DOI 10.1002/pmic.201100214 *Proteomics* 2012, *12*, 300–312

RESEARCH ARTICLE

The proteomic study on cellular responses of the testes of zebrafish (*Danio rerio*) exposed to microcystin-RR

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Received: April 24, 2011
Revised: October 23, 2011
Accepted: October 26, 2011

Microcystin-RR (MC-RR) is a commonly encountered cyanotoxin and receives increasing attention due to the risk of its bioaccumulation in aquatic animals like fish. This study investigated the protein profiles of zebrafish (*Danio rerio*) testes after intraperitoneal injection (i.p.) with 0.5 LD_{50} (2000 μg/kg). MC-RR caused a noticeable damage to testicular ultrastructure, showing widened intercellular junction, distention of mitochondria. The testes showed a rapid response of its defense systems to the oxidative stress caused by MC-RR. This is the first to use a proteomic approach to obtain an overview of the effects of MC-RR on the testes of zebrafish. The proteomic results revealed that toxin exposure remarkably altered the abundance of 24 proteins that were involved in cytoskeleton assembly, oxidative stress, glycolysis metabolism, calcium ion binding and other biological functions. In conclusion, MC-RR damaged the testes and was toxic to the reproductive system of male zebrafish mainly through causing oxidative stress.

Keywords:
2-DE / Animal proteomics / MC-RR / Reproductive toxicity / Testis / Ultrastructure

1 Introduction

Cyanobacterial blooms of freshwaters represent serious problems throughout the world due to the production of cyanotoxins [1]. One of the most studied groups of cyanotoxins is the cyclic heptapeptide hepatotoxins called microcystins (MCs). Up to now, there have been over 80 analogues of MCs [2], among which MC-LR, MC-RR and MC-YR are most commonly present.

MCs are intracellular but may rapidly and massively be released by cell lyses due to natural senescence, herbicides or physical stress [3].The mechanisms of MC toxicity have not been fully elucidated but most likely involve protein phosphatase (PP1 and PP2A) inhibition leading to the disruption of the dynamic equilibrium of protein phosphorylation/dephosphorylation [4]. And MCs could induce production of reactive oxygen species (ROS) [5], followed by oxidative stress and apoptosis or necrosis depending on the exposure concentration and duration [6–8]. Toxicity of MCs has been reported at environmental exposure levels for several fish species, including catfish [9], carp [10], zebrafish [11] and salmon [12]. Both field and laboratory studies indicated that MCs affect various fish organs, such as kidney, liver, heart, gills and intestine and invoke hepatotoxicity and neurotoxicity, kidney impairment and gastrointestinal disorders [13–19]. Recently, several laboratory studies have demonstrated that MCs exert negative effects on the reproductive system of male mammalians [20–22], for example, Ding et al. [21] found that MC-LR exposure at a dose of 6.67 μg/kg body weight (BW) reduced mature sperm in seminiferous tubules of mice and induced testicular atrophy. However, molecular mechanisms underlying such reproductive toxicity of MCs are still unclear.

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Abbreviations: BW, body weight; **LPO,** lipid peroxidation; **MC,** microcystin; **MC-LR,** MC-leucine-arginine; **MC-RR,** MC-arginine-arginine; **MDA,** malondialdehyde

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Proteomics is a relatively large-scale study that boosts our understanding of toxicology by providing global patterns of protein content and activity. In reproductive biology, proteomics is emerging as a tool for defining critical protein and pathways like MCs exposure [23]. Key elements of classical proteomics are the separation of proteins in a sample using 2-DE and subsequent identification by biological MS. Zebrafish is an excellent model to study toxic disturbances produced by pollutants and natural toxins. It has been reported that MCs could damaged the liver and brain of zebrafish [24, 25] and revealed that several proteins involved in different pathways such as oxidative stress, cytoskeleton organization and metabolism, were all modulated by chronic effects of MC-LR. These studies demonstrated that proteomics provides a new insight into MC toxicity in both aquatic organisms and human beings, and some proteins may be biomarker for further MCs toxicity research. However, the effects of MC-RR on aquatic organisms have been studied less extensively, despite of their wide distribution and abundance in the environment. The main purpose of this study was to use proteomics to evaluate cellular responses in zebrafish testes exposed to MC-RR. Ultrastructural and biochemical changes of the testes were examined, and toxin content in testis was also measured. This study is expected to provide proteomic evidences on the reproductive toxicity of MC-RR on animals using a model fish.

2 Materials and methods

2.1 Toxin

MC-RR used in the experiment was extracted and purified from the freeze-dried surface blooms collected from Lake Dianchi, Yunnan, China. The microcystins were extracted by the methods of Wang et al. [26]. MC-RR was separated by semi-performance preparative liquid chromatography system (Waters 600E, USA) and pure MC-RR was obtained. MC-RR was analyzed for MCs content via a reverse-phase HPLC (LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). MC- RR (purity > 95%) concentrations were identified by their UV spectra and retention times, and by using a commercial standard microcystin-LR, RR (Wako Pure Chemical Industries, Japan) to compare the peak areas of the test samples. MC-RR was finally suspended in salt solution (0.8% NaCl).

2.2 LD_{50} of pure microcystin-RR

To determine the median lethal dose (LD_{50}) and to select relevant MC-RR exposure dose for the following experiments, male zebrafish ($n = 100$) were divided into five groups, and fishes were administered by an intraperitoneal injection of MC-RR at different doses of MC-RR equivalent/kg BW. LD_{50} was calculated from a linear regression of log-probit transformations of the dose–response data [27].

2.3 Animals and treatment protocol

Adult healthy male zebrafish (*Danio rerio*) at 18-wk-old (AB strain) were obtained from Institute of Hydrobiology, Academy of Science, China. Zebrafish were maintained at $28 \pm 0.5^{\circ}C$ in charcoal-filtered recirculating aerated tap water; the fish were fed freshly hatched Artemia nauplii twice daily and flake food (Tetra, Germany) once daily. Briefly, zebrafish were maintained in a semi-static system that received charcoal-dechlorinated tap water with a constant temperature of $28 \pm 0.5^{\circ}C$. The photoperiod was adjusted to a 14:10 h (light:dark) cycle.

Healthy male zebrafish weighed almost 0.5 ± 0.1 g were randomly divided into two groups. One of the groups were administrated by intraperitoneal injection of 10 μL MC-RR at about 0.5 LD_{50} (2000 μg/kg BW) MC-RR. Control ones received by the same equivalent volume of 0.8% saline solution. Three sampling points were set during a period of 24 h in the whole experiment (2, 6 and 24 h). For each group, at each sampling point, 27 fish were anaesthetized with 0.03% tricaine (MS-222) and decapitated and the testes were collected for further study. All of the experimental researches on zebrafish were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory and approved by the Animal Ethics Committee in the Institute of Hydrobiology, Chinese Academy of Sciences.

2.4 Biochemical analysis

The testes were homogenized (1:10, w/v) in a cold (4°C) buffer solution (pH 7.5) containing sucrose (250 mM), PMSF (1 mM), DTT (1 mM; Sigma), and EDTA (1 mM). Homogenates were centrifuged at $12\,000 \times g$ (4°C) for 15 min and the supernatants used as the enzyme source.

The activities of SOD, CAT, GST, GPx, and the contents of GSH were assayed by the kits supplied by the Nanjing Jiancheng Bioengineering Institute, China. Superoxide dismutase (SOD) activity assay was based on the method described by Bayer and Fridovich [28]. CAT activity was determined by measuring the rate of disappearance of H_2O_2. GST activity was detected by evaluating the conjugation of GSH with the standard model substrate 1-chloro-2,4-dinitrobenzene (CDNB) according to the method of Habig et al. [29] and Habig and Jakoby [30]. We used H_2O_2 as the substrate to determine the GPx activity according to Drotar et al. [31]. GSH content expressed as μg/mg protein according to Griffith [32] method. Protein contents were determined by the Coomassie blue method using bovine serum albumin (BSA) as a standard.

