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QUANTITATIVE CHANGES IN ANTIOXIDANT ENZYME ACTIVITIES, GLUTATHIONE CONTENT AND MALONDIALDEHYDE IN A FRESHWATER FISH, *ANABAS TESTUDINEUS* (BLOCH), EXPOSED TO SEWAGE

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Abstract: In the present study, the effect of sewage on the antioxidant enzymes such as superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT) were studied along with Glutathione content (tGSH), and lipid peroxidation product, Malondialdehyde (MDA) in the liver, brain and kidney of a freshwater fish, *Anabas testudineus.* Exposure to sewage significantly decreased the activity of liver SOD and GST for 7 and 14 days. But after 21 days of exposure, the activities of liver SOD and GST were restored. Liver CAT activity was increased on 7 and 14 days but restored on 21st day of sewage exposure. While the level of tGSH and MDA were increased after sewage exposure. In kidney, sewage exposure significantly inhibited SOD and CAT activity and inhibited the level of tGSH but GST was increased on 7th and 14th days and restored to control on the 21st day. MDA level was significantly increased only on the 7th day and thereafter found to be decreased. In brain, tGSH was found to be decreasing while, the activities of SOD was significantly increasing on the 14th day and CAT on 7th and 21st days. Brain MDA was found to be significantly elevated from control. Alterations in the antioxidant enzyme activity and amelioration in lipid peroxidation products in the present study reveals that the fish experienced oxidative stress.

Key words: Catalase, glutathione-S-transferase, glutathione, lipid peroxidation, oxidative stress, superoxide oxide dismutase

INTRODUCTION

The unscientific disposal of sewage into water bodies causes grave problems to the aquatic environment as also to animals and human beings world over (Girija *et al.,* 2007). Sewage is a source of chemicals that may trigger generation of reactive oxygen species (ROS) and consequent oxidative stress in aerobic organisms, despite the antioxidant defence mechanisms possessed by them (Davies, 1995; Halliwell and Gutteridge, 1989); Vos *et al.,* 2000). Induction of antioxidants provides sensitive early warning signals of incipient oxidative stress (Benson and Di Giulio, 1992; Cossu *et al.,* 1997; Almroth *et al.,* 2008; Ruas *et al.,* 2008). Lipid peroxidation has been reported as a major contributor to the loss of cell function under oxidative stress (Marnet, 1999).

The present study estimated the quantitative changes of the antioxidant enzymes, superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (CAT) and of glutathione content (tGSH) and the lipid peroxidation product, malondialdehyde (MDA), in the liver, kidney and brain of the freshwater fish, Anabas testudineus in order to gain insight into the antioxidant ability of the subject towards sewage exposure.

MATERIALS AND METHODS

Study site

Parvathyputhenar, an artificial canal in the heart of the Thiruvananthapuram city (Lat- $8^0$29'21" Long- $76^0$55'17"), Kerala, India, dating back was used for navigation, as an avenue of leisure and even the water was used for domestic consumption. But, over years, it has become a major source of environmental pollution, posing a health hazard for the residents of the city. Domestic wastes from the Trivandrum city are brought to the sewage farm at Muttathara established five decades back. These wastes are drained to the nearby grassland and after this, without any treatment, are directly emptied into Parvathy puthenar, which is the study site.

Experimental animal

The fish used in these experiments were collected from local suppliers, brought to the laboratory and kept in large cement tanks with aerated, filtered, dechlorinated and re-circulating tap water (Ahmad *et al.,* 2005) at 26 ± 2°C under natural photoperiod for a month prior to experiment for acclimatization. The fish were fed with 40% protein feed ad libitum. The components of the feed were rice bran, tapioca, fish meal, groundnut cake with adequate amounts of vitamins. Prior to the experiment, adult healthy fish of body weight 40 ± 2g were selected and divided into four groups consisting of 8 fish in each group. Then the fish in groups 1, 2 and 3 were exposed to sewage water brought from Parvathyputhenar for a period of 7, 14 and 21 days respectively in separate aquarium tanks. A static mode without a filtering and re-circulating system was maintained in order to avoid adsorption to filter surface. The 4th group of fish kept in aerated, filtered, dechlorinated and re-circulating tap water served as control. After stipulated periods of exposure, 6 fish from each group were sacrificed by anesthetizing with tricane methane sulfonate (MS-222). A cut was made on the ventral side of fish to expose the viscera and liver, kidney and brain were excised and frozen at -80^0 C (NBS, USA) for enzyme assay.

