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Microcystin-induced variations in transcription of GSTs in an omnivorous freshwater fish, goldfish

Guangyu Li, Ping Xie*, Juan Fu, Le Hao, Qian Xiong, Huiying Li

Donghu Experimental Station of Lake Ecosystems, State Key Laboratory for Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, The Chinese Academy of Sciences, Donghu South Road 7, Wuhan 430072, PR China

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ABSTRACT

The glutathione *S*-transferases are important enzymes in the microcystin-induced detoxication processes. In this experiment, we cloned the full-length cDNA of alpha, pi and theta-class-like glutathione *S*-transferase genes from goldfish (*Carassius auratus* L). Their derived amino acid sequences were clustered with other vertebrate alpha, pi and theta-class GSTs in a phylogenetic tree and the goldfish GST sequences have the highest similarity with those from common carp and zebrafish. Goldfish were i.p. injected with microcystins extract at two doses (50 and 200 μg kg^{-1} BW MC-LR_{eq}) and the relative changes of the mRNA abundance in liver, kidney and intestine were analyzed by real-time PCR. The transcription of GST alpha was suppressed in both liver and intestine, but induced in the kidney. Decreased transcription of GST theta was detected in liver, kidney and intestine in the low-dose group. The transcription of GST pi was suppressed in liver and intestine post-injection in both dose groups. These results suggested that the transcription of GST isoforms varied in different ways within an organ and among organs of goldfish exposed to MCs.

1. Introduction

The occurrence of toxic cyanobacterial blooms in eutrophic lakes, reservoirs, and recreational waters is a worldwide problem (Paerl et al., 2001). Among cyanotoxins, microcystins (MCs) are considered to be one of the most dangerous groups and are known as a group of cyclic polypeptide hepatotoxins of varying potency (Chorus and Bartram, 1999). MCs have been shown to have a far-reaching impact on the aquatic organisms (Codd, 1995; Falconer, 1999). Both field and laboratory studies indicate that MCs accumulate mainly in fish liver (Xie et al., 2005, 2007; Chen et al., 2006, 2007; Williams et al., 1995; Malbrouck et al., 2003; Mohamed et al., 2003), and also in other organs such as kidney (Williams et al., 1995; Mohamed et al., 2003), and intestine (Mohamed et al., 2003; Xie et al., 2004, 2005).

MCs potently inhibit protein phosphatases 1 and 2A in hepatocytes (MacKintosh et al., 1990) and induce production of reactive oxygen species (ROS) (Ding et al., 2000, 2001), followed by destruction of the hepatic cytoskeleton, leading to liver necrosis, apoptosis and hemorrhage (Fischer and Dietrich, 2000; Gehringer, 2004). MCs are also proved to promote renal and intestine alterations and affect renal physiology (Nobre et al., 2003; Moreno et al., 2003). Anemia caused by MC-induced kidney function impairment is suggested to be responsible for the abnormality of swimming behaviors (Zhang et al., 2007). It is known that MCs are taken up into the hepatocyte by multi-specific bile acid transporters (Eriksson et al., 1990) and detoxified in liver through conjugation to glutathione (Kondo et al., 1996) by glutathione *S*-transferase (GST) (Pflugmacher et al., 1998; Takenaka, 2001), subsequently being transported to the kidney and intestine for excretion (Ito et al., 2002).

The glutathione *S*-transferases (GSTs) are a family of phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds (Hayes et al., 2005; Leaver et al., 1997). Such compounds include therapeutic drugs, environmental toxins and products of oxidative stress (Hayes et al., 2005; Leaver et al., 1997). There are commonly 12 reported isoforms of glutathione transferase in mammals, including alpha, mu, pi, theta, sigma, omega, zeta, kappa and microsomal GSTs (Hayes et al., 2005). Although, many novel classes of GST sequences have been identified and classified from non-mammalian organisms, information of fish GSTs is limited. The alpha, pi and theta GSTs are widely found in fishes (George, 1994; Riol et al., 2001) and Fu and Xie (2006) reported five cytosolic GSTs (rho, mu, theta, alpha and pi) from liver of common carp, in which the transcription of GST alpha, theta, and pi changed greatly, while mu isoform was relatively stable and Liang et al. (2007) reported that cyprinid fishes may show an alpha-class predominant expression pattern of liver GST genes. Pérez-López et

* Corresponding author. Tel.: +86 27 68780622; fax: +86 27 68780622.
E-mail address: xieping@ihb.ac.cn (P. Xie).

