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## Effects of mercury on the activities of antioxidant defences in intestinal macrophages of fresh water teleost *Channa punctatus* (Bloch 1793)

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### Abstract

The present study aims to analyse the oxidative stress responses induced by sub-lethal concentrations of mercury and their effect on antioxidant defences in the intestinal macrophages of fresh water teleost *Channa punctatus* (Bloch 1793). The release of pollutants, especially heavy metals, into the aquatic environment is known to cause detrimental effects to the aquatic environment and to the organisms living therein as well as humans by entering the food chain. In the present study, samples of *C. punctatus* (Bloch 1793) were exposed to a sub-lethal concentration of mercury (0.3mg/L). After 4 days and 7 days of exposure, in the treated group, induction of oxidative stress in the intestine was evident from increased respiratory burst activity and lipid peroxidation levels. There were significant ultrastructural changes like fragmented epithelium, lesions in mucosal foldings, disoriented microvilli as evident from transmission electron micrographs. The antioxidants superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) showed changes in a time dependent pattern, suggesting the use of these antioxidants as potential biomarkers of toxicity associated with exposure of freshwater fish to contaminants.

**Keywords:** Mercury toxicity, Respiratory burst, Lipid peroxidation, Superoxide dismutase, Catalase, Reduced glutathione.

### 1. Introduction

The world was introduced to the lexicon of heavy metal pollution with the industrial mercury poisoning at Minamata Bay, Japan in 1956. Since then and more so, in recent years, there has been a growing concern over the increase in heavy metals contaminations affecting the terrestrial and aquatic environments and its ultimately affecting human health. Besides the importance and utility of piscine models in oxidative stress studies<sup>[1]</sup>, it is becoming obvious that oxidative stress significantly affects aquatic organisms that are more exposed to environmental pollutants. Fish accumulate heavy metals in higher concentrations in their tissues, mainly through ingestion of contaminated food or by environmental absorption through the gill surface<sup>[2]</sup>, with metals being accumulated mainly in metabolically active tissues<sup>[3]</sup>, such as the kidney, liver, gills and digestive tract<sup>[4]</sup>. In fact, several studies have reported that some fish species are far more sensitive to heavy metals toxic effects as compared to mammals<sup>[1]</sup>. Heavy metals may alter the structure of the cell membranes by stimulating the lipid peroxidation process with consequent complex sequences of biochemical reactions. Biological membranes are particularly susceptible to reactive oxygen species (ROS) effects. This process is broadly defined as oxidative deterioration of polyunsaturated fatty acids. The peroxidation of unsaturated fatty acids in biological membranes produces a decrease in fluidity, loss of function, disruption of integrity and finally, cell death. Peroxidation results in the production of lipid radicals and in the formation of a complex mixture of lipid degradation products including malonyl di-aldehyde and other aldehydes such as alkanals, hydroxyl-alkanals and ketones<sup>[5]</sup>. Most biological molecules have more than one functions. In particular, many molecules have the ability to directly/indirectly scavenge free radicals and thus act as antioxidants in living organisms. The increased level of these molecules during oxidative stress seems to be a biological

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response that may protect cells from oxidation, in synergy with other antioxidant defence systems. Oxidants fulfil signalling roles during the activation of innate immune Responses and promote the development of adaptive immunity. The cellular defence system against toxicity originating from active oxygen forms includes induction of superoxide dismutase (SOD) which scavenges organism superoxide anion radicals, whereas catalase eliminates hydrogen peroxide [5]. Among heavy metals, mercury has been chosen for the present study because this is a wide spread metal pollutant of high toxicity not only to warm blooded vertebrates, but also to aquatic animals including fishes. The metal is absorbed by fish and other aquatic animals, and passed up the food chain to any other fish-eating species. Several reports have described the effects of mercury in fish [6] and other animals [7]. Nevertheless, the mechanism(s) of its toxicity have yet to be firmly established. Mercury was initially considered to be an enzyme poison [8][9]. Marine or fresh water animals such as fish are able to readily absorb this metal. Mercury is easily stored in fatty tissue and bio-accumulates if the fish is exposed to further contamination. The methylation of inorganic mercury in the sediment of lakes, rivers and other waterways, as well as in the oceans, is a key step in the transport of mercury in aquatic food chains. Mercury accumulated in the tissues of fish is usually in the form of methyl-mercury, while the source is usually inorganic mercury [10]. Several hypotheses of how and where methylation occurs have been proposed. The main hypotheses are: biological methylation, bacterial in origin, which produces methyl-mercury in the environment (methyl-mercury is taken up by fish more readily than inorganic mercury), methylation by microorganisms associated with branchial mucus of the fish or in the fish gut and that in the fish's liver [11].