Biochemical Analysis

The activity of superoxide dismutase (SOD) (EC.1.15.1.1), catalase (CAT) (EC.1.11.1.6), glutathione-S-Transferase (GST) (EC.2.5.1.18), and that of glutathione content (tGSH), malondialdehyde (MDA) and protein were determined using UV-Visible spectrophotometer (Perkin Elmer- Lamda 35, USA). SOD activity was estimated according to the protocol of Kakkar *et al.* (1984). Catalase activity was measured according to Maehly *et al* (1954) and GST according to Habig *et al.* (1974). Glutathione content was estimated according to Benke and Cheevar (1974), and peroxidation product MDA was measured according to the protocol of Nichans and Samuelson (1968). Protein was estimated according to the protocol of Bradford (1976).

For assaying all antioxidant enzymes, 100 mg tissue was homogenized in 2 ml sucrose solution (0.025M) and centrifuged at 10,000g for 10 min and the supernatant was taken as extract.

Assay of SOD

The assay mixture contained 1.2 ml sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1 ml phenazine methosulphate (186 mM), 0.3 ml nitro blue tetrazolium (300 mM), 0.2 ml NADH (780 mM), appropriately diluted enzyme preparation and water in a volume of 3 ml. Reaction was started by the addition of NADH. After incubation at 30^0C for 90 seconds, the reaction was stopped by the addition of 1 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml n-butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was pipetted out. Colour intensity of the chromogen in butanol was measured at 560 nm against butanol (blank) using UV spectrophotometer. One unit of enzyme activity was defined as the enzyme concentration

required to inhibit the optical density at 560 nm of chromogen production by 50% in one minute under the assay conditions. The specific activity was expressed as IU mg protein-1.

Assay of CAT

The reaction mixture contained phosphate buffer (0.01 M, pH 7.0), 2 mM H_2O_2 and 0.1 ml of approximately diluted extract, prepared by homogenizing the tissue in 10 mM buffer. A system devoid of extract constituted blank. Decrease in absorbance was measured at 230 nm for 2 min. The specific activity was expressed in terms of n moles of H_2O_2 liberated/min/mg protein.

Assay of tGSH

The reaction mixture contained 2 ml (0.3 M) phosphate buffer, 500 µl, 0.04 % 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) and 200µl extract. A system devoid of extract served as blank. Change in absorbance was measured at 412 nm within 10 min. The concentration of tGSH was expressed as n mol/100 g tissue.

Assay of GST

The cocktail contained 1 ml phosphate buffer (0.5 M, pH 6.5), 100 µl of 30 mM, 1-chloro, 2, 4, dinitrobenzene (CDNB), 0.1 ml of 30 mM reduced glutathione and 100 µl extract. The increase in optical density of the enzyme was measured against that of the blank at 340 nm for 2 min. Enzyme activity was expressed as n moles of CDNB-GSH conjugate formed/min/mg protein for tissue samples.

Assay of MDA

100 mg tissue was homogenized in 1 ml tris HCl buffer, pH 7.5 and centrifuged at 10,000g for 10 min. The supernatant was taken as extract. 1ml homogenate was added to 2 ml TBA.TCA. HCl and heated in boiling water bath for 15 min. Then it is centrifuged at 1000g for 10 min and absorbance measured at 535 nm. The blank consisted of 2 ml TBA.TCA.HCl reagent. The level of MDA was expressed as µ moles/ g tissue.

Protein was estimated according to the protocol of Bradford (1976).

Chemicals were of analytical grade and purchased from Sigma chemicals U.S.A. Data differences were determined by one-way ANOVA. Significant level used was 0.05. The groups that were not significantly different in Duncan's (1955) multiple range tests were considered homogeneous. Associations between parameters were studied by the use of spearman correlation. Statistical analysis was performed by the use of SPSS 10.0 software.

