC-LR. To address this question, the

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Fig. 4. Effect of MC-LR on the proliferation of spermatogenic cells. Paraffin sections were prepared from testes of rats exposed to saline (A), MC-LR at 50 µg/kg (B) and 100 µg/kg (C) for 7 days. The sections were stained by PCNA and DAPI. The number of proliferating cells (arrow) within the testis which were major spermatogenic cells significantly decreased at the highest dose of MC-LR.

Fig. 5. Relative expression of the differentiation genes in spermatogenic cells. Tissues from testes of rats exposed to saline, MC-LR at 50 µg/kg and 100 µg/kg for 7 days were removed followed by mRNA purification, reverse transcription and Q-PCR for the detection of the c-kit, TH2B and TP2 genes. c-kit was upregulated whereas both TH2B and TP2 were down-regulated. Data are expressed as mean ± S.E. Asterisk denotes a response that is significantly different from the control (* $P < 0.05$, ** $P < 0.01$, $n = 5$).

reproductive toxicity of MC-LR in male rats was examined following daily exposure to 50 or 100 µg/kg MC-LR for 7 days based on the structural damage of the testicular seminiferous tubules. Our results were consistent with previous reports (Chen *et al.*, 2011; Li *et al.*, 2008). These results provide a basis for the subsequent experiments.

Male germ cell death is found most commonly in the spermatogonia, but occurs throughout spermatogenesis (Nakagawa *et al.*, 1997). Degeneration of spermatogenic cells may be a mechanism for eliminating abnormal cells. Recent studies provide evidence that the degeneration of spermatogenic germ cells involves apoptosis

(Nakagawa *et al.*, 1997). Spermatogenic cell apoptosis occurs spontaneously in response to endocrine disruption and exposure to various environmental agents and chemicals (Billig *et al.*, 1995; Brinkworth *et al.*, 1995). According to the study by Russell, there is a positive correlation between seminiferous tubular diameter and spermatogenic activity (FRANÇA LR, 1998). Degenerated tubular diameter could reflect reduced population size of spermatogenic cells. Seminiferous tubular diameter was measured using Image-pro plus to detect whether the number of spermatogenic cells would be affected after exposure to MC-LR for 7 days. Regarding tubular diameter, weight

of the testes is also interested. The lower relative weight of the testes further pronounced the degenerated tubular diameter. Our results showed that the tubular diameter was significantly reduced in male rats exposed to MC-LR especially at 100 µg/kg MC-LR.

MCs are potent inhibitors of specific protein phosphatases (Craig *et al.*, 1996). Recently, many enzymes have been reported to be critically involved in the signal transduction of MC toxicity (Campos and Vasconcelos, 2010). These enzymes are suggested to be involved in both the apoptotic effect and the tumor-promoting activity of MC-LR in hepatocytes and various other cell lines (Boaru *et al.*, 2006; Campos and Vasconcelos, 2010). In our present fluorometric TUNEL analyses, apoptosis was observed mainly in the spermatogenic cells within seminiferous tubules. Apoptosis dramatically increased in response to treatment with MC-LR in a dose-dependent manner. Meanwhile, no Leydig cell apoptosis was detected in the present study. Testosterone is mainly regulated by Leydig cells. However, declines of testosterone after MC-LR exposure were found in our previous study (Chen *et al.*, 2011; Li *et al.*, 2008). Hypothalamic-pituitary-gonadal axis is a most important pathway for endocrine regulation. Hypothalamic Gonadotrophin releasing hormone (GnRH) stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary and plays a key role in the neurohormonal control of reproduction (Counis *et al.*, 2005). LH binds to the receptors in Leydig cells to maintain general metabolic processes and steroidogenic enzymes to regulate the production of testosterone (Dufau, 1988). *In vivo* results showed that the levels of testosterone and GnRH decreased significantly (Wang *et al.*, 2012). In addition, it has been proven that MC-LR has significant toxic effect on hypothalamic GnRH neurons. Furthermore, results from our lab demonstrated that MC-LR affected serum testosterone levels through damaging GnRH neurons (unpublished). Therefore, we speculate that MC-LR may affect the secretion of testosterone by damaging the hypothalamic-pituitary-gonadal axis. Further investigations are required to clarify this. Further investigations are required to clarify this.

