RESPONSE OF ANTIOXIDANT PARAMETERS TO 3,3'-DIMETHYL-BENZIDINE IN GOLDFISH (*CARASSIUS AURATUS*) LIVER

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ABSTRACT

Adaptive responses of the livers of goldfish (*Carassius auratus*) exposed to five concentrations of 3,3'-dimethyl-benzidine (DMBz) (0.9-9.0 mg/L) for 3, 7 or 14-days (d) relative to unexposed goldfish were investigated. A suite of antioxidant parameters including glutathione (GSH) concentration and activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-transferase (GST) were evaluated. DMBz caused measurable oxidative parameters changed in goldfish liver with the following adaptive responses: (1) activities of SOD were significantly less in livers of goldfish exposed to 0.9 mg DMBz/L, but significantly greater when exposed to 4.5, 9.0 mg DMBz/L; (2) activities of GST and GSH were well-correlated with DMBz exposure concentrations; (3) SOD was the primary antioxidant while CAT and GPx activities were not related to concentrations of DMBz, to which the fish were exposed. DMBz caused oxidative parameters changed in livers of goldfish, possibly due to the presence of redox-active metabolites. The concentration of GSH and activities of SOD and GST responded to the effects of DMBz and can be useful functional measures of exposure of goldfish to compounds, such as DMBz, that cause oxidative stress.

KEYWORDS: 3,3'-dimethyl-benzidine, goldfish *Carassius auratus,* superoxide dismutase, catalase, glutathione peroxidase, glutathione-s-transferase.

INTRODUCTION

Aromatic amines are important antioxidants and intermediates in the synthesis of dyes, pesticides, and plastics and, thus, can enter the environment by a number of pathways, such as emission during production of dyes or the manufacture of products, by leaching from consumer products, or following disposal. Aromatic amines have been measured in biotic and abiotic environmental matrices [1, 2]. Given their ubiquity of use and occurrence in the aquatic environment as well as their physical and chemical properties, aquatic organisms are likely to be exposed to aromatic amines. A number of azo-type colorants based on 3,3'-dimethylbenzidine (DMBz), have been synthesized in large quantities [4]. There is also evidence that DMBz is carcinogenic to laboratory animals [5, 6]. The metabolic conversions of bisazobiphenyl dyes, derived from DMBz, are also carcinogenic to mammals, such as the dog and rat [7]. According to current EU regulations, azo-dyes based on DMBz have been classified as carcinogens of a type that "should be regarded as if they are carcinogenic to man". Several epidemiological studies have demonstrated that use of DMBz-based dyes has caused bladder cancer in humans [8]. Several textile dyeing and finishing manufactories still produce DMBz and azo-dyes in China [8], and relatively large quantities of these compounds are discharged into receiving waters and DMBz is frequently detected in surface waters and industrial effluents [1, 2]. Therefore, there is concern about the potential for deleterious effects of DMBz on estuarine and coastal ecosystems, while there were few reports of DMBz on the responses to aquatic animals. It was deemed prudent to study the effects of continual dynamic exposure to DMBz on a model fish. The fish liver is an important detoxification organ, and, thus, was selected on the basis of functional criteria, which made it a preferential target, xenobiotic metabolism.

To minimize cellular damage upon exposure to pollutants, adaptive responses occur in the liver by metabolizing compounds to reduce their toxicity or facilitate depuration. In aerobic organisms, these processes, catalyzed by cytochrome P450 mixed function mono-oxygenases, can produce activated intermediary products as well as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) as byproducts of oxidative metabolism. These intermediate products can result in damage to cells, a process that is referred to as oxidative stress. In particular, $HO^{\bullet}$ can initiate lipid peroxidation in tissues [9] and, especially, damage membranes.

Cells are protected from damage due to oxidative stress by adaptive responses that minimize exposure to these activated, reactive intermediaries and byproducts by antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [10]. Under normal conditions, these antioxidant enzymes maintain relatively small concentrations of the byproducts of oxidative metabolism and minimize their damage to cells. Glutathione-s-transferases (GST) are a family of multifunctional enzymes that combine glutathione with activated intermediates of xenobiotics to facilitate excretion [11]. Other molecular scavengers, such as glutathione (GSH), ascorbate, vitamin E, β-carotene and various proteins located in the cytosol and membranes, or present in the extra cellular fluid, can also react with activated intermediates to facilitate excretion and reduce their damage to cells [12-14]. Reactive electrophilic components can be removed before they covalently bind to tissue nucleophilic compounds which would lead to toxic effects.