RESULTS

In the present study, exposure to sewage significantly reduced the activity of SOD in the liver but was restored on the 21st day. In kidney, the activity was significantly decreased for all time periods compared to control. But brain SOD was significantly increased on the 14th day and decreased on 7th and 21st days. Maximum SOD activity was shown by kidney and minimum by liver (Fig. 1)

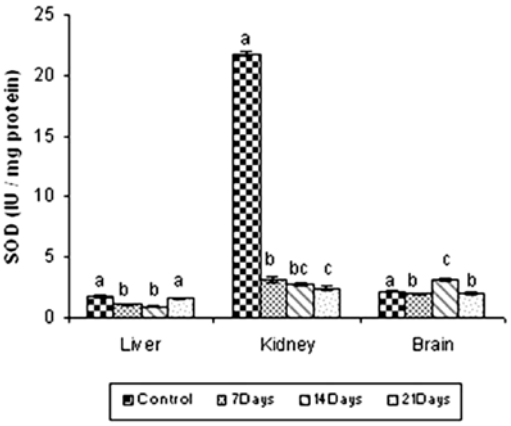

Fig. 1. Effect of sewage on antioxidant enzyme SOD in *Anabas testudineus* for control, 7, 14 and 21 days are plotted. The significant difference between the groups were analysed by one-way analysis of variance. Mean values of different subscript letters (a, b, c) were significantly different ($p<0.05$).

Catalase activity was found to be significantly increasing for liver but restored on the 21st day. For kidney, CAT activity was found to be significantly decreased from control. In the case of brain, the maximum activity was noticed for the 7th day and the activity decreased from control on 14th day. Maximum catalase activity was shown by kidney and minimum by liver (Fig. 2).

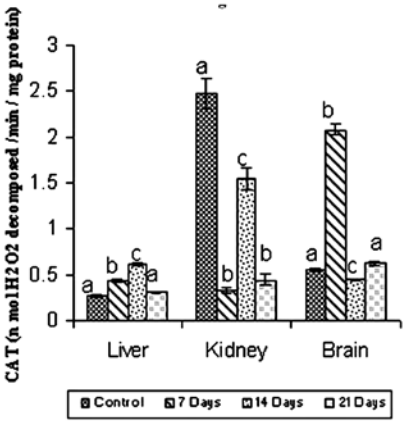

Fig-2. Effect of sewage on antioxidant enzyme CAT in *Anabas testudineus* for control, 7, 14 and 21 days are plotted. The significant difference between the groups were analysed by one-way analysis of variance. Mean values of different subscript letters (a, b, c) were significantly different ($p<0.05$).

GST activity was significantly reduced for liver but restored to normal on 21st day. In Kidney, the activity was increased significantly only on 14th day and then restored. In brain, GST was significantly elevated from control value. Maximum enzyme activity was shown by liver and minimum by brain (Fig. 3).

Liver tGSH was found to be significantly increased from control but kidney and brain tGSH were found to be significantly decreased from control (Fig. 4). Lipid peroxidation product MDA was found to be significantly increased for liver and brain. But kidney MDA was significantly increased only on the 7th day and significantly decreased for other time periods (Fig. 5).

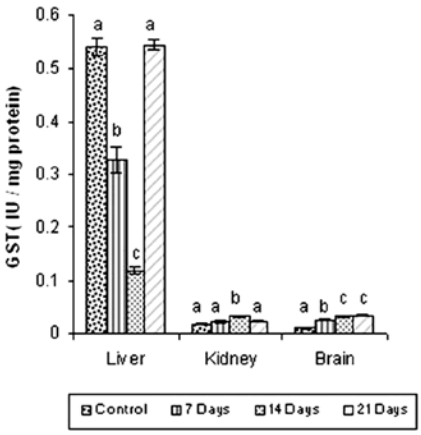

Fig. 3. Effect of sewage on antioxidant enzyme GST in *Anabas testudineus* for control, 7, 14 and 21 days are plotted. The significant difference between the groups were analysed by one-way analysis of variance. Mean values of different subscript letters (a, b, c) were significantly different ($p<0.05$).

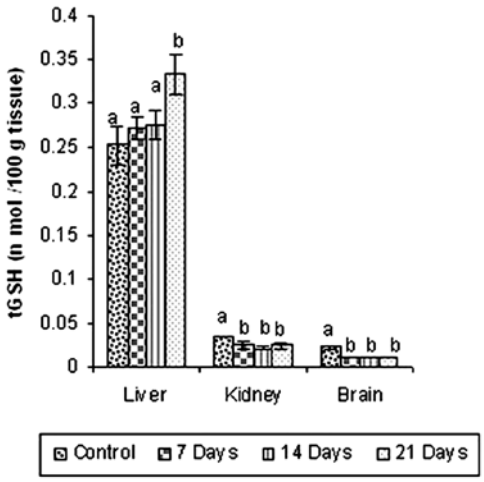

Fig. 4. Effect of sewage on antioxidant enzyme tGSH in *Anabas testudineus* for control, 7, 14 and 21 days are plotted. The significant difference between the groups were analysed by one-way analysis of variance. Mean values of different subscript letters (a, b) were significantly different ($p<0.05$).