Mercury has a high affinity for sulfhydryl (-SH) groups, inactivating numerous enzymatic reactions, amino acids, and sulfur-containing antioxidants with subsequent decreased oxidant defense and increased oxidative stress. Mercury induces mitochondrial dysfunction with reduction in ATP, depletion of glutathione, and increased lipid peroxidation; increased oxidative stress is common. The overall vascular effects of mercury include oxidative stress, inflammation, thrombosis, vascular smooth muscle dysfunction, endothelial dysfunction, dyslipidemia, immune dysfunction, and mitochondrial dysfunction including proteins, lipids, and DNA [10]. In view of the above, and considering the lack of sufficient knowledge about the potential toxic effect of mercuric chloride to freshwater fishes, the objective of this work was to evaluate its effect on antioxidant profiles of *C. punctatus* (Bloch 1793) In the present research, the fresh water fish *C. punctatus* was selected due to its strong immune system and its ready adaptation to a polluted environment.

## 2. Materials and Methodology

### 2.1 Biological material

The live fresh water teleost *C. punctatus* of average length 12.5–15.5 cm and average body weight 20–30 g, were purchased from a local market; upon arrival at the laboratory, the animals were acclimatized to the laboratory condition in glass aquarium with continuously aerated and dechlorinated tap water at least 5 to 6 days prior to the experiment. Only healthy fish that were not diseased, as determined by general appearance (colour, skin lustre, eyes and behaviour), were used for the studies. Water quality characteristics were determined. The mean values for test water qualities were as follows: temperature  $27.8 \pm 1.5$  °C, pH  $7.4 \pm 0.003$ , dissolved

oxygen  $6.5 \pm 0.2$  mg/L, alkalinity  $251 \pm 2.8$  mg/L as CaCO<sub>3</sub>, total hardness  $456 \pm 3.5$  mg/L and salinity (%)  $30.8 \pm 3.5$ . The fishes were fed twice daily with pelleted diet (prawn powder, fish powder and minced liver in 2:2:1) and were maintained on a photoperiod with 12h light and 12h darkness.

### 2.2 Exposure

The heavy metal mercury in the form of mercuric chloride (HgCl<sub>2</sub>) was used in the present study. Stock solution of mercuric chloride was prepared by dissolving analytical grade mercuric chloride HgCl<sub>2</sub> from (Qualigens, India) in double distilled water. Desired concentration of mercuric chloride (0.3 mg/L) {a sub-lethal concentration of LD50 (1.8 mg/L)} [12] was prepared from the HgCl<sub>2</sub> stock solution in the double distilled water. After acclimatization, three groups of fish, one for control and the other for mercury-treated for 4 days and 7 days respectively were established in a gently aerated semi-static system. Each group consisted of three tanks which served as triplicates. Each tank housed five fish (n=5). The toxicant in the test chambers (150 L tank) were renewed completely with fresh solution of the same concentration every 24h. Feeding was allowed to both experimental and control fish throughout the tenure of experiments. All assays were performed in triplicates. No mercury was put in to the chamber containing the control fish. After 4 days and 7 days of exposure five fish from each chamber were dissected by cutting the ventral aorta. The fish were housed, treated and sacrificed following the guidelines of the Institutional Ethics Committee, Assam University.

### 2.3 Isolation of intestinal macrophages

The fish were sacrificed following the guidelines of the Institutional Ethics Committee, Assam University. Clove oil was used to produce unconsciousness followed by exsanguination, to sacrifice the animals. It was ensured that the animals did not regain consciousness before death occurred by observing opercular movement. Unconscious animals were decapitated before aseptic retrieval of tissues and cells. Animal carcasses and blood were exhumed in an incinerator. The fish intestine was then dissected out and homogenized with L-15 media. The fish macrophage were isolated and separated by the method of Secombes [13].

Macrophage cells were collected, then checked for viability and counted using trypan blue exclusion test. More than 95% cells were found to be viable.

### 2.4 Heavy metal analysis by Atomic Absorption Spectrophotometer

The intestine was allowed to dry at 120 °C until reaching a constant weight, concentrated nitric acid and hydrogen peroxide (1:1 v/v) (SD fine chemicals) was added. The digestion flasks were heated to 130 °C until all the materials were dissolved and diluted with double distilled water appropriately. The element mercury (Hg) was assayed using Shimadzu AA 6200 Atomic Absorption Spectrophotometer at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya The results were expressed as ppm/ g tissue.