The concentration of malondialdehyde (MDA) was used as an index of lipid peroxidation (LPO) and was determined according to Ohkawa et al [33]. The content of MDA and H_2O_2 were assayed by the kits supplied by the Nanjing Jiancheng Bioengineering Institute, China. All the experiments were carried out in triplicate.

2.5 Transmission electron microscopic observation and lipid staining

For transmission electron microscopy (TEM), specimens of the testes tissues were prefixed in 2.5% glutaraldehyde solution, diced into 1 mm^3, washed overnight in the 0.1 M phosphate buffer (pH 7.4) and postfixed in 1% OsO_4 buffered for 1 h at 4°C. The fragments were dehydrated in an ethanol ascending series and propylene oxide and embedded in Epon 812. Ultrathin sections were cut with glass knives on an LKB-V ultramicrotome (Nova, Sweden), stained with uranylacetate and lead citrate and observed in a Jeol JEM-1230 TEM.

Testes from control groups and treated groups were fixed in 4% paraformaldehyde and embedded in agarose, and cryosectioned (10 μm). The sections were routinely stained with haematoxylin and eosin. To find out the fatty droplet accumulation the sections were stained with oil red O (Sigma-Aldrich).

2.6 Extraction and determination of MC-RR in testes at 6 h after exposure

Extraction and quantitative analysis of the MC-RR in testes (0.2 g lyophilized sample (about 50 fish) for each groups) of zebrafish basically followed the method of Xie et al. [10].

2.7 Proteomic analysis

Protein extraction was performed basically according to the method reported by Tay et al. [34] with minor modification by Li et al. [35]. Briefly, the frozen gonad tissues of each five fish at 6 h MC-RR exposure were mixed and homogenized in the lysis buffer (2 M thiourea, 7 M urea, 50 mM DTT, 4% CHAPS, 50 mM Tris base, 0.2% carrier ampholyte, 1 mM protease inhibitor cocktail, 1% RNase and 1% DNase) and the supernatant was obtained at 12 000 × *g* for 1 h at 4°C, keeping the supernatant for 2-DE. Three pools were prepared. Protein concentration was determined by the Bradford assay using BSA standards.

The prepared pooled protein samples (450 μg of each protein sample) were mixed with a rehydration buffer to a volume of 450 μL. The first dimension was performed on IPG strips of linear pH gradient 4–7 24 cm (BioRad, USA) in a PROTEAN IEF cell (BioRad) using the following program:30 min at 250 V, 30 min at 500 V, 3 h at 4000 V, 5 h at 4000 V. After completion of IEF, strips were incubated in equilibration buffer (6 M urea, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 2% SDS) containing 2% DTT for 15 min at room temperature, followed by the same buffer with 2.5% iodoacetamide for 15 min. Subsequently, second-dimension vertical 12% SDS-PAGE gel slabs were freshly prepared between glass plates. Electrophoresis was carried out at 100 V/gel for 30 min, followed by about 10 h run at 350 V/gel until bromophenol blue fronts reached the gel bottom. The gels were then visualized by silver staining.

Following electrophoresis, a GS-800 (BioRad) was used to scan the gels. Spot detection and matching were performed using the PDQuest software (BioRad). Triplicate 2-DE gels were performed for each group and then were combined into average gels, which represented spots that were reproducibly present on each set of the triplicate gels. The spots that were changed conspicuously in the control groups or exposure groups were then selected for further MS analysis. The selected spots changed about twice were analyzed with a MALDI TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, USA). The specific processes or functions of the identified proteins were then identified by searching Gene Ontology (http://www.Geneontology.org).

2.8 Western blot analysis

The zebrafish testes exposed 6 h were homogenized for extract proteins in ice-cold protein extraction buffer (Beyotime Institute of Biotechnology, China). Each set of five fish was pooled for protein preparation. The homogenates were centrifuged for 10 min at 12 000 × *g* and supernatants were collected. The concentrations of protein were determined by the Bradford method.

About 20 μg of protein from each sample was denatured, electrophoresed, and transferred onto a PVDF membrane (Millipore). The membrane was blocked and blots were incubated in specific antibody against keratin 18, tubulin β, hsp90, citrate synthase (Abcam, UK) and GAPDH (Bioworld, USA), and then secondary antibodies following the manufacturer's instructions. ECL reagent (Millipore) was applied to the membrane for 1 min. FluorChem Q (Alpha Innotech) system was used to evaluate the protein signal. The results of Western blot were quantified with Quantity One software (BioRad).

2.9 Statistical analysis

Statistical analysis was undertaken using SPSS 13.0 for Windows. All values expressed as mean ± SE were subjected to one-way analysis of variance (ANOVA).Differences in mean values between groups were assessed and were considered statistically different at $p < 0.05$, $p < 0.01$.

3 Results

3.1 LD_{50} of MC-RR in zebrafish

The effect of MC-RR on zebrafish mortality is shown in Table 1. Accordingly, the 24 h LD_{50} of the MC-RR (i.p.) in zebrafish was calculated to be about 4000 μg MC-RR/kg BW.

3.2 Biochemical analysis

H_2O_2 and Levels of LPO (liquid-phase oxidation) in zebrafish testes are shown in Fig. 1A and B. MDA is a well-known oxidation product of polyunsaturated fatty acids in lipoproteins and is often used as a biomarker of oxidative stress. MDA is formed like an end lipid peroxidation product which reacts with TBA reagent under acidic conditions to generate a coloured product. Peroxidative damage may be vulnerable in testes which are rich in unsaturated lipids. The concentration of MDA, as an index of lipid peroxidation, significantly increased after injection of MC-RR. The MDA content reached a maximum at 6 h (183%) after injection, but recovered slowly at 24 h. The maximum level of H_2O_2 (a species of ROS) markedly increase (193%) at 24 h after exposure to MC-RR.

Figure 1C–F also shows the levels of the antioxidant enzyme SOD, CAT, GST and GPx, in testes of zebrafish. SOD significantly increased at all time, and the increases peaked at 2 h (363%). CAT also increased during the whole exposure time, at 6 h the increases reached 236% compared with the control groups. GST activity was elevated to 187% at 6 h post-exposure when compared with the controls. GPx activity increased conspicuously at 2 and 6 h, with a slight recovery at 24 h. While GSH content was reached to 229% at 6 h post-injection of MC-RR (Fig. 1G). GSH content was still higher than in the control groups at 24 h exposure. Based on the above results, 6 h post-exposure was selected for the subsequent research.

3.3 Transmission electron microscopic observation and lipid staining

In zebrafish testes, spermatogonia were randomly distributed along the entire length of the tubule, thus forming an unrestricted spermatogonial testis. Within each cyst, the maturation of the germ cells was synchronous, until the mature sperm was released into the tubular lumen. The cysts contained spermatogonia and spermatocytes at different stages of development (Fig. 2A).

In the control group (Fig. 2A, D, and G), a cluster of spermatogonia were surrounded by cytoplasmic processes of Sertoli cells and had intact plasmalemma. The mitochondria were ovoid or spherical, and their cristae and matrix were clear. The Sertoli–spermatogonia junction was normal. The peripheral membrane of the sperm was integrated.