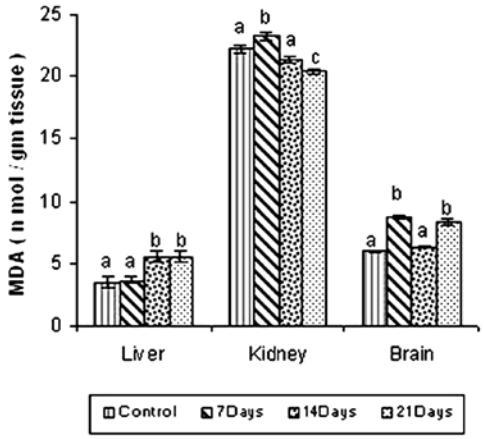

Fig. 5. Effect of sewage on lipid peroxidation product MDA in *Anabas testudineus* for control, 7, 14 and 21 days are plotted. The significant difference between the groups were analysed by one-way analysis of variance. Mean values of different subscript letters (a, b,c) were significantly different ($p<0.05$).

Correlation coefficient (r) between antioxidant enzymes revealed a significant negative correlation between liver SOD and CAT ($r = -0.9475$, $p< 0.05$) and also a significant negative correlation between liver GST and CAT ($r = -0.9919$, $p< 0.01$). In kidney, a positive correlation was found between SOD and CAT ($r = 0.8387$) and a negative correlation between SOD and GST ($r = -0.6778$). In brain, a negative correlation was found between SOD and CAT ($r = -0.5091$) and a negative correlation between GST and CAT ($r = -0.4034$) (Table 1).

Table 1. Correlation coefficient (r) values between different organs

	SOD-CAT	CAT-GST	SOD-GST
Liver	-0.9475 *	-0.9919 **	_
Kidney	+0.8387	_	-0.6778
Brain	-0.5091	-0.4034	_

** shows significance at $p< 0.01$ and * shows significance at $p< 0.05$. The rest proved to be insignificant.

DISCUSSION

The production of ROS, the byproducts of normal oxidative metabolism in eukaryotes, can be increased by conditions such as hypoxia/ hyperoxia, redox cycling xenobiotics and induction of enzymes (Premareur *et al.,* 1986). SOD transforms superoxide anions into less reactive species such as molecular oxygen and H2O2 (Norberg and Arner, 2001). SOD is an 'incomplete antioxidant' that, by scavenging superoxide anion radicals, contributes to overproduction of hydrogen peroxide (Ho *et al.,* 1998). Fall in the activity of SOD in liver and kidney might be due to inactivation by interaction with O_2 radicals or because of overproduction of H_2O_2, as SOD is inactivated by the products of its own reaction (Hodgson and Fridovich, 1975; Pigeolet *et al.,* 1990). Decrease in SOD activity has been reported in the cichlid fish, *Geophagus braziliensis* (Wilhelm Filho *et al*., 2001), in the cyprinid fish, *Barbus meridionalis petenyi* (Velkova-Jordanoska *et al.,* 2005). Brain SOD is significantly increased only on the 14th day of sewage exposure (Fig. 1). The increase in SOD activity indicates that more protein is required to protect cells against superoxide radicals. Significantly higher SOD was reported in the liver of brown bullhead from the contaminated St. Lawrence River than in that from the relatively uncontaminated Lac La Peche river (Heath, 1995). The apparent increase in SOD as noted in the brain of the test fish, might be due to the production of superoxide anions.

Catalase converts H_2O_2 to water and molecular oxygen, thus preventing the formation of extremely dangerous hydroxyl radical from H_2O_2 (Kehrer, 2000). The increase in CAT activity in liver and brain may be a response to the hydrogen peroxide produced by SOD activity since CAT is responsible for the detoxification of hydrogen peroxide to water (Pandey *et al.,* 2003). Higher CAT activity was reported by Sturve *et al.* (2008) in *Onchorynchus mykiss* exposed to sewage

treatment plant effluent and Elia *et al.* (2006) in the liver of *Cyprinus carpio* exposed to three disinfectants. In the present study, decreased CAT activity in kidney may be due to the flux of superoxide radicals, which inhibits CAT activity (Stanic *et al.,* 2005). Similar results has been observed in Cyprinidae fish living in polluted Seyhan dam lake of Turkey (Gul *et al.,* 2004) and carp kidney exposed to heavy metals (Vinodhini and Narayanan, 2009).