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al. (2002) found that only some of the rainbow trout GST isoenzymes were specifically induced by the polychlorinated biphenyls treatment, rendering these isoenzymes as valuable biomarkers for exposure to these environmental pollutants. However, information of fish GSTs is still limited, although fish are a large heterogeneous group of lower vertebrates.

Based on these facts, a better understanding of GST isoenzymes is necessary to improve the knowledge on mechanisms involved in toxicity of MCs and responses of different organs to such toxins. In this study, we compared the changes in transcriptional level of GST isoenzymes of goldfish (*C. auratus*) exposed to extracted MCs. The main purposes were to evaluate the time-dependent responses of three GST isoforms (alpha, pi and theta) in diverse organs of goldfish exposed to MCs and to discuss the possible mechanisms underlying these observations.

2. Materials and methods

2.1. Fish

Goldfish (*C. auratus* L), an omnivorous fish, is a dominant freshwater species in China. This carp can ingest a significant portion of toxic cyanobacteria in eutrophic lakes, leading to MC accumulation in its tissues (Xie et al., 2004). Goldfish, with mean weight of 265 ± 22.6 g, were obtained from fish farm and acclimatized in aerated freshwater tanks for 15 days before being used in the experiment. Water temperature was controlled at 25 ± 1 °C and dissolved oxygen was 6.8 ± 0.7 mg L^{-1}. Feeding of food pellets at a rate of 1% of the body weight per day was terminated 2 days before initiation of our experiment. No food was supplied to fish during the whole experimental period.

2.2. Toxin

Cyanobacteria (mainly composed of *M. aeruginosa*) were collected from surface bloom scum of Lake Dianchi, Yunnan Province, China. Crude microcystins in the cyanobacteria were extracted. Determination of cellular MC was carried out according to Li et al. (2005). In this study, algal cells were freeze-dried and microcystins were extracted with methanol. The total microcystin content was detected as 1.41 mg g^{-1} DW and MC-RR, -LR and -YR contents were 0.84, 0.50 and 0.07 mg g^{-1} DW, respectively. Crude microcystins were then suspended in distilled water, 0.5 ml suspension, amounting to equivalent of 100 and 400 μg MC-LR + MC-RR + MC-YR kg^{-1} BW, and directly injected (i.p.) along the ventral midline into the peritoneum using syringes. Since i.p. LD_{50} in mice for MC-RR and -YR is about five times and 2.5 times higher than MC-LR, respectively (Gupta et al., 2003), coefficients of 0.2 and 0.4 were used to convert MC-RR and -YR into MC-LR equivalent ($MC\text{-}LR_{eq}$), respectively.

2.3. Experimental protocol

A total of 180 acclimated goldfish were injected i.p. under ventral fin into the peritoneum by syringe using physiological saline solution as vehicle for injection. Fish in the low-dose group ($n = 60$) and high-dose group ($n = 60$) were injected i.p. with crude extracted microcystins (mainly MC-RR and -LR) at 50 and 200 μg kg^{-1} BW $MC\text{-}LR_{eq}$, respectively; the control fish ($n = 60$) were injected i.p. with equal volume of physiological saline solution. The 60 fishes in each dose group and the control were divided in equal numbers into 12 aquariums with the temperature maintained at 25 ± 1 °C during the experiment. Six sampling points were set during a period of 7 days in the experiment (1, 3, 12, 24, 48 h and 7 days = 168 h post-treatment).