In the present study, the goldfish, which is widespread and common in freshwaters and often used as a model laboratory organism [15, 16], was used to evaluate the toxicity of DMBz, with particular attention to hepatic antioxidant modulation. This study investigated responses of goldfish hepatic antioxidant parameters changed with DMBz exposure, including the phase II enzyme (GST), ROS scavenger (GSH) and antioxidant enzymes (SOD, CAT, GPx). Goldfish were exposed to 5 concentrations of DMBz for 3, 7 and 14 d.

MATERIALS AND METHODS

Chemicals

The GSH assay kit was obtained from the Nanjing Jiancheng Bioengineering Institute Company (Nanjing, China). Other standard enzyme chemicals were obtained from Sigma Chemical Company. DMBz (analytical grade) was purchased from the Beijing Chemical Company (Beijing, China). Stock solutions of 10 g/L DMBz were prepared in acetone and maintained at 4 °C.

Fish collection and treatment

Goldfish (*Carassius auratus*) with mean body-length and weight 12±2 cm and 25±2 g, respectively, were purchased from Heilongtan Fish Ponds (Nanjing, China). Fish were acclimated for 10 d to aerated, dechlorinated water prior to DMBz exposure. There were no mortalities in the unexposed fish during acclimation or during exposures. Fish were fed commercial fish food (0.1g dry pellets) per fish daily in the morning during acclimation and exposures.

After acclimation, fish were divided into six groups of six fish each (n=6). Since the 96-h LC_{50} was approximately 18 mg DMBz/L [17], fish were exposed to 9.0, 4.5, 3.0, 1.8, or 0.9 mg DMBz/L for 3, 7, or 14 d, respectively. The water was always aerated and renewed every 24 h by adding DMBz test solution. The study stock solutions were prepared by dissolving DMBz in less than 0.01% of acetone to avoid co-solvent effects. One blank was set as control, which was maintained in tap water. During the experiment, water pH was 7.3±0.3 and water temperature was 22±1 °C, on a 14:10 light: dark photoperiod, with hardness of 100 mg/L as $CaCO_3$.

At the end of the exposure, fish were weighed, dissected and their livers were separated after rinsing in physiological salt water (0.9% NaCl). Approximately 0.30 g of liver was homogenized after adding 3.0 ml of 10 mmol/L Tris-HCl (pH 7.5) containing 0.25 mol/L sucrose, 0.1 mmol/L EDTA using a glass-teflon homogenizer for detection of enzyme activities. Approximately 0.10 g of liver was homogenized after adding1.0 ml of 1.0 mmol/L EDTA and 10 µl $HClO_4$ for measurement of GSH (reduced glutathione). The extracts were centrifuged at 1×10^4 rpm for 10 min at 4 °C, and then preserved at -85 °C until analysis.

Enzyme, glutathione and protein assays

SOD activity was determined using a modification of the method of Maklund and Marklund [18], which is based on inhibition of the auto-oxidation of pyrogallol. Samples were assayed in a solution of 8.7 ml of 50 mmol/L phosphate buffers, and 0.3 ml of 3 mmol/L pyrogallol (dissolved in 10 mmol/L HCl). The media final pH value was 8.24. The rate of pyrogallol auto-oxidation was measured with a UV-2450 spectrophotometer (Shimadzu, Japan) at 325 nm. One unit of enzyme activity was defined as the amount of enzyme which gave 50% maximal inhibition of the auto-oxidation rate of 0.1 mmol/L pyrogallol in 1 ml of solution at 25 °C.

CAT activity was determined by use of the method of Xu et al. [19], which is based on ultraviolet spectrophotometry. 10 µl of sample was added to 3.0 ml of H_2O_2 phosphate buffer, pH 7.0 (0.16 ml of 30% H_2O_2 to 100 ml of 0.067 mol phosphate buffer), and the change in H_2O_2 absorbance within 60 s was measured at 250 nm with an UV-2450 spectrophotometer (Shimadzu, Japan). One unit of enzyme activity was defined as the amount of the enzyme that decreased 1 µmol H_2O_2 per min.

GPx activity was measured with a modification of the methods of Hafeman et al. [20]. The reaction mixture contained GSH, NaN_3-PBS, H_2O_2, TCA (trichloroacetic acid),

Na_2HPO_4 and DTNB (5,5'-dithiobis (2-nitrobenzoic acid)). The rate of reaction was measured by the decrease in GSH, which was determined by measuring the reaction products of DTNB and GSH (absorbance of the ions at 423 nm). One unit of enzyme activity was defined as a decrease of 1 µmol/L of GSH concentration at 37 °C and pH 6.5, while non-enzymatic reactions were excluded.

