Acute Effects of Microcystins Exposure on the Transcription of Antioxidant Enzyme Genes in Three Organs (Liver, Kidney, and Testis) of Male Wistar Rats

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ABSTRACT: Microcystins (MCs) induce the production of reactive oxygen species (ROS) in various tissues in mammals, whereas the endogenous antioxidant enzymes are responsible to scavenge the ROS. ROS can modulate the antioxidant enzyme activities by regulating the mRNA levels. The present study was undertaken to find out the relationship between the transcriptional alterations of antioxidant enzymes and MCs stimulation in rats. The time-dependent changes of relative transcription abundance of catalase (CAT), Mn-superoxide dismutase (Mn-SOD), Cu,Zn-superoxide dismutase (Cu,Zn-SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and gamma-glutamylcysteine synthetase (γ-GCS) were investigated in three organs (liver, kidney, and testis) of male Wistar rats injected intravenously (i.v.) with 80 μg MC-LR$_{equivalent}$/kg body weight using the quantitative real-time PCR (qPCR) method. We found that MCs could affect the transcriptional activities of these antioxidant enzymes in liver, kidney, and testis of MCs-treated rats and we speculated the possible causation of the transcriptional change. The altered transcription of antioxidant enzymes may play an important role in counteracting the potential deleterious effects of elevated oxidative stress induced by MCs, and this will provide us new insights into the possible role of antioxidant enzymes in the toxicological mechanisms of MCs at molecular level. © 2010 Wiley Periodicals, Inc. J Biochem Mol Toxicol 24:361–367, 2010;

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INTRODUCTION

Eutrophication of aquatic environments leads to excessive proliferation of cyanobacteria. Some cyanobacteria can produce a variety of toxins, of which microcystins (MCs) are the most widespread distributed [1]. Microcystins are cyclic heptapeptides, composed of seven amino acids. More than 80 variants of microcystin have been reported [2,3], with microcystin-LR (MC-LR) being the most toxic [4].

Nowadays, accumulating evidences imply that MC-dependent damage is accompanied by oxidative stress in liver and kidney in mammals [5-7]. Recently, some studies found that MCs-induced oxidative stress may exert negative effects on male reproductive system [8–10]. MCs' concentration was detected in various tissues of rats within 24 h postintravenous injection (i.v.) of MCs' extracts at 1 LD_{50}, using liquid chromatography-mass spectrometry (LC-MS). The highest concentration of MCs was found in kidney (0.034–0.295 μg/g dry weight). Rapid accumulation and degradation of MCs was found in the liver of rats, which was similar to the results observed in fishes. The content of MCs (RR+LR) remained relatively stable (mean 21–37 ng/g) in the testis within 24 hours postexposure (hpe), suggesting the difficulty of MCs elimination from gonad [11–13].

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The metabolism of MCs in animal frequently results in the formation of reactive oxygen species (ROS), such as superoxide radical (O^{2-}) and hydrogen peroxide (H_2O_2), which oxidize unsaturated lipids in cell membranes, proteins, and DNA, significantly contributing to their toxicity [15,16]. The ROS generated in tissues could be effectively scavenged by the antioxidant enzymes and glutathione (GSH)-related enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and gamma-glutamylcysteine synthetase (γ-GCS) [17].

ROS can modulate the antioxidant enzyme activities by regulating the mRNA levels through activation of signaling pathways [18]. The induction of antioxidant enzyme mRNA levels coincides with an increase in oxidative damage of proteins, suggesting the positive correlation between oxidative stress and antioxidant enzyme mRNA expression [19]. However, most of the studies concerning about the oxidative stress induced by MCs have focused on the response of activities of antioxidant enzymes [7,14,20–23], and less is known about the effects of MCs on antioxidant enzyme genes at transcriptional level [24–27].

The primary aim of the present study was to evaluate MCs-induced acute response of antioxidant enzymes in liver, kidney, and testis of male rats through analyzing the transcription abundance of antioxidant enzyme genes using quantitative real-time PCR (qPCR), so as to provide new insights into the possible role of antioxidant enzymes in the toxicological mechanisms of MCs at the transcription level.