In the MC-RR-treated group (Fig. 2B, C, E, F, and H) the mitochondria proceeded to lose cristae and matrix, and lost the metrical density with highly hydropic changes at 6 h. Dilation and vesiculation of cisternae of endoplasmic reticulum were prominent after 6 h exposure. And large digestive vacuoles found in the testes of the MC-RR-treated zebrafish. The widening of Sertoli–spermatogonia cell junction was conspicuously observed. The swelling, disruption and lysis of the mitochondria were observed. The

Table 1. The effect of MC-RR on zebrafish mortality

Groups	Number of fish	Dose (μg/kg)	Number of death	Mortality
1	20	8000	20	1
2	20	5656	16	0.8
3	20	3999.9	8	0.4
4	20	2828	6	0.3
5	20	1999.8	0	0

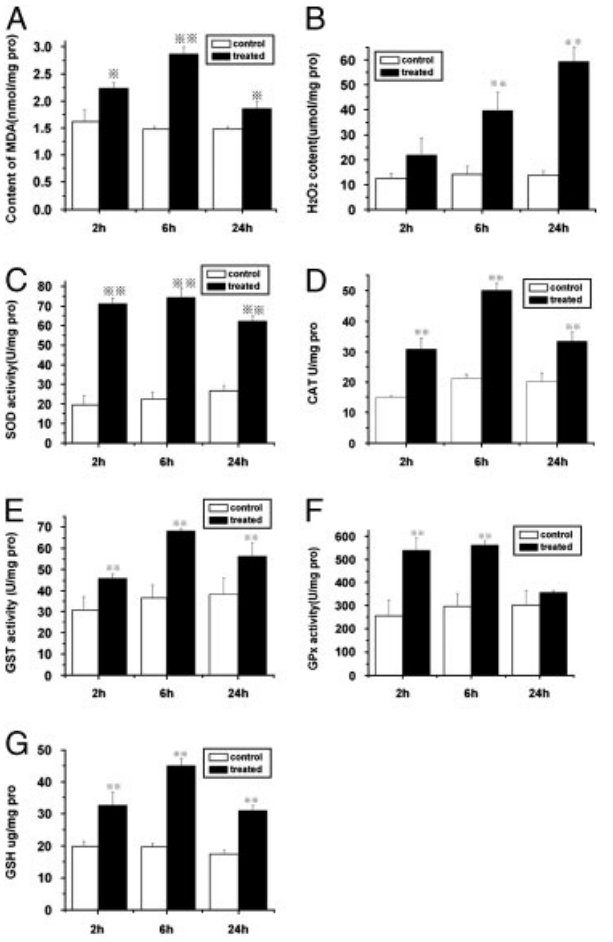

Figure 1. The MDA,H_2O_2 and GSH level and the activities of antioxidant enzymes (SOD, CAT, GST, GPx) in testes after an i.p. injection with 0.5LD_{50} MC-RR until 24 h. The values are expressed as mean ± SE ($N = 3$). Each value is mean ± S. D. $p < 0.05$ (※), $p < 0.01$ (※※).

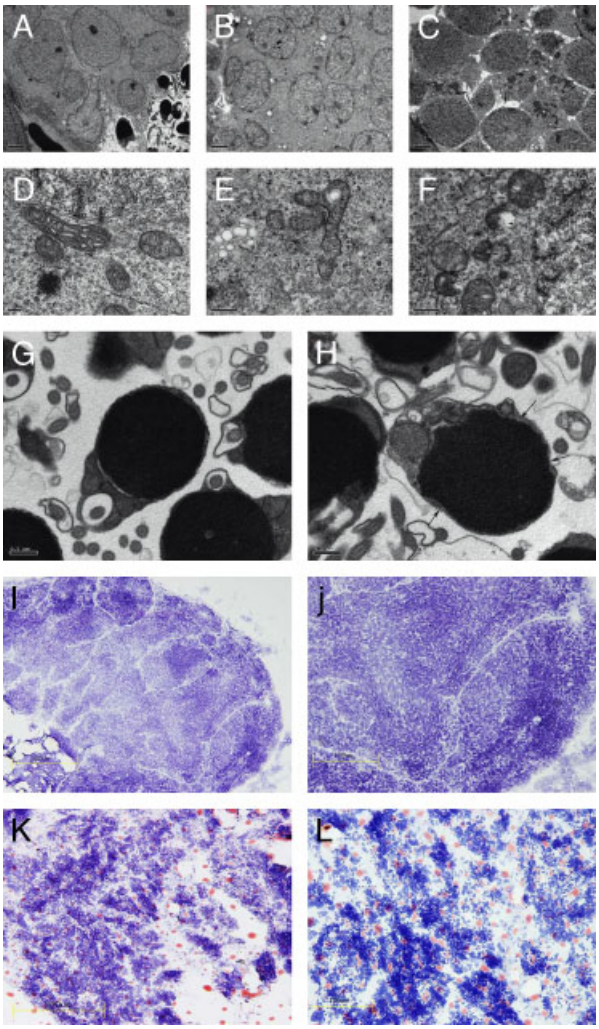

Figure 2. Ultrastructure changes in testes of zebrafish after an i.p. injection with $0.5LD_{50}$ MC-RR for 6 h. (A) Control $\times$5000 Sg, spermatogonia; Sp, sperm; Se, Sertoli cell; Bc, basal cell; (B) Treated $\times$5000 Large lipid droplets found in the testes of MCRR-treated zebrafish (arrow heads). (C) Treated $\times$5000 showing the widening of the cell junctions. (D) Control $\times$40 000 showing normal mitochondria. (E) Treated $\times$30 000 showing the swollen mitochondria and large lysosomal elements (arrow-heads). (F) Control $\times$20 000 nomal sperm cells. (G) Treated $\times$20 000 showing irregular peripheral membrane of the sperms (arrowheads) Lipid accumulation was confirmed by oil red O staining. control (I, 300 $\times$; J, 600 $\times$), treated (K, 300 $\times$; L, 600 $\times$).

peripheral membrane of the sperm was irregular and damaged. The oil red O staining (Fig. 2I, J, K, and L) revealed that the vacuoles contain lipids. The lipid droplet accumulated in the testis cells.

3.4 Effect of MC-RR on toxin accumulation

In the control group, no MC-RR was detected in testes tissues. At 6 h post-injection, MC-RR content in testes were

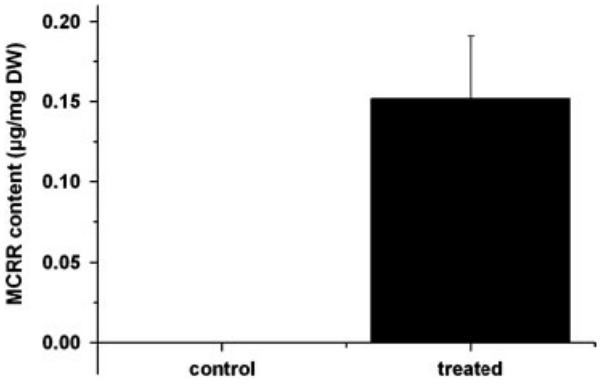

Figure 3. MC-RR contents in the testes tissues of zebrafish *Danio rerio* after 6 h of $0.5LD_{50}$ MC-RR exposure. Data are expressed as mean values $\pm$ SD ($n = 3$).