GST activity was measured by use of the methods of Habig et al. [21] with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Assays were performed in a reaction mixture containing 1.05 ml of 100 mmol/L Tris buffer (pH 7.4), 50 µl CDNB (1 mmol/L), 50 µl GSH (1 mmol/L) and 50 µl tissue homogenate. Before use, the GSH required for the assay was dissolved in Tris buffer, and CDNB was dissolved in ethanol. In all cases, final concentration of ethanol in the assay mixture did not exceed 5% (v/v). Blanks had the same conditions replacing the sample with Tris buffer. Enzyme activity was determined by monitoring changes in absorbance at 340 nm, which related to the rate of CDNB conjugation with GSH (E_{340}CDNB-GSH conjugation = 9.6 $mmol^{-1}cm^{-1}$) for 2 min at constant temperature. The GST activity is expressed as µmol/min/mg protein.

GSH levels were measured by the method of Hissin and Hilf [22]. To 0.5 ml of the original tissue supernatant, 4.5 ml of the phosphate-EDTA buffer, pH 8.0, was added. The final assay mixture (2.0 ml) contained 100 µl of the diluted tissue supernatant, 1.8 ml of phosphate-EDTA buffer, and 100 µl of the OPT (*O*-phthalaldehyde) solution, containing 100 µg OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with the activation at 350 nm. GSH concentration was estimated from a standard curve and reported as µmol GSH/mg protein.

Protein in the samples was determined by the method of Gomall et al. [23], and comparison to bovine serum albumin as a standard. This method is based on dye-binding, using concentrated Coomassie Brilliant Blue (G-250), in which a red colored dye is bound to the protein producing a blue colored product which can be quantified by measuring absorbance at 595 nm.

Statistical analyses

Values are reported as means ± SD and analyzed using the SPSS for Win 12.5 computer program. Analysis of variance (ANOVA) was used to determine differences among treatments. If the overall ANOVA indicated the presence of statistically different treatment effects, Dunnett's *t* test was used to determine which groups were significantly different from the control.

RESULTS

Exposure to DMBz for 14 d resulted in differential responses among antioxidant parameters. SOD activity ({SOD}) was inhibited by exposure to 0.9 or 1.8 mg DMBz/L, especially after 7 d (Fig. 1). There was no difference between the {SOD} in livers of goldfish exposed to 3.0 mg/L and controls, while exposure to the three highest concentrations of DMBz resulted in higher {SOD} relative to that of the livers of unexposed controls. SOD activity was 365% that of controls in fish exposed to 9.0 mg DMBz/L for 14 d.

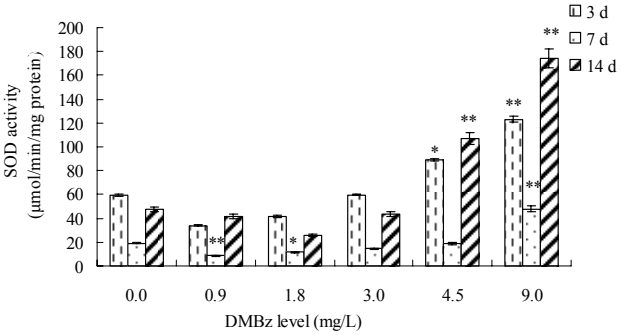

FIGURE 1 - Effects of DMBz on SOD activity in livers of *Carassius auratus*. Each value represents the mean ± SD (n = 6). (*) Significantly different from control, p < 0.05. (**) Highly significantly different from control, p < 0.01.

Relative to the control group, CAT activity ({CAT}) was greater following exposure to DMBz for 3 d, especially in those exposed to 1.8 mg DMBz/L for which CAT activity was 319% higher than that of controls (Fig. 2). However, there was not a monatonic relationship between {CAT} and concentration of DMBz. When exposed for longer periods (7 d and 14 d), the relationship between {CAT} and exposure to DMBz was opposite to that of the shorter duration of exposure.