To determine whether there are any changes in the proliferation of spermatogenic cells, we used immunohistochemical staining with antibodies against PCNA, a marker for cell proliferation (Katerina Kovačević, 2006; Nakagawa *et al.*, 1997). Spermatogenic cells and Sertoli cells both can proliferate postnatally. Sertoli cells proliferate only postnatally and their proliferation normally ceases by day 18 (RM, 1994). A previous study has reported that all Sertoli cells are negative for this marker on day 19 postnatally (Katerina Kovačević, 2006). Therefore, Serto-

li cells were basically no longer proliferating due to rat aging around day 19 in our experiment. MC-LR treatment at 100 µg/kg clearly reduced PCNA positive cells which included mainly spermatogenic cells. These results were consistent with the observation in liver where cell apoptosis and proliferation are shown to be affected by MC-LR (Brzuzan *et al.*, 2009; Dias *et al.*, 2010; Huang *et al.*, 2011).

Biochemical studies have demonstrated that male germ cell differentiation is achieved through sequential expression of genes that can be used as markers for different types of germ cells (Dym *et al.*, 1995; Wu *et al.*, 2000). The c-kit receptor as a specific spermatogonia maker is used frequently in many studies (Schrans-Stassen *et al.*, 1999). The presence of pachytene spermatocytes is confirmed by detection of the TH2B gene (Marret *et al.*, 1998). The detection of a high level of transition protein 2 (TP2) mRNA confirms the differentiation of germ cells into round spermatids (Alfonso and Kistler, 1993; Marret *et al.*, 1998). Therefore, c-kit, TH2B and TP2 were chosen to be differentiation markers for investigating whether MC-LR exerts toxicity on the specific stage differentiation of spermatogenic cells. We found that the gene expression levels of TH2B and TP2 were both decreased, while c-kit was increased after exposure to MC-LR. It has been reported that c-kit is highly expressed in undifferentiated spermatogonia but undetectable in differentiated spermatogonia. Elevated c-kit expression may suggest that MC-LR can block spermatogonial differentiation. Down regulation of the genes TH2B and TP2 was accompanied by reduction of pachytene spermatocytes and round spermatids, respectively. It is reported that at nanomolar concentrations MC-LR stimulates cell cycle progression in the Vero-E6 kidney cell line via the activation of the pro-proliferative ERK1/2 pathway (Dias *et al.*, 2010). MCs affect the expression of cellular differentiation proteins in human amniotic epithelial cells (Fu *et al.*, 2009). These findings support our data and we may infer that the formation of spermatocytes and round spermatids was hindered by MC-LR. It is speculated that MC-LR influences sperm quality through its effect on spermatogenic cells. Our *in vitro* study on spermatogonia has showed that MC-LR might lead to cytotoxicity through oxidative stress, which may play an important role in cell injury (Zhou *et al.*, 2012). Our *in vivo* experiments are more in line with the natural exposure, which confirms the *in vitro* findings regarding the reproductive toxicity of MC-LR on spermatogonia. It is suggested that MCs can cause damage to Sertoli cells directly through the Fas/FasL system *in vivo* (Xiong *et al.*, 2009). We may also hypothesize that MC-LR may also damage spermatogen-

ic cells via poisoning Sertoli cells because these cells provide nutrition and support to spermatogenic cells in various developmental stages.

In conclusion, we confirmed that MC-LR affected the apoptosis, proliferation and differentiation of spermatogenic cells, which may present a mechanistic account for the declines in sperm quantity.

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