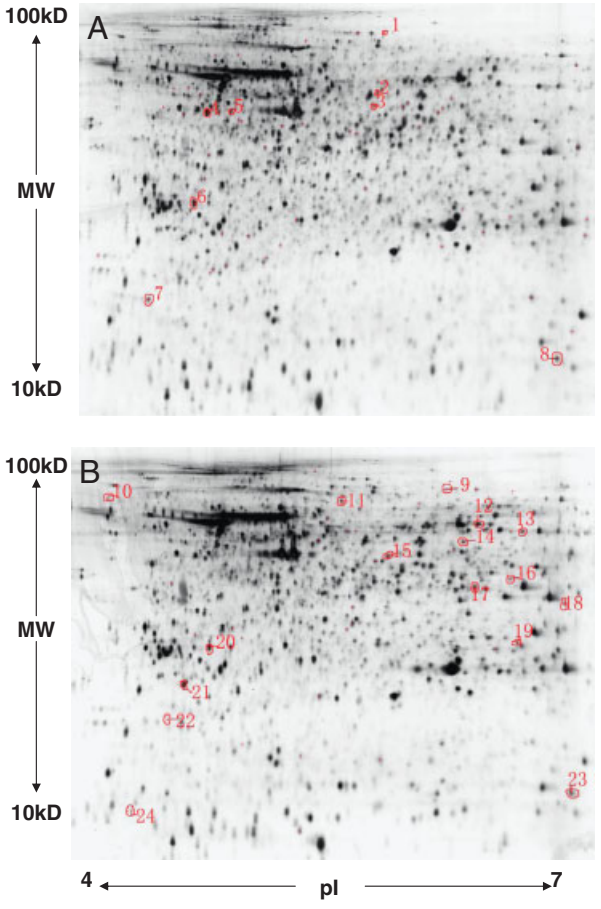

Figure 4. Representative 2-DE gels of control and treated samples. (A) Control (B) 0.5 LD_{50} 2000 μg/kg. Whole soluble proteins from zebrafish testes were separated by 2-DE and visualized by silver staining. The protein spots altered by MC-RR exposure are labeled with numbers. The spots circled were analyzed by MALDI-TOF. Each gel is representative of three independent replicates.

$0.152 \pm 0.04\,\mu g/g$ (Fig. 3) dry weight. The results could be applied to evaluate toxicological effects of natural MC-RR exposure on the health of freshwater fish.

3.5 Testes protein profiles of zebrafish exposed to MC-RR

In 6 h exposure, the gonad of zebrafish was significantly damaged, and the representative 2-DE gels of the testes in the MC-RR treated and control zebrafish are shown in Fig. 4, and gel spots showing significant changes were detected from 2-DE gels. On average, more than 1000 proteins spots were detected in each gel using silver staining by the PDQuest software. Among these altered proteins, one hundred spots displayed statistically noticeable changes in their expression pattern with 46 of them being upregulated and 54 downregulated (Fig. 5). Compared with the 2-DE gels of the nonexposed zebrafish groups, significantly altered expression of 33 protein spots from the MC-RR-exposed zebrafish testes was detected in abundance (fold change $\geq$1.75-fold; $p<0.05$) (Table 2). Totally, 24 proteins of these differentially expressed protein spots were manually excised and submitted for identification using MALDI-TOF/TOF MS analysis, 16 of which seemed to correspond to an upregulation and 8 to a downregulation (Table 3). Other 9 proteins were not identified. The apparent change ranged from a 13-fold increase to 5-fold decrease but most of the spots showed an increase or decrease $<$5-fold. The results with CI values greater than 95% were considered to be a successful identification and the matched proteins came from the NCBI database for the zebrafish *D. rerio*. Of them, eight proteins which were respondent to stress of MC-RR were involved in metabolism, six proteins in oxidative stress, and three in calciumion binding. Two proteins were characterized as cell cytoskeleton and cell structure, corresponding to keratin and tubulin β. And the remaining five proteins were involved in other functions.

3.6 Verification of differential expressed protein by Western blot analysis

Four proteins, keratin 18, tubulin β, hsp90, citrate synthase were selected for Western blot analysis due to their correlation with the known MC-RR toxic mechanisms. As shown in Fig. 6, the expression changes of the selected proteins were consistent with the 2-DE and silver-staining results.

4 Discussion

It is known that MCs exposure damages reproductive systems of mammalians. The mean absolute weight of the testes and epididymides is decreased and motility and viability of the sperm are reduced in microcystin-treated mice [20]. Liu et al. [36] reported that the MCs could actively induce the antioxidant enzyme system and the abundant GSH could against oxidative stress to protect the testis. Therefore, the quick and significant increases in activities of antioxidant enzymes and the GSH content might be a rapid response to oxidative stress in our study. In the present study, substantial MC-RR accumulated in testes of zebrafish, and the exposed testes presented hydropic changes of mitochondria, widened intercellular junction and numerous electronlucent membrane-bound vacuoles, suggesting that MC-RR were able to pass through the blood barrier to damage the testes.

The present study first investigated the potential effects of MC-RR on the protein expression profiles of zebrafish testes. A proteomic approach to zebrafish testes covers many different aspects of the changes in proteins. The cytoskeleton is the basic structural element of all cell types and plays key roles in the maintenance of cell architecture, adhesion,

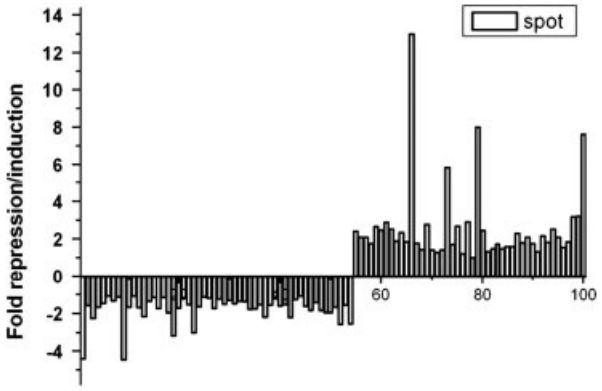

Figure 5. Volume variations for protein expression. Fold range variation was calculated for each spot as the ratio of the higher volume intensity value (between treated or control sample) divided by the lower volume intensity value (between treated or control sample). This value is affected with a plus sign when the protein is overexpressed in treated sample compared with control sample. It is affected with a minus sign in the reverse situation (underexpressed protein in the treated compared to control sample). Each bar corresponds to an individual spot. Spots with significant variation were ordered according to their fold range variation between treated and control samples.

Table 2. Variations of spot volumes

	Downregulation				Upregulation				
Expression	$-1<X<-2$	$-5<X<-2$	Min	Max	$1<X<2$	$5<X<2$	$X>5$	Min	Max
100 spots	44	10	−1.07	−4.43	23	19	4	1.01	12.9

Note: Variations were calculated as treated/controls ratio and if the result was below 1, it is reported as – controls/treated ratio.

Table 3. Identification of the differentially expressed proteins in zebrafish (*D. rerio*) testis following MC-RR exposure