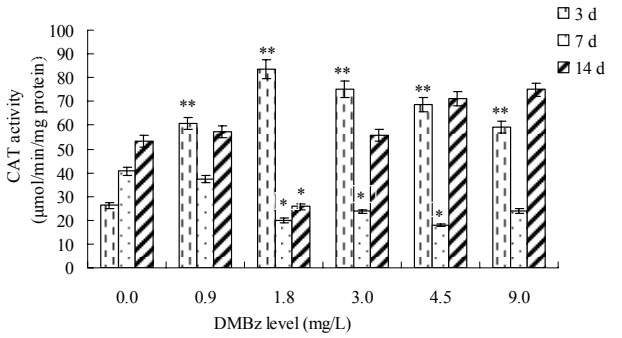

FIGURE 2 - Effects of DMBz on CAT activity in livers of *Carassius auratus*. Each value represents the mean ± SD (n = 6). (*) Significantly different from control, p < 0.05. (**) Highly significantly different from control, p < 0.01.

GPx activity ({GPx}) was greater in exposed goldfish, relative to that of controls, and the response was similar between 3 d and 7 d of exposure (Fig. 3). After 14-d exposure, the change of {GPx} was not statistically significant in livers of fish exposed to the lesser concentrations (0.9, 1.8 mg DMBz/L) but significantly higher in livers of fish exposed to the greater DMBz concentrations.

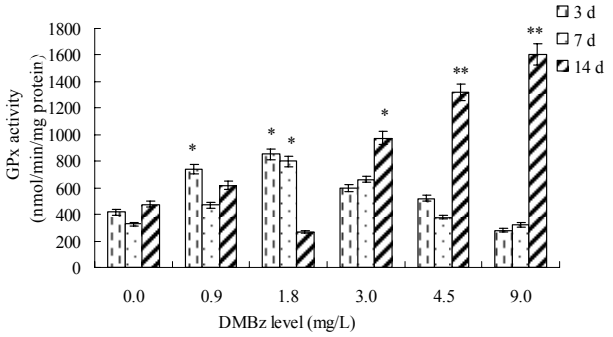

FIGURE 3 - Effects of DMBz on GPx activity in the livers of *Carassius auratus*. Each value represents the mean ± SD (n = 6). (*) Significantly different from control, p < 0.05. (**) Highly significantly different from control, p < 0.01.

GST activity ({GST}) was directly proportional to DMBz concentrations after exposure for 3, 7, or 14 d (Fig. 4), a trend which was demonstrated by the degree of correlation observed between {GST} and DMBz concentrations. {GST} in livers of goldfish exposed to 0.9 mg DMBz/L was significantly greater than that of the control. {GST} was significantly higher ($p< 0.05$) in livers of fish exposed to all concentrations of DMBz. {GST} was 545 % that of control, when goldfish were exposed to 9.0 mg DMBz/L for 3 d. There was no statistically significant relationship between {GST} after 3 d, but after 7 d exposure, {GST} were correlated ($r^2 = 0.9590$) with and could be predicted from DMBz concentration (Equation 1). GST activities were activated after 7 d, and then were depressed gradually after 14 d. {GST} was 1344% and 1165% greater than that of the control when goldfish were exposed to 9.0 mg DMBz/L for 7 or 14 d, respectively.

$$\{GST\}= 189.93\ [DMBz] + 492.67 \quad (1)$$

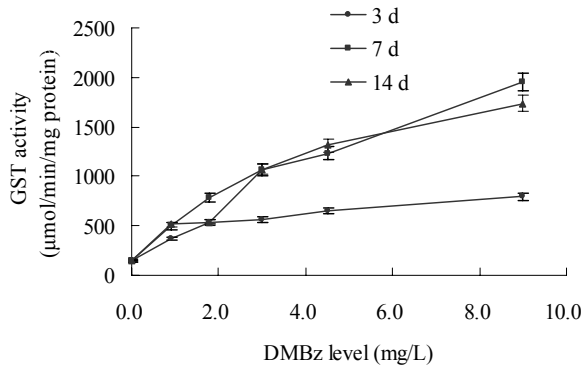

FIGURE 4 - Effects of DMBz on GST activity in livers of *Carassius auratus*. Each value represents the mean ± SD (n = 6).