MATERIALS AND METHODS

Toxin

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

Animals

Male Wistar rats weighing 200 ± 20 g were supplied by Hubei Laboratory Animal research Center (Hubei, People's Republic of China). The rats were housed under controlled conditions of 12 h light/dark cycle, $50 \pm 5\%$ humidity and $23 \pm 1^{\circ}$C. The animals were allowed free access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory.

LD_{50} of Cyanobacterial Crude Extracts

Male Wistar rats weighing 200 ± 20 g ($n = 40$) were divided into five groups, and rats in each group were administered by an i.v. injection of MCs at different doses of MC-$LR_{equivalent}$/kg body weight. While calculating the death number and mortality of rats in each group during 24 hpe, we obtained the LD_{50} level for 24 hpe by using the formula $LD_{50} = \log^{-1}[Xm - I(\Sigma p - 0.5)]$, where Xm: the log of max dose; p: mortality; Σp: the sum of mortality in each group; I: the difference between the log of adjacent two group dose. The LD_{50} studies were approved by the IACUC.

MCs Exposure

Healthy male Wistar rats weighing 200 ± 20 g were divided in equal numbers into two groups randomly. One group received i.v. injection of 1 mL MCs extracts at LD_{50} of 80 μg MC-$LR_{equivalent}$/kg body weight. An equivalent volume of 0.9% saline solution was applied to control ones. Six sampling points were set during a period of 24 h in the experiment (1, 2, 4, 6, 12, and 24 hpe). Five rats from each group were killed at each time point, and the livers, kidneys and testes were quickly removed, minced, and stored frozen at liquid nitrogen for later analysis.

Total RNA Isolation

Total RNA was isolated from 50-100 mg sections of liver, kidney, and testis using Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by determination at OD_{260}. RNA was extracted according to the manufacturer's protocol, resuspended in 50 μL RNase-free water, and stored at -80°C. Quantification was done using

TABLE 1. Real-Time PCR Primers Used in This Experiment

CAT	(+) 5′-ATTGCCGTCCGATTCTCC-3′	60	105
	(−) 5′-CCAGTTACCATCTTCAGTGTAG-3′		
MnSOD	(+) 5′-GCTCTAATCACGACCCACT-3′	62	147
	(−) 5′-CATTCTCCCAGTTGATTACATTC-3′		
ZnSOD	(+) 5′-GAGCAGAAGGCAAGCGGTGAA-3′	60	202
	(−) 5′-CCACATTGCCCAGGTCTC-3′		
GR	(+) 5′-GGGCAAAGAAGATTCCAGGTT-3′	60	101
	(−) 5′-GGACGGCTTCATCTTCAGTGA-3′		
GPX1	(+) 5′-CAGTTCGGACATCAGGAGAAT-3′	64	139
	(−) 5′-AGAGCGGGTGAGCCTTCT-3′		
GPX3	(+) 5′-AAACAGGAGCCAGGCGAGAA-3′	62	237
	(−) 5′-CTCAAAGTTCCAGCGGATGTCAT-3′		
GPX4	(+) 5′-AACGTGGCCTCGCAATGA-3′	60	101
	(−) 5′-GGGAAGGCCAGGATTCGTAA-3′		
γ−GCS	(+) 5′-ATCTGGATGATGCCAACGAGTC-3′	62	129
	(−) 5′-CCTCCATTGGTCGGAACTCTACT-3′		
GAPDH	(+) 5′-ATGGAGAAGGCTGGGGCTCACCT-3′	60	209
	(−) 5′-AGCCCTTCCACGATGCCAAAGTTGT-3′		

Eppendorf Biophotometer (Germany). The purified total RNA (2 μg) was then reverse transcribed. Reverse transcription was performed with oligo (dT) 18 primer using first strand cDNA synthesis kit (TOYOBO, Osaka, Japan). The resultant cDNA was then diluted 20 fold and was kept at −20°C.

Quantitative Real-Time PCR (qPCR)

All the primers used in qPCR were listed in Table 1. The primers were designed based on the gene sequences of *Rattus norvegicus* present on the GenBank. The specification of each pair of primers was confirmed by randomly sequencing six clones and was further confirmed by the melting curve analysis using qPCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid. Only primers with similar amplification efficiency were used in this experiment. GAPDH was used as the internal control gene for qPCR assay. qPCR was conducted by amplifying 1.0 μL of diluted cDNA with the SYBR Green qPCR kit (Finnzymes, Finland) on a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA). Cycling conditions were as follows: 3 min at 95°C, 44 cycles of 15 s at 95°C, 20 s at 62°C or 64°C, and 15 s at 72°C. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in three tubes, and PCR reactions without the addition of the template used as blanks. After completion of the PCR amplification, data were analyzed with the Option Monitor software 2.03 version (MJ Research, Cambridge, MA).