### 2.5 Ultrastructural analysis of tissue by Scanning Electron Microscope

The intestine was excised and rinsed in heparinized saline, rinsed in 0.1 M cacodylate buffer at pH 7.5, infiltrated with

2.5% glutaraldehyde for 24 h fixation at 4°C, rinsed in buffer, trimmed into 8.0 mm squares and subjected to post-fixation in 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer at pH 7.5 for 2 h and dehydrated through graded acetone. The mucosal surface of each tissue was mounted on metal stubs, coated with gold using a JFC-1100 (Jeol) ion sputter. Finally, the tissues were scanned with a JSM-6360 (Jeol) Scanning Electron Microscope at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya.

## 2.6 Ultrastructural analysis of tissue by Transmission Electron Microscope

The anterior portion of the intestine was excised immediately after perfusion, cut into small blocks of 1.0-1.5 mm cube size and incubated in perfusion fixative for at least 30 min at 4°C. The fixation was continued in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.6) containing 4 % PVP and 0.05 % calcium chloride for 20 min at 4°C. After rinsing in cacodylate buffer, tissue block was post fixed at 4°C for 1 h with 1% osmium ferrocyanide [14]. After repeated rinsing in 0.1 M cacodylate and 0.05 M maleate buffers (pH 5.2), the tissue was stained en bloc with 1 % uranyl acetate in maleate buffer for at least 1 h at 4 °C. Specimens were dehydrated in a graded series of ethanol and embedded in Spurr' s medium [15]. Ultrathin sections of 60 to 80 nm thickness were stained with alkaline lead citrate for 1 min and examined in a JEM-2100, 200 Kv (Jeol) transmission electron microscopes at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, India.

## 2.7 Respiratory Burst Activity

Respiratory burst activity of intestinal macrophages of control and treated fish was measured by the method of Fujiki and Yano [16], with some modifications. The respiratory burst activity was expressed as A<sub>630</sub> nm per 10 [16] cells.

## 2.8 Estimation of lipid peroxidation (LPO)

The LPO activity was determined by the procedure of Utley [17], with some modifications. The rate of lipid peroxidation was expressed as nanomoles of thiobarbituric acid reactive substance (TBARS) formed per hour per milligram of protein using a molar extinction coefficient of 1.56×10<sup>5</sup> M<sup>-1</sup>cm<sup>-1</sup>. Protein content of each sample was determined using method of Lowry [18].

## 2.9 Reduced glutathione (GSH) assay

Non-enzymatic antioxidant, reduced glutathione, was assayed by the method of Ellman [19]. The amount of glutathione was calculated using a GSH standard curve and expressed as micrograms of GSH formed/mg protein.

## 2.10 Superoxide Dismutase (SOD) activity

One unit of SOD activity was determined as the amount of enzyme that inhibited the auto-oxidation of pyrogallol by 50%. The activity was expressed as U/mg protein [20].

## 2.11 Catalase (CAT) activity

Catalase activity was measured by the method of Claiborne [21] with some modifications. One unit (U) of Catalase activity is defined as the amount of enzyme catalyzing 1μM of H<sub>2</sub>O<sub>2</sub> per minute at 25 °C.

## 2.12 Statistical Analysis

All the values were expressed as mean ± standard error mean (SEM). The data were compared by using the 'Student t' test. All the differences were considered significant at P<0.05. All treatments were assayed in triplicate for each fish.

## 3. Results

### 3.1 Analysis of mercury accumulation by Atomic Absorption Spectrophotometer (AAS)

The amount of mercury accumulated in the intestinal tissue was found to be higher in the treated group as compared to the control fish (Table 1).