Glutathione-S-Transferase is a family of enzymes catalyzing conjugation of a large variety of foreign compounds to Glutathione (Deleve and Kaplowitz., 1991). GST is involved in the detoxification of xenobiotics and aldehydic products of lipid peroxidation (Barata *et al.,* 2005). GST activity is correlated inversely with Catalase activity and may be this is responsible for decreased activities in liver. GST activities were found lower in cichlid fish, *G. braziliensis* found at polluted sites (Wilhelm Filho, 2001) and in *Anguilla anguilla* found in polluted fresh water ecosystem (Ahmad *et al.,* 2006). Increase in GST activity in kidney and brain indicates corresponding defence mechanism to the exogenous compounds established in the fish body to eliminate the increased oxidation products (Shao Peng *et al.,* 2010). GST activity was significantly increased in the kidney of African Cat Fish, *Clarias gariepinus* found in Nigeria Ogun river (Farombi *et al.,* 2007).

Glutathione (GSH) a tripeptide thiol consisting of Glysine, Cystine and Glutamic acid moieties, present in high concentration in all types of living cells (Comporti, 1987). Glutathione content, tGSH (GSH + GSSG) was found to be significantly increasing in liver. Increase in Glutathione content has been described as one of the protective mechanisms that fish adopt in the initial phases of exposure to aquatic pollutants (Stephenson *et al.,* 2002). In Atlantic cod Gadus morhua, exposed to alkyl phenols (Hasselberg *et al.,* 2004), *C. carpio,* exposed to disinfectants. (Elia *et al.,* 2006), and *Oreochromis niloticus* exposed to effluents (Lima *et al.,* 2006), tGSH increased significantly. GSH biosynthesis is regulated by feedback inhibition and increased GSH consumption will lead to an increase in synthesis to keep the glutathione homeostasis (Meister and Anderson, 1983). But in kidney and brain, tGSH was inhibited for all time periods. In the cerebrum (Bano and Bhatt, 2007), myometrial cells (Caruso *et al.,* 2005) and also in the heart of lindane intoxicated mice (Ananya *et al.,* 2005), decreased tGSH were found.

The most widely used assay for lipid peroxidation is MDA formation as a secondary lipid peroxidation product. Increase in LPO levels in liver and brain in spite of increased catalase activities and glutathione content in the present study could be due to increased production of oxygen free radicals than the capacity of antioxidant enzymes (Koshy *et al.,* 2003). An increase in MDA level rapidly ruptures the lysosomal enzymes; stimulate cellular necrosis and destruction of the parenchymal tissues (Arun *et al.,* 2006). Lower level of antioxidant enzymes can justify higher levels in liver lipid peroxidation. Increased MDA was reported by Almroth *et al.* (2008) in rainbow trout, *O. mykiss* caged outside a sewage treatment plant, in the fresh water fish *Channa punctatus* (Bloch) exposed to herbicide atrazine (Nwani *et al.,* 2010) and in the common carp, C. carpio exposed to heavy metals (Vinodhini and Narayanan, 2009). In kidney, MDA was found to be elevated only on the 7th day. A short-term increase in lipid peroxidation products which are the result of oxidative stress was proposed to trigger enhanced protection against stressful conditions (Baraboy and Sutkovoy, 1997).

From the correlation studies it is revealed that the combined action of the enzymes, SOD, CAT and GST in liver may be responsible for combating the stressful conditions and restoration to control value.While in kidney and brain, the enzymes

were ineffective in the combined action may be due to oxidative stress experienced by the animal beyond the activity of antioxidant enzymes.

Alterations in the antioxidant enzymes activity and amelioration in lipid peroxidation products following sewage exposure clearly reveals that the fish experienced oxidative stress. Oxidative stress is known to play a large role in the pathology of several human diseases, including atherosclerosis, pulmonary fibrosis, neurodegenerative diseases and cancer, as well as aging pathology. The untreated sewage need to be properly treated at least by less-cost conventional treatment technologies like the extended aeration activated sludge treatment, prior to its release; so that the burden of organic pollution can be reduced in the canal to be minimal

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