Spot ID	Accession number	Protein identity	MASCOT score	MW/p*I*	Fold change	*p*-Value	Total ion score	MS/MS peptide sequence	SC[a] (%)	Function category
Cytoskeleton										
2	gil30410758	Keratin	119	48.6/5.53	−4.43	0	58	FQPIVDELR	16	Type I cytoskeletal 18, structural molecule activity
7	gil37681963	Tubulin β tubb2c	216	6.9/9.86	−2.556	0	205	ISEQFTAMFR	56	Protein polymerization, microtubule-based movement
Response to stress										
3	gil41053732	Aldehyde dehydrogenase 2 family (Mitochondrial) a	171	57.2/5.93	−2.150	0	110	TFVQESIYDEFVER	17	oxidoreductase activity
4	gil1865782	Heat shock protein 8	187	71.4/5.18	−1.95	0	102	AQIHDIVLVGGSTR	13	ATP binding
11	gil226823315	Heat shock protein 90	185	74.2/7.06	2.54	0	83	NAVVTVPAYFNDSQR	19	Response to stress
12	gil19882271	Aldh1a2 Aldehyde dehydrogenase 1 family, member A2	241	57.1/5.95	12.99	0	57	IHGSTIPIDGEFFTLTR	32	Catalytic activity
14	gil18202431	Similar to ferrodoxin-NADP(+) reductase	259	53.5/7.52	5.855	0	25	SLPIEPTVPFDPR	43	Aldehyde dehydrogenase (NAD) activity
17	gil190336734	coch Coagulation factor C homolog, cochlin	210	60.9/8.53	7.988	0	104	IGAIQFTYDQR	16	Coagulation factor
Metabolism										
1	gil56090150	NADH-ubiquinone oxidoreductase 75 kDa subunit	163	80.4/5.82	−4.4	0	163	KPIVVVGSSALQR	60	ATP synthesis coupled electron transport
5	gil41055387	zgc:63663	93	71.1/5.23	−3.173		57	FEELNADLFR	33	ATP binding
13	gil68086449	eno3 Enolase	313	47.8/6.2	2.776	0	195	AAVPSGASTGVHEALELR	31	Glycolysis
16	gil41054571	cs Citrate synthase, mitochondrial	104	51.9/7.68	2.923	0	75	GLVYETSVLDPDEGIR	16	Citrate (Si)-synthase activity
18	gil42734425	Aldolase B 23 kDa protein	180	22.9/5.5	2.453	0	96	INVENTEENRR	31	Catalytic activity, fructose and mannose metabolism
19	gil41152026	pgam1b Phosphoglycerate mutase	394	28.9/6.2	2.288	0	314	NLKPVKPMQFLGDEETVR	38	Intramolecular transferase activity
20	gil41152453	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein	232	28/4.76	2.1	0.03153	138	AVTEGDIELSNEER	31	Monooxygenase activity
23	gil41053595	Nucleoside diphosphate kinase -Z2	178	17.2/6.75	3.17	0.00676	82	TFIAVKPDGVQR	45	Nucleoside diphosphate kinase activity
Calcium ion binding										
10	gil117606250	clgn Calmegin	277	71.8/4.27	2.885	0	148	DLELEQFHDR	20	Calcium ion binding, protein binding

Table 3. Continued

Spot ID	Accession number	Protein identity	MASCOT score	MW/p*I*	Fold change	*p*-Value	Total ion score	MS/MS peptide sequence	SC[a] (%)	Function category
22	gil47086015	Myosin, light polypeptide 9	99	20.1/4.76	2.519	0	45	GNFNYVEFTR	29	Calcium ion binding
24	gil47174755	zgc:153867	244	20.1/5.65	3.206	0	169	EAFLLFDR	32	Calcium ion binding
Other function										
6	gil66773134	Ubiquitin carboxyl-terminal hydrolase isozyme L3	136	20.1/4.88	−1.75	0	72	WLPLEANPEVMNQFLR	16	Ubiquitin thiolesterase activity
8	gil162139048	Hypothetical protein LOC100002523	132	18.7/6.42	−2.535	0	81	CAEMGFGDEQR	36	Sugar binding
9	gil39795310	Ceruloplasmin	157	12.5/6.4	2.07	0.00456	114	AVYHQYTDATYR	16	Oxidoreductase activity
15	gil50344784	ppid Peptidylprolyl isomerase D	320	41.7/5.75	2.678	0.02886	117	VFFDVEIGAER	51	Peptidyl-prolyl *cis–trans* isomerase activity
21	gil126540682	Novel pentaxin family domain containing protein	237	25.1/4.81	2.171	0	160	TSEVDELNVWR	30	Unkown

a) SC indicates the sequence coverage of the protein in percentage obtained by MS/MS identification.

migration, differentiation, division, and organelle transport. It is known that cytoskeleton disruption is one of the first remarkable cytotoxic events following MC-LR exposure [37], and our observation also demonstrated that MC-RR induced a prominent disruption of cytoskeleton organization in the testes, which might affect testes metabolism. We found that abundances of two cytoskeleton proteins, β-tubulin and intermediate filaments keratin, were remarkably altered in exposed testes. The most common members of the tubulin family are α-tubulin and β-tubulin [38]. Microtubule dynamics in cells rely in part on α and β tubulin post-translational modifications such as phosphorylation, which is under the control of a balance of protein phosphatases and kinases [39]. Fu et al. [40] showed that some cells lose microtubules after MC treatment except the reorganization and aggregation of microtubules. It was also shown that MCs could disrupt the cytoskeleton [41, 42] and particularly modify the phosphorylation state of microtubule-associated proteins, which subsequently disrupted the microtubule polymerization balance [41, 43]. One protein of the intermediate filaments, keratin, was significantly altered in the present study. Several studies have reported that the disruption of the intermediate filaments could be attributed to MC-LR-induced hyperphosphorylation of keratins 8 [44]. The exposed testes tissues present lost cristae and matrix with hydropic changes of the mitochondria and numerous large digestive vacuoles. We also observed that the widening of cell junction was conspicuous in our studies. All the cytoskeleton organization changed because the cytoskeleton protein changed and then disrupted of the whole net of cell cytoskeleton. In the present study, MC-RR substantially accumulated in the MC-RR-treated testes and induced noticeable damage to ultrastructure which is in agreement with our studies, demonstrating that MC-RR caused a significant disruption of cytoskeleton organization in the testes, which might interrupt reproductive metabolism. Together with these results, we may assume that MC-RR may affect the normal microfilament network in testis due to the disrupted cytoskeleton organization and the cytoskeleton disruption can be a marker in MCRR-induced reproductive toxicity.

Another significant point of our study is that the activity levels of some important antioxidative enzymes and the protein related to oxidative stress were significantly changed. Cellular oxidative stress occurs when the production of endogenous ROS (e.g. hydroxy radical, superoxide anion) overwhelm the antioxidant defenses, resulting in lipid peroxidation (LPO), protein oxidation and DNA strand breaks [45]. In the current study, the levels of MDA and H_2O_2 increased remarkably, indicating that MC-RR resulted in oxidative stress. The MDA which is often used as a biomarker of oxidative stress increased and the oil red O staining also revealed that the vacuoles contain lipids. In recent studies, MC-induced fatty/lipid acid transport and metabolism of the activities of several enzymes in the liver of MC-LR have been described [24]. The excessive ROS