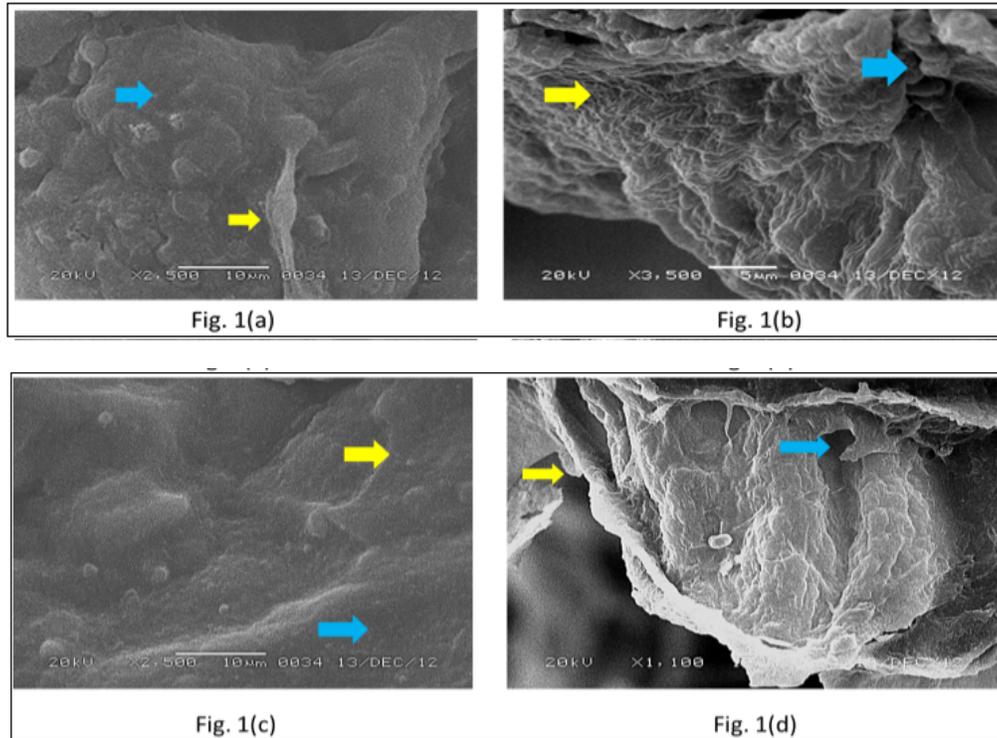
**Table 1:** Mercury accumulation in the intestinal tissue of fresh water teleost *C. punctatus* after 4 days and 7 days of exposure (ppm/g tissue).

Sl. no.	Group	Mercury accumulation in the intestinal tissue
1.	Control	0.072±0.001
2.	4 day treated	0.180±0.009
3.	7 day treated	1.065±0.06

**Note:** The values were statistically significant at p < 0.05.

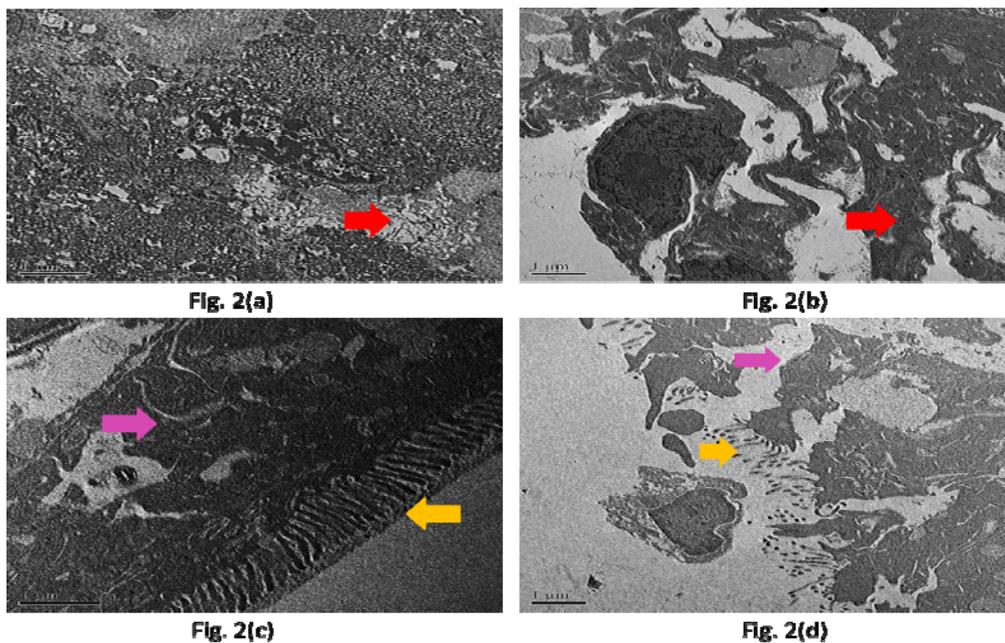
### 3.2 Ultrastructural analysis of tissue by Scanning Electron Microscope

The scanning electron micrograph of intestinal tissue isolated from mercury treated fish showed fragmented, degenerated epithelium, lesions and disarrangements of mucosal folding as compared to the control fish.



**Fig 1(a) & Fig 1(c):** Ultramicroscopic photographs of control *C. punctatus* (Bloch 1793) showing normal epithelium (light blue arrow) and prominent mucosal folding's (yellow arrow). Fig. 1(b), 1(d): Ultramicroscopic photographs of mercury treated *C. punctatus* (Bloch 1793) showing damaged and degenerated epithelium (light blue arrow), disarrangement and fragmentation of mucosal folding's (yellow arrow) after 4 days {fig: 1 (b)} and 7 days {fig: 1 (d)} of exposure.