Exposure to all concentrations of DMBz resulted in higher levels of the free radical scavenger, glutathione (GSH) in the liver of goldfish (Fig. 5). Exposure to DMBz for 3 d and 7 d resulted in significant positive correlations between concentrations of GSH and DMBz after 3 d ($r^2 = 0.9628$) (Equation 2) and 7 d ($r^2 = 0.9454$) (Equation 3), but there was no statistically significant correlation after 14 d of exposure ($p = 0.05$).

$$[GSH] = 0.0349\ [DMBz] + 0.0373 \quad (2)$$

$$[GSH] = 0.0051\ [DMBz] + 0.037 \quad (3)$$

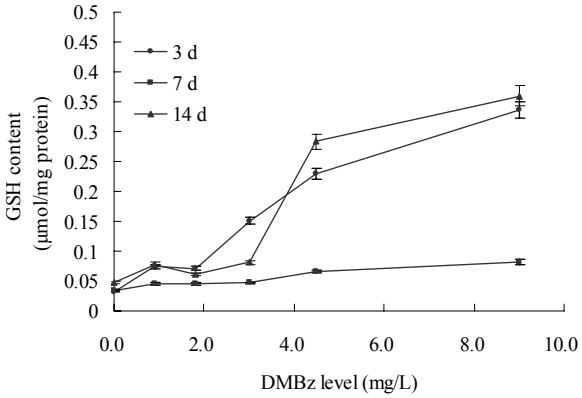

FIGURE 5 - Effects of DMBz on GSH level in the livers of *Carassius auratus*. Each value represents the mean ± SD (n = 6).

DISCUSSION

The three major antioxidant defense enzymes are inducible enzymes. They can be induced by even slight oxidative stress due to compensatory responses. However, a severe oxidative stress suppresses the activities of these enzymes due to oxidative damage and a loss in compensatory mechanisms [24]. The possibility that change in antioxidant enzymes could be due to the presence of xenobiotics in organisms has been considered. The increased activities of SOD, CAT, GPx, and GST are known to serve as protective response to eliminate reactive free radicals. Reduction of superoxide radicals by SOD and of H_2O_2 and ROOH by CAT and GPx, respectively, prevent the formation of radical intermediates by oxygen reduction mechanisms. GSH is involved in scavenging free radicals, and, thereby, blocking the propagation of lipid peroxidation [25]. At first, SOD converts superoxide anion to hydrogen peroxide in a cellular antioxidant reaction. The same trends of SOD activity of DMBz at different time were noticed as an initial decrease (0.9 mg DMBz/L), followed by an increase in exposure concentration. Studies carried out with primary cell cultures of mussel digestive gland and specific inhibitors of SOD, CAT, and GPx indicate a central role for SOD in protecting cells from oxidative stress [26]. A concentration of 0.9 mg DMBz/L can be suggested as the threshold value for response of goldfish, but at higher concentrations of DMBz (3.0 mg/L), SOD activity returned to normal. This pattern indicates that formation of an adaptable equilibrium for SOD. Increases in SOD activity were a probable early adaptation response to oxidative stress, and may be a potential biomarker of fish to DMBz exposure.

CAT is mainly located in the peroxisomes and responsible for the reduction of hydrogen peroxide produced from the metabolism of long-chain fatty acids; while GPx catalyzes the reduction of both hydrogen peroxide and lipid peroxides, and is considered to be an efficient protective enzyme against lipid peroxidation [27]. In previous studies, CAT was found to be induced in response to exposure to PAH- or PCB-contaminated sediments in fish [28, 29] and in marine mussels [30]. Parallel induction of glutathione peroxidase was also observed in those studies. But in another study [31], a decrease in CAT activity was observed in carp (*Cyprinus carpio* L.) and catfish (*Ictalurus nebulosus*) exposed to the organophosphate insecticide dichlorvos, which is known to induce oxidative damage.

In the study on which we report here, CAT activity was significantly greater in livers of goldfish exposed to 0.9 mg DMBz/L after 3 d, which demonstrated that a relatively small concentration of DMBz can induce CAT in a relatively short period of time. The fact that exposure to the three higher levels of DMBz suggests the accumulation of H_2O_2, but not enough to poison CAT activity. CAT is a prospective biomarker of exposure of fish to DMBz. Wu et al. [32] studied 922 papers in the biomarker literature, and then analyzed that many stress responses may decline with time after induction (i.e. adaptation), even if the level of stress remains constant, i.e. although significant induction of CAT activity was observed after 3-days exposure, but depression was also found after 7 days.