Table 1
PCR conditions used in this paper

RT-PCR	RACE-PCR	Real-time PCR
94 °C for 3 min	95 °C for 3 min	94 °C for 4 min
94 °C for 30 s (30 cycles)	95 °C for 30 s (30 cycles)	94 °C for 20 s (40 cycles)
94 °C for 30 s (30 cycles)	62 °C for 30 s (30 cycles)	60 °C for 20 s (40 cycles)
72 °C for 1 min (30 cycles)	72 °C for 1 min (30 cycles)	72 °C for 25 s (40 cycles)
72 °C for 5 min	72 °C for 5 min	72 °C for 5 min

Three fish from each group were killed at every sampling time and the livers, kidneys and intestines were quickly removed, minced, and stored frozen at liquid nitrogen until RNA isolation.

2.4. Total RNA extraction, reverse transcription and rapid amplification of cDNA ends (RACE)

Total RNA was isolated using Trizol reagent (Invitrogen) and quantified by determination at OD260. 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 mRNA sequences of mammalian GSTs were used to search in zebrafish and pufferfish genome and database for homologous genes, and degenerate primers were designed from conserved regions to clone partial GST cDNA sequences of goldfish by PCR. The PCR cycling conditions are listed in Table 1. PCR products were cloned and sequenced. Gene-specific primers were designed based on the cloned PCR fragments of goldfish GST cDNA for 5′-RACE and 3′-RACE.

2.5. Bioinformatics

The sequence identity of carp GST genes with their homologues in other animals was analyzed by using the DNAstar software. Multiple sequence alignments were performed using the CLUSTALX program. The phylogenetic tree was constructed based on the results of alignments using the Mega 3.0 program. A bootstrap analysis was performed using 1000 replicates to test the relative support for particular clades. The GenBank accession numbers of sequences used are listed in Table 2.

2.6. Real-time PCR

All the primers used in the real-time PCR are listed in Table 3. The specification of each pair of primers was confirmed by randomly sequencing six clones, and further confirmed by the melting curve analysis using real-time PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid and only primers with similar amplification efficiency were used in this experiment. One commonly used housekeeping gene GAPDH was analyzed in samples. Based on the results obtained, GAPDH level was stable in the present experiments and therefore it was used as the internal control gene for the quantitative RT-PCR assay. Semiquantitative real-time RT-PCR was conducted by amplifying 1.0 μl of cDNA with the SYBR Green qPCR kit (Finnzymes, Finland) on a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA). Amplification conditions are listed in Table 1. 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).

Table 2
GST sequences used for phylogenetic tree construction

Species	Protein	Accession no.
Homo sapiens	Hs Alpha1	CAI13812
	Hs Alpha2	AAH02895
	Hs Alpha3	NP_000838
	Hs Alpha4	AAH15523
	Hs Kappa1	AAH01231
	Hs Mu1	AAV38750
	Hs Mu2	AAI05067
	Hs Mu3	NP_000840
	Hs Mu4	AAI08730
	Hs Mu5	AAH58881
	Hs Pi	AAV38753
	Hs Theta1	NP_000844
	Hs Theta2	AAG02373
Mus musculus	Mm Alpha1	AAH61134
	Mm Alpha2	AAH30173
	Mm Alpha3	AAH09805
	Mm Alpha4	AAH12639
	Mm Kappa1	NP_083831
	Mm Mu4	AAH30444
	Mm Mu6	AAH31818
	Mm Pi	AAH61109
	Mm Theta1	AAH12254
	Mm Theta2	Q61133
	Mm Theta3	AAH03903
Danio rerio	Dr Alpha	AAH60914
	Dr Kappa1	XP_698521
	Dr Kappa1	XP_686115
	Dr Kappa1	XP_708257
	Dr Mu	NP_997841
	Dr Mu1	XP_690427
	Dr Pi	AAH83467
	Dr Pi2	NP_001018349
	Dr Theta	NP_956878
	Dr Theta3	XP_692427
	Dr Rho	CAK10882
	Dr Rho	XP_693045
Tetraodon nigroviridis	Tn Alpha	CAG09409
	Tn Kappa1	CAF97858
	Tn Mu	CAG07510
	Tn Theta	CAG09655
Cyprinus carpio	Cc Alpha	ABD67507
	Cc Kappa	ABD67508
	Cc Mu	ABD67509
	Cc Pi	ABD67510
	Cc Theta	ABD67512
	Cc Rho	ABD67511
Pagrus major	Pm Rho	BAD98443
Pleuronectes platessa	Pl Rho	CAA64493

2.7. Statistics

All values are expressed as mean ± standard deviation. One-way ANOVA was used to elucidate if there are significant differences between the treatment groups and the control group ($P < 0.05$ was accepted as statistically significant change).

