Oxidative Stress Biomarkers in the Freshwater Fish, *Heteropneustes fossilis* (Bloch) Exposed to Sodium Fluoride: Antioxidant Defense and Role of Ascorbic Acid

Shiv Shankar Yadav, Rajesh Kumar, Puneet Khare\(^1\), Madhu Tripathi

Department of Zoology, Aquatic Toxicology Research Laboratory, University of Lucknow, \(^1\)Developmental Toxicology Division (CSIR-IITR), Lucknow, Uttar Pradesh, India

**ABSTRACT**

The present study highlights fluoride-induced toxicity and the protective role of ascorbic acid in the liver and ovary of freshwater fish, *Heteropneustes fossilis*. The fish specimens were exposed to different concentrations (35 mg F/L and 70 mg F/L) of fluoride. Parameters related to oxidative stress were studied at the end of the experiment. The biomarkers selected for the study were thiobarbituric acid reactive substances for assessing the extent of lipid peroxidation (LPO) and antioxidant defense system such as reduced glutathione (GSH), superoxide dismutase (SOD) catalase (CAT) glutathione peroxidase (GPx), and glutathione S-transferase (GST) activities. The fluoride exposure significantly elevated the level of LPO, CAT, SOD, and GST in the tissues of treated group as well as modulated the activities of GSH and level of GPx after exposure as compared to the control. A significant decrease in GPx activity was found in these tissues suggesting that fluoride exposure increases the level of free radical, as well as CAT activity. Pre- and post treatment with ascorbic acid decreased the LPO, SOD, CAT, GST level, and increased GSH, GPx levels in the liver and ovary.

**Key words:** Ascorbic acid, biomarkers, *Heteropneustes fossilis*, oxidative stress, sodium fluoride

**INTRODUCTION**

In biological systems, oxidative stress has become an area of significant interest for aquatic toxicological studies. The problems associated with fluoride exposure indicate the biochemical stress in the body by generating imbalance between reactive oxygen species (ROS) and antioxidants thereby inducing oxidative stress. Fluorosis is a disease arising due to excess intake of fluoride. There is an evidence that fluoride consumption causes crippling disease in man.\(^1\)\(^,\)\(^2\) There are several routes of fluoride intake such as water, food, air, medicaments, and cosmetics. Out of these, water is the main source of fluoride exposure.\(^3\)

There are strong evidences suggesting the involvement of oxidative stress in the mechanism of fluoride toxicity. Lipid peroxidation (LPO) is one of the main manifestations of oxidative damage induced by various compounds,
including metals\cite{4} hence, it has been used as a biomarker of pollution.\cite{5-7} Superoxide dismutase (SOD) is the enzyme that catalyzes the dismutation of the superoxide anion to \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) and catalase (CAT) reacts with \( \text{H}_2\text{O}_2 \) to form water and oxygen molecule.\cite{8} SOD is a group of metalloenzymes that plays a crucial antioxidant role and constitutes the primary defense against the toxic effects of superoxide radical in aerobic organisms.\cite{9} CAT is one of the sensitive enzymes and its activity is modulated by various factors including overproduction of superoxide radicals.\cite{10,11} Glutathione (GSH) plays an important part in modulating metal-induced LPO through its role as a reducing substrate in oxidative reactions and its sequestering capacity of metals.\cite{12} The antioxidant defense system includes enzymes such as SOD, CAT, glutathione peroxidase (GPx), glutathione S-transferase (GST), and other low molecular weight scavengers such as GSH.\cite{13} The GPx detoxifies \( \text{H}_2\text{O}_2 \) or organic hydroperoxides that are produced in LPO.\cite{14} Reduced GSH is an important antioxidant, which is readily oxidized by ROS to oxidized GSH.\cite{15} GST form a family of multifunctional Phase II biotransformation enzymes present in the cytosol of most cells, which catalyze the conjugation of GSH to a variety of compounds and also involved in the transport and elimination of reactive compounds that carry other indirect antioxidant functions.\cite{16,17}

Ascorbic acid is a water-soluble ROS scavenger with high potency. The role of antioxidants is to balance the cellular production of ROS, maintaining the intracellular redox balance by preventing the cellular damage caused by ROS. Adequate amount of Vitamin C is another important content which can ameliorate fluoride toxicity.

**MATERIALS AND METHODS**

**Experimental animals and chemicals**

The freshwater catfish *Heteropneustis fossilis* (mean weight 28.04 ± 0.20 g and length 17.09 ± 0.20 cm) were purchased from the local market in Lucknow and used for the experiment. They were acclimated to laboratory conditions for 1-month prior to the experiments. The specimens were given prophylactic treatment by bathing them twice in 0.05% \( \text{KMnO}_4 \) solution for 2 min to avoid any dermal infections. The \( \text{NaF} \) (AR grade) obtained from Qualigens Fine Chemicals Limited, Mumbai, India. Ascorbic acid was purchased from HiMedia.

**Experimental plan**

Fishes were divided into five groups with 12 fish in each group. Group A served as the control (without F added to the water) and Group B was exposed to a concentration of fluoride 35 mg F ion/L (one-tenth of 96-h \( \text{LC}_{50} \) value).