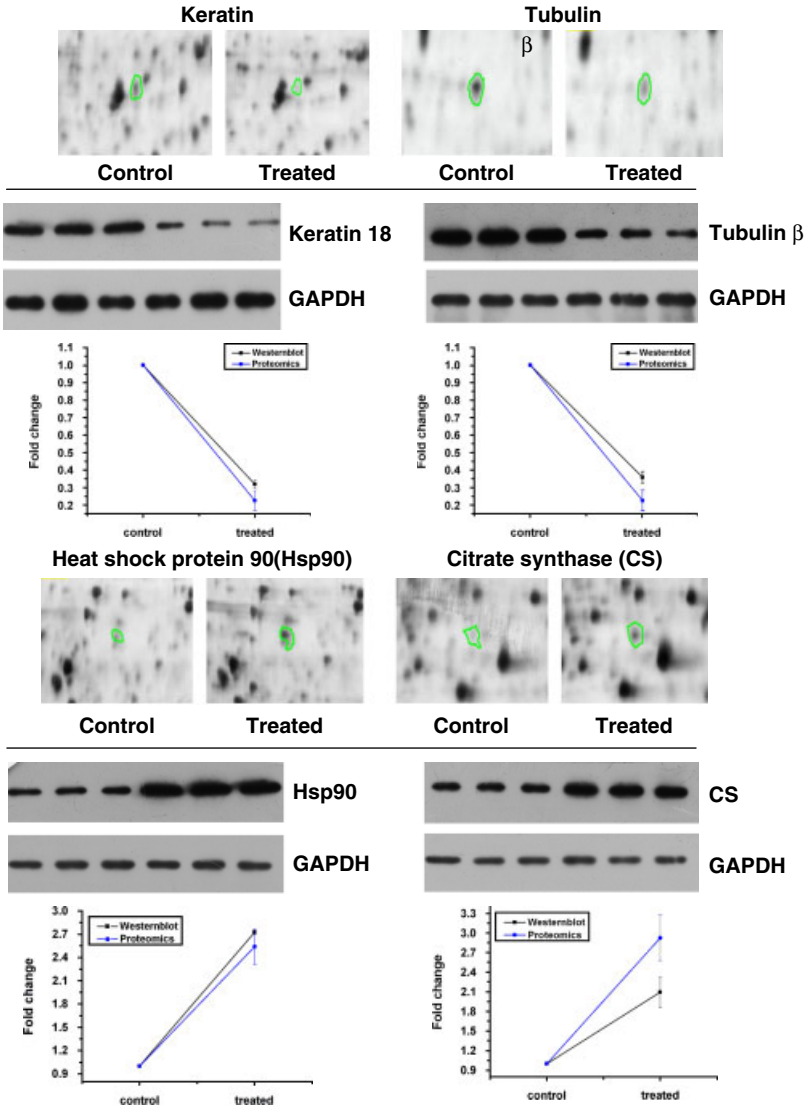

Figure 6. The alteration of proteomic and Western blot of selected altered proteins in the zebrafish testes following $0.5LD_{50}$ MC-RR treatments. The magnified images of protein spots from the 2-DE gels are shown in the upper part of each panel. The line charts show the protein levels based on 2-DE, Western blot results. The values represent the average fold changes. The values of protein abundance are the average %Vol of spots in three replicated gels.

triggered the antioxidant defense systems such as SOD, CAT, GST, and GPx, which alleviates the toxic effects of ROS by scavenging free radicals and ROS with the increase in exposure time [46]. This is in line with the proteomic analysis that oxidative stress resulted in several proteins related to the MCs stress. In particular, in a proteomic study Aldehyde dehydrogenase 2 (ALDH2) describing as MC-sensitive [47], presented an apparent decrease in abundance in this study. ALDH2 participates in aldehyde metabolism via oxidizing aldehyde to carboxylic acid [48].The accumulation of acetaldehyde is known to produce ROS [49]. ALDH2 dysfunction may lead to aldehyde-induced ROS generation and, in turn, apoptosis [47]. In addition, Aldehyde dehydrogenase 1 family, member A2 (ALDH1A2) could catalyze the synthesis of retinoic acid (RA) from retinaldehyde. The abnormal expression of ALDH1A2 correlated closely to carcinogenesis. Aldehydes are highly reactive and cytotoxic which are harmful to various important physiological processes, consequently leading to DNA damage, protein modification and enzyme inactivation [50, 51]. Retinol and its derivatives have been shown to reverse hyperplasia in carcinogen-induced prostate cancer mouse models [52]. These effects are due to the regulation retinoids exert on cell growth and differentiation [53]. The alterations in ALDH enzyme expression in prostate cancer suggest that these enzymes may contribute to abnormal retinoid levels during carcinogenesis [54]. Thus, massive aldehyde accumulated in the treated testes and subsequently oxidative stress occur in fish (i.e. increasing antioxidant activity, lipid peroxidation), indicating that MC-RR caused reproductive toxicity.

Another four proteins, similar to ferrodoxin-NADP(+) reductase, coagulation factor C and two heat shock proteins, were also involved in the oxidative stress response.

Ferredoxin is the primary soluble acceptor and is known to directly transfer electrons to a wide range of proteins for use in metabolism and regulatory processes [55]. Coagulation factor C that belongs to the coagulation system was clearly up-regulated which is consistent with the organs like liver hemorrhage observed in our study. Additionally, heat shock proteins induction has been suggested as early marker of oxidative stress [56]. Heat shock protein 90 increased in protein expression after MC-RR exposure. Heat shock protein 8 functions as an ATPase during transport of membrane components through the cell. Unlike canonical heat shock proteins, Hsp8 is constitutively expressed and performs functions related to normal cellular processes [57]. After exposure to MC-RR, Hsp8 abundance decreased might leads to an impairment of ATP production, affecting all cellular process that depends on ATP. Therefore, it is possible that oxidation stress represents alternative mechanisms for stimulating cellular responses relevant to MC-RR toxic effects.

Furthermore, protein expression profiles defined an impact on carbohydrate metabolism that could be related with the described MC-RR exposure stress responses, and these manifestations are schematically presented in Fig. 7. The proteomic analyses showed an accelerating speed of glycolysis and the tricarboxylic acid (TCA) cycle. MC-RR caused an upregulation of aldolse B 23 kDa protein, phosphoglycerate mutase, enolase, citrate synthase and nucleoside diphosphate kinase-Z2 which are very important in glycolysis pathway. The proteome results profiled an acceleration of glycolysis and demonstrated a metabolic flux

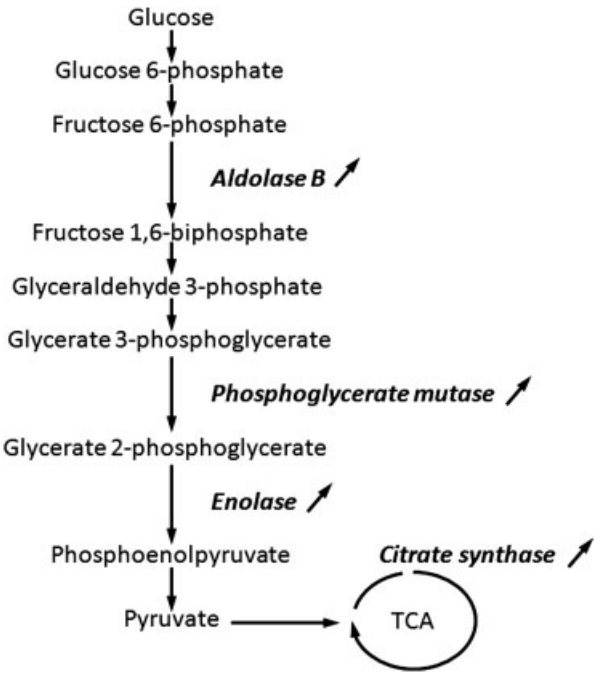

Figure 7. Schematic presentation of the observed interferences with carbohydrate metabolism.

redistribution that shifted glucose derived glucose-6-phosphate towards the TCA cycle to address an increase in the production of NADPH, which is needed by many antioxidant enzymes, thus confirming indications of oxidative stress [58]. Hereby, MC-RR caused a dysregulation of glycogen and induced dysfunction of carbohydrate metabolism. However, Ding et al. [21] assumed that MCs could lead to decreased weight in mice, with the likely reason that MCs affected the appetite of mice and inhibited some metabolic enzyme activity and anabolism, especially lowering the ATP synthesis rate. In our study, the upregulation of some metabolic enzymes indicates that the increased expression may be a compensatory effect to counteract an impairment of the energetic pathways in response to an increased energy acquirement with stress, which is in accordance with a previous study. In this paper the decrease of hepatocyte glycogen content was evoked by MC-LR attack, concomitantly increasing demand for energy and an incidental cellular metabolic exhaustion [59]. Overall, the abnormal expression poteins related to the energy generation and substance metabolism might be taken into account for the dysfunction in the zebrafish testes caused by MC-RR.