3. Results

3.1. Multiple alignment and phylogenetic tree

The full open reading frames of GST isoforms of alpha, pi and theta have been cloned (The Genbank accession numbers of these sequences were EU182590, EU183358 and EU183359). Alpha and theta GST isoforms have not been reported in goldfish before. To investigate the phylogenetic relationship of goldfish GST alpha, theta and pi with different classes of GST enzymes from other vertebrates, a phylogenetic tree was constructed with deduced amino acid sequences (Fig. 1). The goldfish GST sequences have the highest similarity with those from common carp and zebrafish.

3.2. Variation of transcription in different GST isoforms

The time-dependent transcriptional changes of GST mRNAs in different organs of goldfish are shown in Fig. 2. Overall, the transcription of GST isoforms in different organs exhibited different responses. The transcription of GST alpha was suppressed in both liver and intestine in the two dose groups, but in kidney it was induced. Decreased transcription of GST theta was detected in liver, kidney and intestine post-injection with 50 $\mu g\,kg^{-1}$ BW MC-LR_{eq}. The transcription of GST pi was suppressed in liver post-injection with 50 $\mu g\,kg^{-1}$ BW MC-LR_{eq}, and in intestine post-injection with 200 $\mu g\,kg^{-1}$ BW MC-LR_{eq}.

The transcription of these GST isoforms also showed different temporal kinetics. In liver, the transcription of GST alpha was suppressed from 3 to 168 h post-injection with 50 $\mu g\,kg^{-1}$ BW MC-LR_{eq} and showed no tendency to recover even after 168 h post-injection. The transcription of the theta isoform was relatively stable and only decreased at 48 h. The transcription of GST pi increased at 3 h and was suppressed at the following time and reverted to the original level at 168 h. The transcription of alpha isoform was suppressed from 1 to 48 h post-injection with 200 $\mu g\,kg^{-1}$ BW MC-LR_{eq}. Increased transcription level of GST theta was detected at 1 and 3 h and similarity was detected at 3 h of GST pi.

In kidney, the transcription of the alpha, theta and pi isoform was relatively stable at 1 h post-injection with 50 $\mu g\,kg^{-1}$ BW MC-LR_{eq}. However, different transcription among these isoforms was observed from 3 to 168 h post-injection. Alpha isoform was first induced transcriptionally at 3 h post-injection but then reverted to the original level. Theta isoform was suppressed transcriptionally at 3, 12 and 48 h but showed a tendency to recover at 168 h and decreased transcription of pi isoform was detected at 3 h but increased transcription at 24 h. Increased transcription of alpha, theta and pi isoform were detected in the initial period post-injection with 200 $\mu g\,kg^{-1}$ BW MC-LR_{eq}. The transcription of alpha isoform was first induced from 3 to 24 h but suppressed at 48 h; theta isoform was induced from 1 to 3 h but reverted to the original level at 12 h; pi isoform was induced at 1 h but inhibited at 3 and 24 h.

In intestine, a strong inhibition was detected in the transcription of GST alpha from 3 to 24 h with 50 $\mu g\,kg^{-1}$ BW MC-LR_{eq} injected,

Table 3
Real-time PCR primers used in this experiment

Target gene	Primer sequence (5′–3′)		Size (bp)
	Forward	Reverse	
Alpha	AGCAGGTGCCTTTGGTGGAAATCG	ACTGAGAGTTCGCAAGACCCCTTTCA	276
Pi	CCTGGTCCTGTATCAGTCCAATGCC	AATGGTTTGAGGTGTTTGGGCAGAT	210
Theta	TGAGAAGTTCCACACTCCAGACCAC	CCACAATAGCAACCAAATCAGCCAG	261
GAPDH	GCCAGTCAGAACATTATCCCAGCCT	GGTCCTCAGTGTATCCCAGAATGCC	235