Statistical Analysis

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

A Pearson's correlation was used to present the relationship between the GSH-related enzymes (GPx, GR, and γ-GCS) throughout their fluctuation during the 24 hpe ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$).

RESULTS

Figure 1 shows the transcriptional changes of several antioxidant enzymes in liver, kidney, and testis of male MCs-treated rats within 24 hpe.

We found that the expression of CAT was significantly decreased in all the three organs. In liver, it showed a tendency to decline from 4 hpe onward, and the expression level was only 1/10 of the control level at 24 hpe. In testis, it was generally depressed, but a significant induction was observed at 6 hpe.

The mRNA expression of Mn-SOD was significantly induced in all the three organs tested. The expression of Cu,Zn-SOD was induced in liver at 1, 2, 4, 6, and 12 hpe, but at 24 hpe it was significantly suppressed. In kidney, it was induced evidently at first 2 h, then it was suppressed significantly at 6 hpe and did not show a tendency to recover until 24 hpe. In testis, it was suppressed at first 6 h, but at 12 and 24 hpe time point it was significantly induced.

The transcription of GR was suppressed in liver at all time points. It was evidently induced at 2 hpe in kidney and at 4 hpe in testis, respectively. But from 12 hpe onward, it was significantly depressed in both organs and did not show a tendency to recover until 24-hpe postinjection.

γ-GCS mRNA expression was generally suppressed in liver, kidney, and testis. But significant induction of γ-GCS mRNA expression was observed at 2 hpe in liver and at 4 hpe in kidney.

In liver, the transcription of GPx1 was induced at 2 and 4 hpe, but suppressed from 12 hpe onward. In kidney, the GPx1 expression was significantly depressed from 2 to 6 hpe, whereas GPx3 expression was significantly elevated in the first 12 h. GPx4 was evidently induced in testis from 6 to 24 hpe and peaked at 6 hpe.

Correlation analysis between GSH-related enzymes (GPx, GR, γ-GCS) showed different patterns in the three organs. In liver, there were positive correlations between GPx1 and γ-GCS ($r = 0.973$, $P < 0.01$). In kidney, there were negative correlations between GPx1 and GPx3 ($r = -0.901$, $P < 0.05$). In testis, the GSH-related enzymes showed no obvious correlation between each other.