The Group C was exposed to a concentration of fluoride 70 mg F ion/L (one-fifth of 96-h \( \text{LC}_{50} \) value). Group D and E pretreatment (100 mg/L ascorbic acid + low and high dose fluoride) and Group F and G posttreatment (low and high dose fluoride + 100 mg/L ascorbic acid). At the end of the experiment, the fish were sacrificed. The liver and ovary of both control and treated fish were dissected. The organs were washed in ice-cold physiological saline solution and stored at – 80°C until analysis (Table 1).

**Preparation of homogenate**

The liver and ovary were separately homogenized in 10% (w/v) ice-cold chilled sodium phosphate buffer (0.1 M, pH 7.4) using a Potter-Elvehjem homogenizer. A part of this homogenate was used for biochemical estimations and the other part was centrifuged at 9000 rpm for 30 min at 4°C to using a Sigma Refrigerated Centrifuge (model 3K30) to obtain the supernatant which was further used for SOD, CAT, GPx, GST, and protein estimations.

**Biochemical and enzyme estimation**

LPO was estimated by the method of Ohkawa et al.\cite{17} GSH was assayed by the method of Ellman.\cite{18} SOD activity was analyzed using the method of Kakkar et al.\cite{19} The activity of CAT was measured according to the method of Sinha.\cite{20} GPx activity was measured using the method of Rotruck et al.\cite{21} GST was estimated by the method of Habig et al.\cite{22} Protein estimation was determined using a colorimetric method and BSA as standard.\cite{23}

**Statistical analysis**

The results were expressed as mean and standard error (mean ± standard error mean) and determined for all the parameters. The data were analyzed employing the analysis of variance (ANOVA) using Graph Pad in Stat Software Inc., version 3.06, San Diego, USA. The Dunnett’s test for multiple comparisons of groups against the control was performed, to determine the significant differences among the groups.

| **Table 1: Experimental plan** |
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| **Experimental groups** | **Doses** |
| Group A control | Normal diet and water for 90 days |
| Group B exposure | Low dose exposure (35 mg/L) of fluoride for 90 days |
| Group C exposure | High dose exposure (70 mg/L) of fluoride for 90 days |
| Group D and E pretreatment | Ascorbic acid (100 mg/L) for 45 days followed by low and high dose exposure of fluoride (35 mg/L and 70 mg/L) for 45 days |
| Group F and G posttreatment | Low and high dose exposure of fluoride (35 mg/L and 70 mg/L) for 45 days followed by ascorbic acid (100 mg/L) for 45 days |
RESULTS

Lipid peroxidation and nonenzymatic antioxidant

The LPO level was significantly increased ($P < 0.01$) in exposure Group B and C of the liver and ovary as compared to the control Group A. A nonsignificant ($P > 0.05$) decrease in LPO was observed in pre- and post-treatment in Groups D, E, F, and G [Figure 1].

In liver, the GSH level was decreased nonsignificantly ($P > 0.05$) in Group B and C, while significantly ($P > 0.05$) increase in Group D, E, and F, whereas increase significantly ($P < 0.05$) in Group G as compared to control. In ovary, the GSH level was nonsignificantly ($P > 0.05$) decrease in Group B while increase significantly ($P < 0.01$) in Group C. In pretreatment group the GSH level was increased nonsignificantly ($P > 0.05$) in contrast significant ($P < 0.01$) increased level was recorded in posttreatment group as compared to control group respectively [Figure 2].

Enzymatic antioxidants

The SOD activity increase insignificantly ($P > 0.05$) in Group B, whereas a significant increase ($P < 0.01$) in Group C of liver, while a nonsignificant ($P > 0.05$) increase was seen in Group B and C of ovary as compared to control. In both organ liver and ovary of Group D, E, F, and G decrease nonsignificantly ($P > 0.05$) as compare to control group [Figure 3]. The CAT activities were increase very significantly ($P < 0.01$) in Group B and C of liver whereas in Group D, E increase insignificantly ($P < 0.01$) and Group F, G decreased nonsignificantly ($P < 0.05$) as compare to control. The CAT activity was increased nonsignificantly ($P > 0.05$) and ($P < 0.01$) in Group B and C of ovary, while Group D and E decreased significantly ($P < 0.01$) and ($P < 0.05$) whereas in Group F and G decreased nonsignificant ($P > 0.05$) and ($P < 0.05$) as compare to control [Figure 4]. The GPx activity in Groups B and C of the liver was decreased significantly ($P < 0.01$), while a nonsignificant ($P > 0.05$) increase in Group D of the liver. In Group E, F and G of liver increase significantly ($P < 0.05$) as compared to the control. The GPx level in ovary increase significantly ($P < 0.01$) in Group B and C, whereas...
decreased nonsignificantly \((P > 0.005)\) in Group D, E, F, and G as compared to control group [Figure 5].