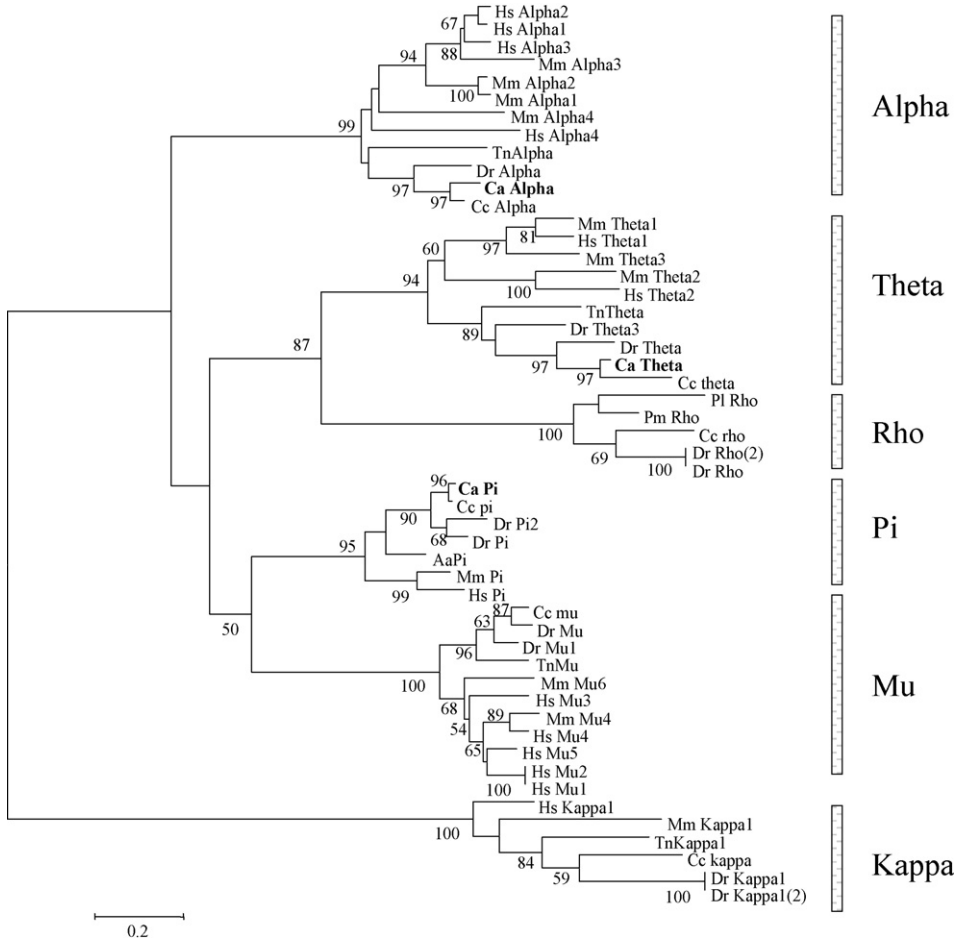

Fig. 1. Phylogenetic tree of piscine GSTs and their homologous molecules from mammals. Multiple sequence alignments were performed using the CLUSTALX program. The phylogenetic tree, based on the results of alignments, was obtained by using the Mega 3.0 program. A bootstrap analysis was performed using 1000 replicates to test the relative support for particular clades. The GenBank accession numbers of sequences used are listed in Table 2.

but the recovery was detected after 24 h. The transcription of theta isoform decreased at 12 and 24 h with a recovery at 48 h. The transcription of pi isoform decreased at 1 and 3 h but increased at 24 h. When injected with 200 $\mu g\,kg^{-1}$ BW MC-LR_{eq}, the transcription of alpha and theta isoform were similar to the control at 1 h and reverted to the original level at 24 h, while the transcription of pi GST isoform in intestine showed a time-dependent reduction after 1 h of injection.