In addition, other special proteins were also be disturbed by MC-RR. Calmegin is a testis-specific endoplasmic reticulum chaperone protein. It may play a role in spermatogeneisis and infertility [60]. Some studies show that sperm motility is decreased and sperm abnormality increases when exposed to MCLR in a dose-dependent manner [21]. It is consistent with our studies that the over expression of Calmegin maybe one of the reasons for infertility and MC-RR would be anticipated to impair male fertility. Additionally, NADH-ubiquinone oxidoreductase has NADH dehydrogenase activity and oxidoreductase activity. It transfers electrons from NADH to the respiratory chain. The protein belongs to the complex I 75 kDa subunit family which is a potent source of ROS in the previous studies [61]. In our study, the decrease in NADH-ubiquinone oxidoreductase abundance might decrease ROS production to protect the testes from oxidative attack. Meanwhile, some protein degradation was influenced by MC-RR. Ubiquitin carboxyl-terminal hydrolase isozyme L3, one of the members of the ubiquitin-proteasome system which is a cellular pathway responsible for degradation of misfolded and damaged proteins, is involved in collaborating to affect the turnover of cellular misfolded and damaged proteins. [62]. In livers of zebrafish it has been showed microcystin could also affect proteolysis [24]. Together with the earlier discussed oxidative damage and subsequent protein degradation, liberated amino acids can be re-used for protein synthesis or to address increased metabolic demands associated with an oxidatively stressed cell [63].

We followed up in proteomic analysis with Western blot to investigate the protein expression of four proteins. The Western blot results correlated well with the proteomic analysis, indicating that the proteomic analysis was convincing.

To summarize, our present study was aimed at understanding the effects of MC-RR on fish testes by proteomic approach in combination with other approaches. A number of proteins were altered in abundance and these proteins were involved in multiple functions and took part in a variety of biology processes. MC-RR induced a dysfunction of cytoskeleton assembly, oxidative stress with a concomitant interference with macromolecule metabolism and other functions. The reproduction toxicity of MC-RR might initiate the production of ROS, and the antioxidative enzymes may play a key role in deintoxication process. These indicate that MCs were toxic to male reproduction. It is quite likely that animals or humans routinely ingest food or liquids contaminated with MCs, MCs would enter testis and accumulate, and consequently damage male reproduction.

This study was jointly supported by the National Basic Research Program of China (973 Program) (2008CB418101), the water special project (the Water Pollution Control and Management Project) launched by China's Ministry of Environmental Protection (Grant No. 2008ZX07101-001) and State Key Laboratory of Freshwater Ecology and Biotechnology (2008FBZ01).

The authors have declared no conflict of interest.

5 References

[1] Chorus, I., Bartram, J., *Toxic Cyanobacteria in Water – A Guide To Their Public Health Consequences, Monitoring and Management*. E&FN Spon, London 1999, p. 416.

[2] Dietrich, D., Hoeger, S., Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach. *Toxicol. Appl. Pharm.* 2005, *203*, 273–289.

[3] Ross, C., Santiago-Vazquez, L., Paul, V., Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium *Microcystis aeruginosa*. *Aquat. Toxicol.* 2006, *78*, 66–73.

[4] MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P., Codd, G. A., Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* 1990, *264*, 187–192.

[5] Wiegand, C., Pflugmacher, S., Ecotoxicological effects of selected cyanobacterial secondary metabolites: A short review. *Toxicol. Appl. Pharmacol.* 2005, *203*, 201–218.

[6] Ding, W. X., Ong, C. N., Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol. Lett.* 2003, *2*, 1–7.

[7] Li, X. Y., Chung, I. K., Kim, J. I., Lee, J. A., Oral exposure to *Microcystis* increases activity-augmented antioxidant enzymes in the liver of loach (*Misgurnus mizolepis*) and has no effect on lipid peroxidation. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* 2005, *141*, 292–296.

[8] Li, X. Y., Wang, J., Liang, J. B., Liu, Y. D., Toxicity of microcystins in the isolated hepatocytes of common carp (*Cyprinus carpio L.*). *Ecotoxicol. Environ. Saf.* 2007, *67*, 447–451.

[9] Zimba, P. V., Khoo, L., Gaunt, P. S., Brittain, S. et al., Confirmation of catfish, *Ictalurus punctatus* (*Rafinesque*) mortality from *Microcystis* toxins. *J. Fish Dis.* 2001, *24*, 41–47.

[10] Xie, L., Xie, P., Ozawa, K., Honma, T. et al., Dynamics of microcystin- LR and -RR in the phytoplanktivorous silver carp in a subchronic toxicity experiment. *Environ. Pollut.* 2004, *127*, 431–439.

[11] El Ghazali, I., Saqrane, S., Carvalho, A. P., Ouahid, Y. et al., Compensatory growth induced in zebrafish larvae after pre-exposure to a *Microcystis aeruginosa* natural bloom extract containing microcystins. *Int. J. Mol. Sci.* 2009, *10*, 133–146.

[12] Anderson, R. J., Luu, H. A., Chen, D. Z. X., Holmes, C. F. B. et al., Chemical and biological evidence links microcystins to salmon ''netpen liver disease''. *Toxicon* 1993, *31*, 1315–1323.

[13] Xie, L., Xie, P., Guo, L. G., Li, L. et al., Organ distribution and bioaccumulation of microcystins in freshwater fishes with different trophic levels from the eutrophic Lake Chaohu, China. *Environ. Toxicol.* 2005, *20*, 292–300.

[14] Xie, L., Yokoyama, A., Nakamura, K., Park, H., Accumulation of microcystins in various organs of the freshwater snail *Sinotaia histrica* and three fishes in a temperate lake, the eutrophic Lake Suwa, Japan. *Toxicon* 2007, *49*, 646–652.

[15] Chen, J., Xie, P., Zhang, D., Lei, H., In situ studies on the distribution patterns and dynamics of microcystins in a biomanipulation fish – bighead carp (*Aristichthys nobilis*). *Environ. Pollut.* 2007, *147*, 150–157.

[16] Malbrouck, C., Trausch, G., Devos, P., Kestemont, P., Hepatic accumulation and effects of microcystin-LR on juvenile goldfish *Carassius auratus L. Comp. Biochem. Physiol. C: Pharmacol. Toxicol.* 2003, *135*, 39–48.

[17] Fischer, W. J., Dietrich, D. R., Pathological and biological characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). *Toxicol. Appl. Pharmacol.* 2000, *164*, 73–81.

[18] Best, J. H., Eddy, F. B., Codd, G. A., Effects of purified microcystin- LR and cell extracts of *Microcystis* strains PCC 7813 and CYA 43 on cardiac function in brown trout (*Salmo trutta*) alevine. *Fish Physiol. Biochem.* 2001, *24*, 171–178.

[19] Funari, E., Testai, E., Human health risk assessment related to cyanotoxins exposure. *Crit. Rev. Toxicol.* 2008, *38*, 97–125.

[20] Li, Y., Sheng, J., Sha, J., Han, X., The toxic effects of microcystin- LR on the reproductive system of male rats in vivo and in vitro. *Reprod. Toxicol.* 2008, *26*, 239–245.

[21] Ding, X. S., Li, X. Y., Duan, H. Y., Chung, I. K. et al., Toxic effects of *Microcystis* cell extracts on the reproductive system of male mice. *Toxicon* 2006, *48*, 973–979.

[22] Xiong, Q., Xie, P., Li, Y., Hao, L. et al., Involvement of Fas/FasL system in apoptotic signaling in testicular germ cells

of male Wistar rats injected i.v. with microcystins. *Toxicon* 2009, *54*, 1–7.

[23] Merrick, B. A., Toxicoproteomics in liver injury and inflammation. *Ann. NY Acad. Sci.* 2006, *1076*, 707–717.

[24] Wang, M. H., Chan, L. L., Si, M. Z., Hong, H. S. et al., Proteomic analysis of hepatic tissue of zebrafish (*Danio rerio*) experimentally exposed to chronic microcystin-LR. *Toxicol. Sci.* 2010, *113*, 60–69.

[25] Wang, M. H., Wang, D. Z., Lin, L., Hong, H. S., Protein profiles in zebrafish (*Danio rerio*) brains exposed to chronic microcystin-LR. *Chemosphere* 2010, *81*, 716–724.