The GST activity increased significantly \((P > 0.05)\) and \((P < 0.01)\) in Groups B and C of liver while decreased nonsignificantly \((P < 0.05)\) in Group D, E, F, and G as compared to control. The GST level in ovary increased significantly \((P < 0.01)\) in Group B and C, while GST level was decreased significantly \((P < 0.05)\) in Group D, E, F, and G as compared to control [Figure 6]. The protein level decreased nonsignificantly \((P > 0.05)\) in Group B and C of liver and ovary as compared to the control. A nonsignificant \((P > 0.05)\) increase in protein activity was observed in Group D, E, F, and G.

**DISCUSSION**

A significant increase in the LPO level was observed after fluoride exposure in the liver and ovary of fishes. Increase in MDA was an indicator of enhanced oxidative stress.[24] Fluoride induced increase in LPO and disturbance in the integrity of the cell membranes leading to inhibition of the membrane-bound enzymes has already been reported.[25] Similar result shows dichlorvos causes a significant increase in MDA content in *H. fossilis*. [26] Increased MDA level observed with similar results of the study carried out by [27] in *Cyprinus carpio* and *Ictalurus nebulosus* following the exposures to dichlorvos. Similar result has been reported with Diazinon exposure in the liver of *C. carpio*. [28]

Increase in ROS and LPO resulted in the depletion of GSH in the liver and ovary of exposed fish, in this study GSH is an endogenous, peptidal, antioxidant, which prevents damage to the cellular components by ROS and peroxides.[29] In addition to working as a direct free-radical scavenger, GSH also functions as a substrate for GPx and GST. The reduction in GSH level may be due to the direct conjugation of GSH with electrophiles generated after due to fluoride exposure. Decrease in the GSH level has already been reported after exposure to other organophosphate methyl parathion in freshwater fish.[30]

SOD is a class of enzymes that catalyze the binding of ROS with water to generate \(\text{H}_2\text{O}_2\), and it is the first enzymatic defense against the superoxide anion. CAT is responsible for the breakdown of \(\text{H}_2\text{O}_2\) to water and oxygen, protecting the cell from the damaging action of \(\text{H}_2\text{O}_2\) and the hydroxyl radical. In the present study, SOD, CAT, and GST level increased significantly in liver and ovary with increased concentration of fluoride and exposure duration. Similar result was found with most investigations.[31,32] Dabas et al.[33] reported a similar result was an assessment of tissue specific effect of cadmium in freshwater fish *Channa punctatus*. GPx catalyzes the reaction of hydroperoxides with reduced GSH to form glutathione disulfide and the reduction product of the hydroperoxide. In the present study, different doses of fluoride concentration decreased the GPx activity in liver and ovaries of fish as compared to control group. It may be due to reduced activity of GSH which is used as a substrate for GPx. Similar results were reported by Sharma et al.[34]

Ascorbic acid has been reported to exhibit good antioxidant and free-radical scavenging activities. These antioxidants exhibit LPO lowering effect by radical scavenging activity.[35] The MDA level was brought to a normal level by pre- and post-treatment of ascorbic acid, in the present study pre- and post-treatment with ascorbic acid replenished the decreased GSH level, possibly due to its capability of scavenging the free-radical generated after fluoride exposure. Scavenging of ROS by ascorbic acid may be responsible for the decrease in SOD and CAT activity and replenishment of GSH may have helped in regaining the GPx activity. Pre- and post-treatment with ascorbic acid helps in decreasing the GST level. Tripathi and Shasmal,[36]

**Figure 5:** Effect of ascorbic acid and fluoride on the glutathione peroxidase level. The value represents the mean ± standard error mean \((n = 6)\). Group A: Control, Group B: Fluoride (35 mg F ion/L), Group C: Fluoride (70 mg F ion/L), Group D and E: Pretreatment, Group F and G: Post treatment

**Figure 6:** Effect of ascorbic acid and fluoride on the glutathione-S-transferase level. The value represents the mean ± standard error mean \((n = 6)\). Group A: Control, Group B: Fluoride (35 mg F ion/L), Group C: Fluoride (70 mg F ion/L), Group D and E: Pretreatment, Group F and G: Post treatment
also reported that Vitamin C can repair the impairment of antioxidant enzymes when induced with chlorpyrifos in *H. fossilis*. This study shows that ascorbic acid possesses moderate antioxidant activity which is clearly evident by the investigations in the liver and ovary of fluoride exposed fish. Exposure of normal fish with ascorbic acid does not exhibit any type of oxidative stress-related toxicity.

**CONCLUSION**

The present study revealed that fluoride is responsible for oxidative stress in various tissues of the fish. It has been shown by the increase in LPO and in response the antioxidant defense mechanisms were induced. The effect of chronic exposure of fluoride on LPO, nonenzymatic antioxidant, and enzymatic antioxidant in liver and ovary, SOD, CAT, and GST level increase significantly while GSH and GPx decrease nonsignificantly and significantly, respectively. Pre- and post-treatment was observed after 90 days duration decrease LPO, SOD, CAT, and GST level in both organ while increase GPx and GSH level.

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**Conflicts of interest**

There are no conflicts of interest.

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