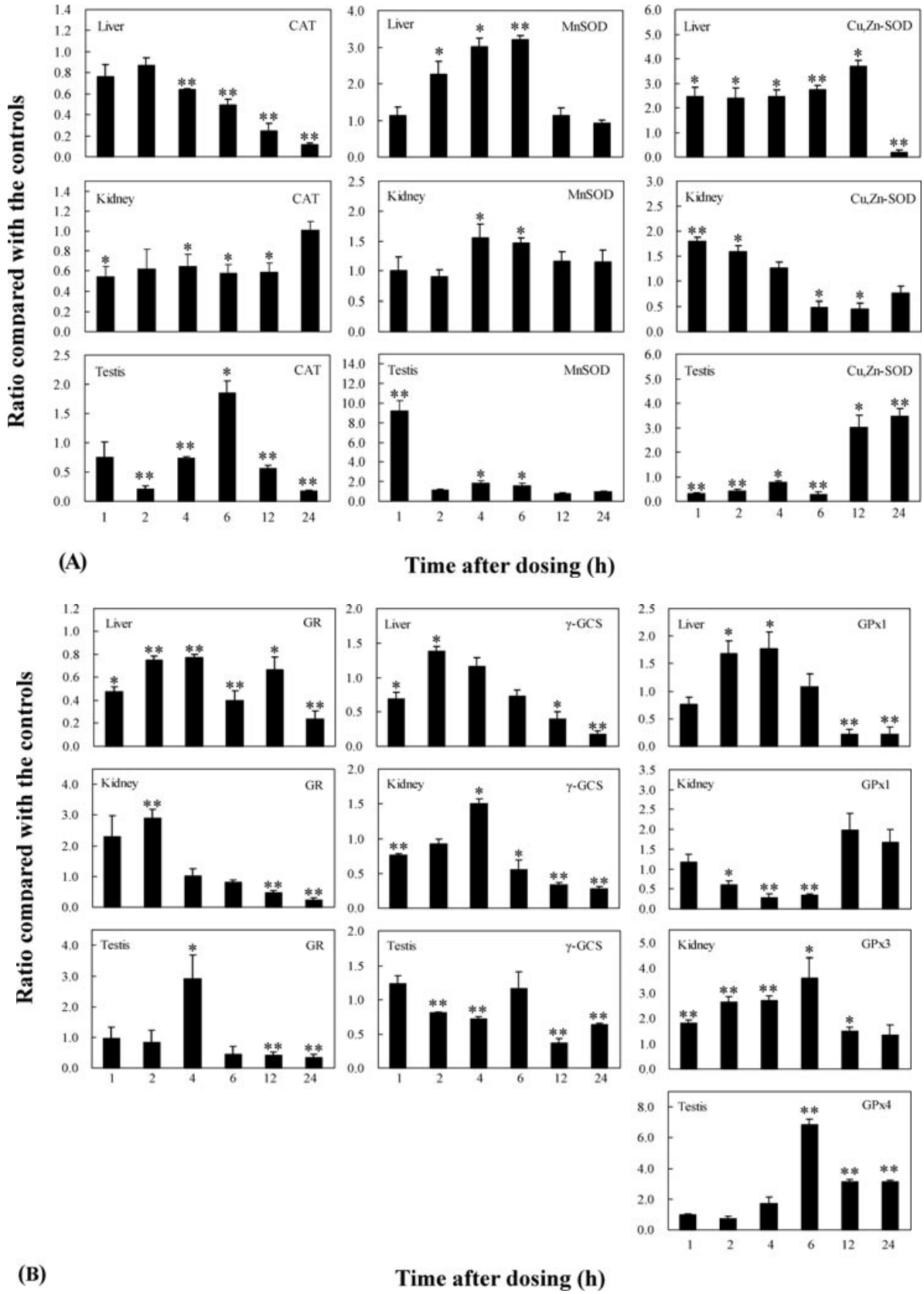

FIGURE 1. Time course of antioxidant enzyme mRNA expression by Q-PCR analysis in liver, kidney and testis of Wistar rat after treatment with 80 μg MC-$LR_{equivalent}$/kg body weight. (* indicates significant change at $P < 0.05$ and ** indicates significant change at $P < 0.01$).

DISCUSSION

MCs can induce the production of ROS, namely O^{2-} and H_2O_2 [5,14], which lead to increased formation of lipid peroxides (ROOH) [5,24]. ROOH are known to be reduced by the action of GPx to alcohols (ROH) using GSH [28]. Then the oxidized glutathione dimer (GSSG) is reduced to two GSH molecules by GR. Previous studies showed that the activity of GPx is transcriptionally regulated, and increased activity of GPx corresponded with increased transcription of this enzyme in mice or rats administered with MCs [24,25,27]. In this study, the transcription of cytosolic GPx (GPx1), which distribute ubiquitously in various tissues in mammalian species [29], was significantly induced in liver at 2 and 4 hpe. The upregulation of GPx1 expression at first was supposed to counteract oxidative attack induced by MCs. However, decreased transcription of GPx1 was observed from 12 hpe onward when the expression of γ-GCS, the rate-limiting enzyme of GSH synthesis also declined. Thus, the lower GPx1 transcription, which may lead to lower GPx1 activity, could be due to a decline in GSH concentration induced by lower expression of γ-GCS [14,30–32]. In the present study, correlation analysis also showed that there were positive correlations between GPx1 and γ-GCS in liver ($r = 0.973$, $P < 0.01$). Similar to GPx1, the mRNA expression of GR was significantly suppressed at 12 and 24 hpe in the three organs when the expression of γ-GCS also declined. Decreased transcript activities of GR could also be due to reduced GSH concentration induced by lower expression of γ-GCS [7,25,33].