[26] Wang, Q., Xie, P., Chen, J., Liang, G. D., Distribution of microcystins in various organs (Heart, Liver, Intestine, Gonad, Brain, Kidney and Lung) of Wistar rat via intravenous injection. *Toxicon* 2008, *52*, 721–727.

[27] Gad, S. C., Weil, C. S., *Statistics and Experimental Design for Toxicologists*. Telford Press, NJ 1988, p. 435.

[28] Bayer, W. F., Fridovich, J. L., Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* 1987, *161*, 559–566.

[29] Habig, W. H., Pabst, M. J., Jakoby, W. B., Glutathine-*S*-transferases: the first enzymatic step in mercapturic acid formation. *Biol. Chem.* 1974, *249*, 7130–7139.

[30] Habig, W. H., Jakoby, W. B., Assays for differentiation of glutathione *S*-transferases. *Methods Enzymol.* 1981, *77*, 398–405.

[31] Drotar, A., Phelps, P., Fall, R., Evidence for glutathione peroxidase activities in cultured plant cells. *Plant Sci.* 1985, *42*, 35–40.

[32] Griffith, O., Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 1980, *106*, 207–212.

[33] Ohkawa, H., Ohishi, N., Yagi, K., Assay for lipid peroxides in animal tissues bythiobarbituric acid reaction. *Anal. Biochem.* 1979, *95*, 351–358.

[34] Tay, T. L., Lin, Q., Seow, T. K., Tan, K. H. et al., Proteomic analysis of protein profiles during early development of the zebrafish, *Danio rerio*. *Proteomics* 2006, *6*, 3176–3188.

[35] Li, G.Y., Chen, J., Xie, P., Jiang, Y., Wu, L.Y. et al., Protein expression profiling in the zebrafish (*Danio rerio*) embryos exposed to the microcystin-LR. *Proteomics* 2011, *11*, 2003–2018.

[36] Liu, Y., Xie, P., Qiu, T., Li, H. Y. et al., Microcystin extracts induce ultrastructural damage and biochemical disturbance in male rabbit testis. *Environ. Toxicol.* 2010, *25*, 9–17.

[37] Wickstrom, M. L., Khan, S. A., Haschek, W. M., Wyman, J. F. et al., Alterations in microtubules, intermediate filaments, and microfilaments induced by microcystin-LR in cultured cells. *Toxicol. Pathol.* 1995, *23*, 326–337.

[38] Howard, J., Hyman, A. A., Dynamics and mechanics of the microtubule plus end. *Nature* 2003, *422*, 753–758.

[39] Westermann, S., Weber, K., Post-translational modifications regulate microtubule function. *Nat. Rev. Mol. Cell. Biol.* 2003, *4*, 938–947.

[40] Fu, W. Y., Yu, Y. N., Xu, L. H., Identification of temporal differentially expressed protein responses to microcystin in human amniotic epithelial cells. *Chem. Res. Toxicol.* 2009, *22*, 41–51.

[41] Ding, W. X., Shen, H. M., Ong, C. N., Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes. *Environ. Health Perspect.* 2000, *108*, 605–609.

[42] Batista, T., de Sousa, G., Suput, J. S., Rahmani, R. et al., Microcystin-lr causes the collapse of actin filaments in primary human hepatocytes. *Aquat. Toxicol.* 2003, *65*, 85–91.

[43] Fourest-Lieuvin, A., Peris, L., Gache, V., Garcia-Saez, I. et al., Microtubule regulation in mitosis: tubulin phosphorylation by the cyclin- dependent kinase cdk1. *Mol. Biol. Cell* 2006, *1*, 1041–1050.

[44] Toivola, D. M., Goldman, R. D., Garrod, D. R., Eriksson, J. E., Protein phosphatases maintain the organization and structure interactions of hepatic keratin intermediate filaments. *J. Cell Sci.* 1997,*110*, 23–33.

[45] Winston, G. W., Di Giulio, R. T., Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* 1991, *19*, 137–161.

[46] Sies, H., Strategies of antioxidant defense. *Eur. J. Biochem.* 1993, *215*, 213–219.

[47] Chen, T., Cui, J., Liang, Y., Xin, X. B. et al., Identification of human liver mitochondrial aldehyde dehydrogenase as a potential target for microcystin-LR. *Toxicology* 2006, *220*, 71–80.

[48] Marchitti, S. A., Deitrich, R. A., Vasiliou, V., Neurotoxicity and metabolism of the catecholamine-derived 3,4-dihydroxyphenylacetaldehyde and 3,4- dihydroxyphenylglycolaldehyde: the role of aldehyde dehydrogenase. *Pharmacol. Rev.* 2007, *59*, 125–150.

[49] Wright, R. M., McManaman, J. L., Repine, J. E., Alcohol-induced breast cancer: A proposed mechanism. *Free Radical. Bio. Med.* 1999, *26*, 348–354.

[50] Lindahl, R., Aldehyde dehydrogenases and their role in carcinogensis. *Crit. Rev. Biochem. Mol. Biol.* 1992, *27*, 283–335.

[51] O'Brien, P. J., Siraki, A. G., Shangari, N., Aldehyde sources, metabolism,molecular toxicity mechanisms, and possible effects on human health. *Crit. Rev. Toxicol.* 2005, *35*, 609–662.

[52] Chopra, D., Wilkoff, L. J., Activity of retinoids against benzo(a)pyrene-induced hyperplasia in mouse prostate organ cultures. *Eur. J. Cancer* 1979, *15*, 1417–1423.

[53] Mongan, N. P., Gudas, L. J., Diverse actions of retinoid receptors in cancer prevention and treatment. *Differentiation* 2007, *75*, 853–870.

[54] Kim, H., Lapointe, J., Kaygusuz, G., Ong, D. E. et al., The retinoic acid synthesis gene ALDH1a2 is a candidate tumor suppressor in prostate cancer. *Cancer Res.* 2005, *65*, 8118–8124.

[55] Hanke, G. T., Satomi, Y., Shinmura, K., Takao, T., Hase, T., A screen for potential ferredoxin electron transfer partners uncovers new, redox dependent interactions. *Biochim. Biophys. Acta* 2010, *1814*, 366–374.

[56] Peng, J., Graham, J., Kenneth, W., Stress proteins as biomarkers of oxidative stress: effects of antioxidant supplements. *Free Radical. Bio. Med.* 2000, *28*, 1598–1606.

[57] Goldfarb, S., Kashlan, O., Watkins, J., Suaud, L. et al., Differential effects of Hsc70 and Hsp70 on the intracellular trafficking and functional expression of epithelial sodium channels. *Proc. Natl. Acad. Sci. USA* 2006, *103*, 5817–5822.

[58] Dowling, V. A., Sheehan, D., Proteomics as a route to identification of toxicity targets in environmental toxicology. *Proteomics* 2006, *6*, 5597–5604.

[59] Mezhoud, K., Bauchet, A. L., Château-Joubert, S., Praseuth, D. et al., Proteomic and phosphoproteomic analysis of cellular responses in medaka fish (*Oryzias latipes*) following oral gavage with microcystin-LR. *Toxicon* 2008, *51*, 1431–1439.

[60] Watanabe, D., Yamada, K., Nishina, Y., Tajima, Y. et al., Molecular cloning of a novel Ca(2+)-binding protein (calmegin) specifically expressed during male meiotic germ cell development. *J. Biol. Chem.*1994, *269*, 7744–7749.

[61] Murphy, M. P., How mitochondria produce reactive oxygen species. *Biochem. J.* 2009, *417*, 1–13.

[62] Ciechanover, A., Brundin, P., The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. *Neuron* 2003, *40*, 427–446.

[63] Grune, T., Reinheckel, T., Davies, K. J. A., Degradation of oxidized proteins in mammalian cells. *FASEB J.* 1997, *11*, 526–534.