4. Discussion

Evaluation of mRNA levels is an established method to measure xenobiotic induction and studies show that GST mRNA levels correlate closely with GST protein and enzyme activity levels (Buetler et al., 1995). Increased mRNA expression of GST has been found accompanying a concomitant increase in catalytic activity (Gehringer et al., 2004). Our experiment demonstrated that the phase II detoxification systems were modified by MC administration with altered transcript level of GST isoenzymes, suggesting different roles for GST isoenzymes in the detoxification of MCs-induced toxicity. Our results also showed varied trends of GSTs alteration in liver, kidney and intestine of goldfish subjected to the acute exposure to MCs. Liver has been described as the most important organ involved in the regulation of redox metabolism (Klaassen, 2001), due to synthesis of key enzymes responsible for reactive oxygen species clearance and production of the most important systemic antioxidant agent, glutathione (GSH). On the other hand, it has been proven that MC-LR promotes renal and intestinal alterations and affects renal physiology (Nobre et al., 2003; Moreno et al., 2003). Thus, it could be assumed that MCs affected the functions of the detoxification system of these organs.

The transcription of GST isoforms in this study varied in different ways within an organ and between organs of goldfish exposed to MCs. In both dose groups, the transcription of GST alpha was suppressed in both liver and intestine, but induced in kidney. Decreased transcription of GST theta was detected in liver, kidney and intestine in the low-dose group. The transcription of GST pi was suppressed in liver and in intestine post-injection within the two dose groups. The possible explanations for the observed variations in transcription of GST isoforms could be attributed to (1) that accumulation of MCs in different organs were different (Li et al., 2007) and different concentrations of MCs might influence transcription factors, e.g. Nrf2 (NF-E2 p45-related factor 2) (Itoh et al., 2004) and further influence the transcription of GST isoenzymes; and (2) that GST isoenzymes were expressed tissue-specifically, with different binding abilities to GSH-complexes (Lee et al., 2006).

MCs showed different effects on the transcription of GST isoforms in our experiment, which was similar with pervious studies on mammals and other fish species. Levels of transcription of hepatic GST alpha, rho and mGST3 isoforms increased in common carp after exposed to MC-LR at 50 $\mu g\,kg^{-1}$, while the transcription of mu, theta and mGST isoforms were relatively stable and all the GST isoforms except GST kappa and rho recovered to original lev-