GPx independently detoxifies produced hydrogen peroxide [33]. GPx removes hydrogen peroxide and lipid hydro-peroxides in mammals, and its role has recently been investigated in different types of aquatic animals [26]. The reason that there was no significant relationship between GPx activity and exposure to DMBz is unknown. However, antioxidant activity is not always directly proportional to the concentration of xenobiotics to which organisms are exposed. For instance, Doyotte et al. [34] pointed out that a decreased enzyme activity's response may accompany a first exposure to pollutants, which can be followed by an induction of antioxidant systems. Thus, the existence of an inducible antioxidant system may reflect an adaptation of organisms. Abcisic acid and gibberellic acid can cause increased lipid peroxidation and fluctuated antioxidant defense systems of various tissues in rats [35]. In the text, authors said another possibility is that the chemical may lead to the inhibition or stimulation of the enzymes` synthesis in rats' tissues by effects of mRNA transcription mechanisms [36]. Nevertheless, the physiological roles of these antioxidant enzymes in the cell are poorly understood because of complex interactions and interrelationships among individual components [35].

Since activities of SOD, CAT, and GPx were all increased by exposure to DMBz, oxy-radials are removed by these antioxidant enzymes. It is known that activities of these enzymes in mammals and other aquatic organisms may be increased by conditions of enhanced oxy-radical generation [27, 36]. Glutathione-s-transferase (GST) is one of these enzymes [37]. GST has been reported as a bio-indicator for assessing the environmental impact of organic xenobiotics that generate oxidative stress [38]. GST is a group of multifunctional enzymes involved in biotransformation and detoxification of xenobiotics. GST catalyze the conjugation of various electrophilic compounds with the tripeptide glutathione, the resulting conjugates being water-soluble and, thus, more easily excretive. In addition, GST serves to reduce the likelihood of electrophilic compounds covalently binding to important cellular macromolecules, such as DNA.

In this study, among the measured antioxidant parameters, {GST} exhibited the greatest coefficient of determination with DMBz concentrations, regardless of duration of exposure ($r^2 = 0.9590$). Since GST is involved in xenobiotic detoxification and excretion of xenobiotics and their metabolites, its increased activity in liver may indicate development of a defensive mechanism to benzidine congeners. Increasing in GST activity was concomitant to increasing in glutathione content in liver. A remarkable sensitivity of GST to a large variety of pollution conditions, either organic or inorganic, was identified by several authors [39]. However, these enzymes exhibit high sensitivity, but low specificity as noted by McLoughlin et al. [37]. In depth investigation has been dictated of GST for its importance in cancer susceptibility and prevention [40].

GSH, as an oxy-radical scavenger, is important in antioxidant defense. GSH also acts as a reactant in conjugation with electrophilic substances. Thus, a change in GSH levels may be a very important indicator of the detoxification ability of an organism. GSH concentrations have rarely been studied in aquatic invertebrates exposed to trace organic compounds, although a few authors have studied the effects of heavy metals on GSH levels [41]. Ibrahim Orun et al. [42] studied effects of various sodium selenite concentrations on Se-GSH-Px of rainbow trout (*Oncorhynchus mykiss*), and they found that Se-GSH-Px activity generally increased in all groups for brain, heart and spleen tissues with respect to control group, but the only statistically significant difference ($p<0.05$) was found in liver tissues of trouts exposed to 4 and 6 ppm sodium selenite (SS). Similarly, the treatment of the channel catfish (*Ictalurus punctatus*) and brown bullhead (*Ameriurus nebulosus*) with a redox active quinone, likemenadione, led to significantly higher constitutive levels of hepatic GSH [43]. The fact that concentrations of GSH were directly proportional to the concentration of DMBz to which fish were exposed ($r^2 = 0.9454, 0.9628$) may be due to the scavenging ability of GSH, which offers a first step protection against oxy-radicals. GSH can react with hydrogen peroxide (H_2O_2) and organic hydro-peroxides (ROOH) to transform them into H_2O and ROH, respectively.

CONCLUSIONS

The antioxidant parameters were measured, including SOD, CAT, GPx, GST activities and concentration of GSH

all responded to DMBz exposure, which indicates that DMBz causes oxidative stress and that these parameters can be used as functional measures of hepatic antioxidant homeostasis of fish. SOD activity was the most responsive parameter while CAT and GPx activities exhibited poor responses to exposure levels. The possible mechanism of toxic action of DMBz to goldfish can be explained as an oxidative stress mechanism. As DMBz is transformed in the liver, oxidation and reduction reactions take place such that activated transformation products and reactive oxygen is generated, which can result in damage to the membranes of cells and organelles in the liver. In addition, GST, SOD activity and GSH level were sensitive to DMBz contamination and, thus, can be likely used in the early assessment of DMBz–dominant polluted aquatic ecosystems. In the future, tests including the chronic toxicity test, the long-term toxicity test in low dose exposure, and molecular toxicological tests will be used to assess the toxicology of DMBz more accurately.

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