Plasma GPx (GPx3) and phospholipid GPx (GPx4), mainly expressed in kidney and testis, respectively [34,35], were significantly induced in the present study. GPx3 is proposed to be a major scavenger of ROS in the extracellular space and within the vasculature [36]. GPx4 was discovered as a factor preventing lipid peroxidation [37] and primarily discussed as the GPx-protecting biomembranes against oxidative stress [38]. So it was probably that GPx3 and GPx4 play important roles in counteracting the oxidative stress induced by MCs in kidney and testis, respectively. In kidney, the expression of GPx3 was significantly induced since 1 hpe, followed by the decreased expression of GPx1. Correlation analysis showed that there were negative correlations between GPx1 and GPx3 ($r = -0.901$, $P < 0.05$) in kidney. So it is supposed that the decreased expression of GPx1 is caused by the upregulation of GPx3 mRNA.

SOD is the endogenous scavenger that catalyzes the dismutation of the highly reactive O^{2-} to H_2O_2 [39]. The mRNA expression of Mn-SOD was induced in the three organs tested. The marked increase in Mn-SOD mRNA was probably attributed to the high metabolic demand induced by MCs and indicated an enhanced demand for new synthesis of this enzyme. The transcriptional activation of Mn-SOD can be mediated by activator protein 1 [40,41], a class of transcription factors involved in oxidative stress response. AP-1 exists either as a heterodimer, composed of the protein products of the *fos* and *jun* immediate-early response gene families (Jun/Fos), or as a homodimer of Jun proteins (Jun/Jun). Both c-Jun and c-Fos are quickly induced in cells in response to oxidants [42,43]. Our early study revealed that both c-Jun and c-Fos proteins were induced in liver, kidney, and testis of rats administered a 1 LD_{50} dose of MCs i.v. [44]. So it was probably that the induction of Mn-SOD expression in liver, kidney, and testis might be resulted from the increased expression of c-Jun and c-Fos.

CAT can catalyze the increasing $H_2O_2$to H_2O. Decreased activities of CAT have been shown in MC-LR-treated rats and mice in earlier study [7,25]. Decreased activity of CAT was also reported in the liver of MCs-treated domestic rabbits [23]. In our study, we found that the expression of CAT mRNA was decreased at almost all time points in the three organs. So we deduce that the decreased activity of CAT may parallel the decreased expression of CAT mRNA. High production of superoxide anion radical was indicated to inhibit CAT activity [45]. Decreased transcription of CAT, which probably led to reduced activity of the enzyme, could be due to the excess of superoxide anion radical caused by MCs.

Increased mRNA expression of CAT, Mn-SOD, and GPx1 in heart muscle of rats at 24 h postinjection i.v. with MCs at the 1 LD_{50} dose was reported, which paralleled the increased activities of these enzymes [27]. But in the present study, the mRNA expression of these three genes was not significantly induced at 24 hpe in all the three organs; on the contrary, the expression of CAT was significantly suppressed in liver and testis, and decreased expression of GPx1 was also detected in liver. Previous study also reported the response of antioxidant enzymes in mice administered i.p. 1 LD_{50} of MC-LR at the transcriptional level. The gene expression profile of GPx, SOD, CAT, and GR did not show any significant difference compared to control [25]. At variance with their results, the present study demonstrates that the transcription of antioxidant defense systems is modified by MCs' administration. Therefore, it seems that the transcriptional activity of antioxidant enzymes may differ with exposure routes, time, toxin level, sampling interval, composition of MCs, and physiological characters of the selected tissues and animals.

In conclusion, we found that MCs could affect the transcriptional activities of CAT, Mn-SOD, Cu,Zn-SOD,

GR, GPx, and γ-GCS in liver, kidney, and testis of male Wistar rats using the qPCR method for the first time. What is more, we speculated the possible causation of the transcriptional alteration of these enzymes. The transcriptional responses of antioxidant enzymes to oxidant stress differ in an organ-specific manner. A possible explanation is that the accumulation and distribution of MCs in male Wistar rats were different in these organs [11], and the different concentrations of MCs might influence some transcriptional regulating factors of these enzymes in different ways, resulting in the different mechanisms of transcriptional regulation of these enzymes. The transcriptional alterations of these antioxidant enzymes in MCs-treated rat could suggest an adaptative response to combat oxidative injury induced by MCs, confirming that oxidative stress in involved in the damage induced by MCs and providing us new perception of the possible role of antioxidant enzymes in the toxicological mechanisms of MCs at the transcription level.

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