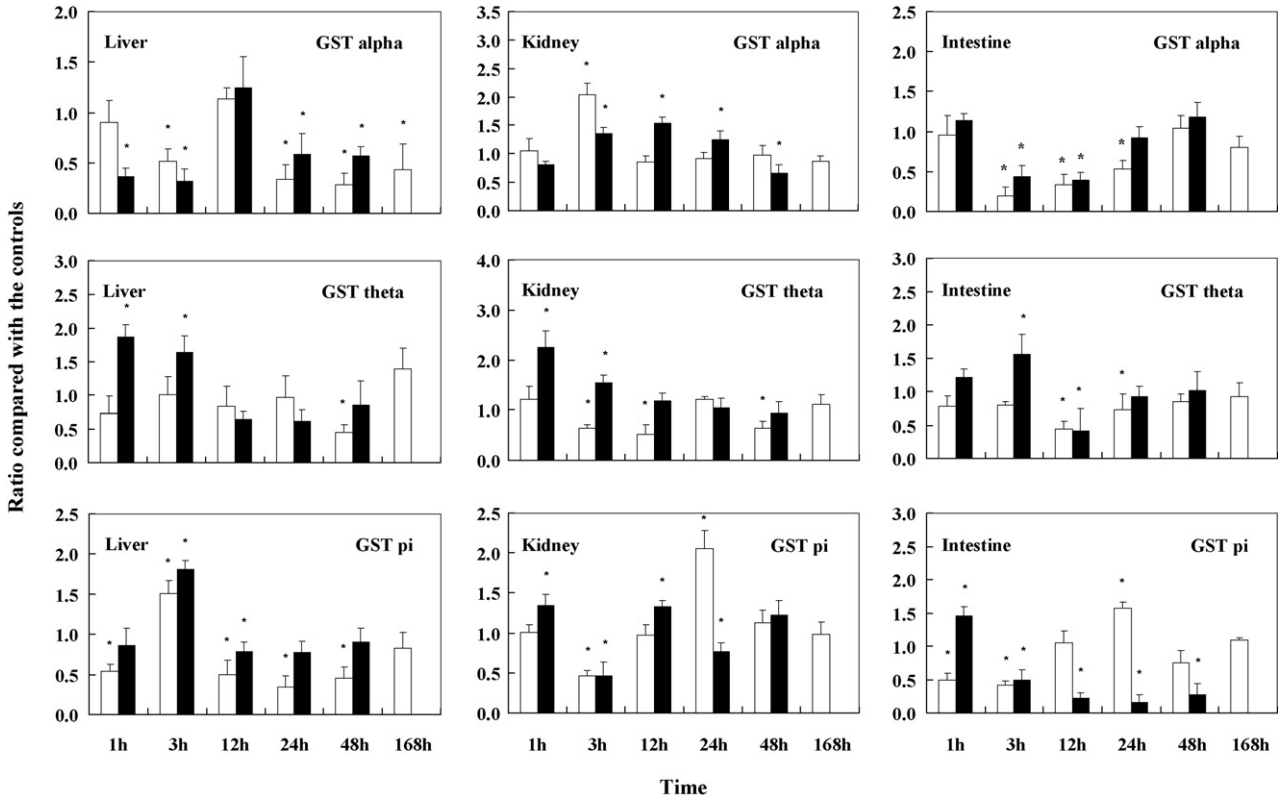

Fig. 2. The temporal changes of GSTs transcripts after microcystin exposure compared with controls. Black columns represent high-dose group and white columns represent low-dose group (* indicates significant change at $P < 0.05$).

els compared with controls at 72 h in carp (Fu and Xie, 2006). The transcription of GST alpha and mu in rats exposed to MC-LR at 50 $\mu g\,kg^{-1}$ was elevated at 3 h and mGST1 decreased at 6 h, while pi had no change at 3 and 6 h post-injection (Bulera et al., 2001). In a micro-array analysis, when mice were exposed to MC-LR at 75% LD_{50}, transcription of the GST pi was elevated at 8 h, and GST Mu (Mu2 and Mu5 isoform) increased during 8–24 h (less than twofold compared with controls) (Gehringer et al., 2004).

Either induced or inhibited activity of GSTs after exposure to MCs was reported in different fish species. Inhibition of GST activity was reported in zebrafish embryos exposed to a mixture of MC-LR and cyanobacterial lipopolysaccharides (Best et al., 2002) and in diverse organs of *Corydoras paleatus* exposed to microcystin-RR (Cazenave et al., 2006). However, GST activity was either elevated in the early life stages of the zebrafish (Wiegand et al., 1999) or unaffected in bighead carp injected with extracted microcystins and in juvenile goldfish exposed to microcystin-LR. (Li et al., 2005; Malbrouck et al., 2003). The ambiguous variation in the activity of GSTs in different fish species might be due to different experimental conditions and/or interspecies differences in susceptibility to the acute effects of MCs (Fu and Xie, 2006). In our experiment, real-time PCR was used instead of enzyme kinetic analysis based on the fact that GSTs can form homodimers or heterodimers. It is difficult to define a distinction between GST classes by specific substrates because of broad and overlapping substrates (Fu and Xie, 2006; Sheehan et al., 2001). The real-time PCR method provides relatively accurate quantification of gene transcripts at low copy number of small samples and variations in transcription of GST isoforms by real-time PCR could reflect more accurately the influence of MC exposure on the phase II detoxification systems than GST activities by enzyme kinetic analysis.

The present experiment showed a diverse variation in gene transcription of GST isoforms (alpha, theta and pi) in various organs of goldfish exposed to two different doses of MCs. The toxicity of MCs depends on the balance between accumulation and metabolism (Ito et al., 2002) and GSTs play important roles in the detoxification of MCs (Pflugmacher et al., 1998). The inhibited and/or induced transcription of GSTs implicated an increased health risk for fish. It is reasonable to assume that, if fish are not able to eliminate MCs effectively, the variations of GSTs might affect capability of MC metabolization and subsequent cellular damages, physiological alterations, behavioral changes and other effects (Kotak et al., 1996; Baganz et al., 1998, 2004; Fischer and Dietrich, 2000; Malbrouck et al., 2003).

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