### 3.3 Ultrastructural analysis of tissue by Transmission Electron Microscope

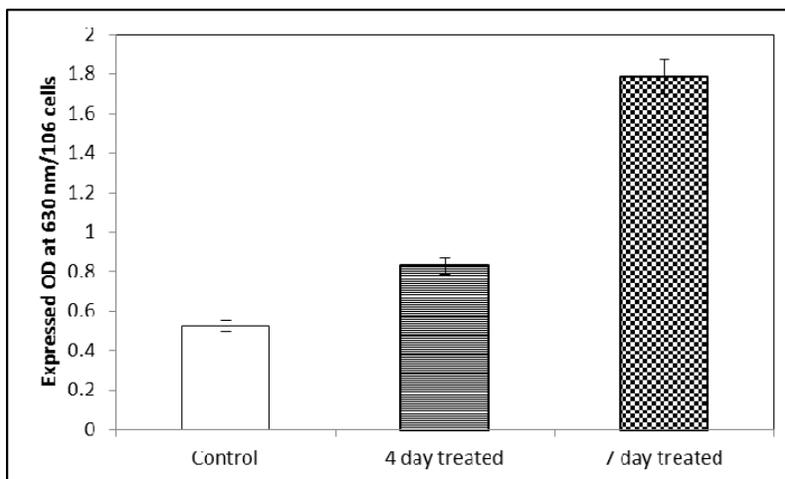


**Fig 2:** (a) Transmission electron micrograph (TEM) of the control fish showing intact rough endoplasmic reticulum (RER) of epithelial cells (red arrow), Fig. 2(b): Transmission electron micrograph (TEM) of *C. punctatus* (Bloch 1793) after 4 days of mercury treatment showing disrupted RER (red arrow) as compared to the control group. Fig. 2(c): Transmission electron micrograph (TEM) of the intestinal tissue of control fish showing regularly arranged microvilli (yellow arrow) and electron dense material (pink arrow). Fig. 2(d): Transmission electron micrograph (TEM) of *C. punctatus* (Bloch 1793) after 7 days of mercury treatment showing disrupted microvilli (yellow arrow) and less electron dense material (pink arrow) as compared to the control fish.

### 3.4 Effect of mercury on Respiratory Burst Activity in Intestinal Macrophages

There was a significant increase in respiratory burst activity in

mercury treated group after 4 days and 7 days of exposure which depicts the capability of mercury in stimulating cells to produce large amount of ROS leading to cell damage.

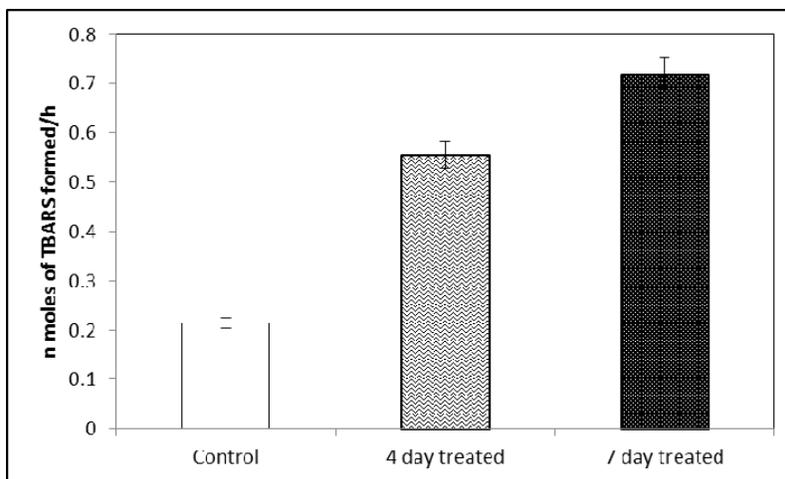


**Fig 3:** Respiratory burst activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± SEM. Significant difference from control value is P < 0.001.

### 3.5 Effects of mercury on lipid peroxidation in intestinal macrophages

The results in this figure showed the lipid peroxidation which is expressed as nanomoles of TBARS formed per hour. The lipid peroxidation of the treated group was found to be

significantly increased as compared to the control group after 4 days and 7 days of mercuric chloride exposure. Increased production of lipid peroxides signifies that mercury can indeed disturb the integrity of plasma membrane, which is essential for cell viability, making cells prone to damage.

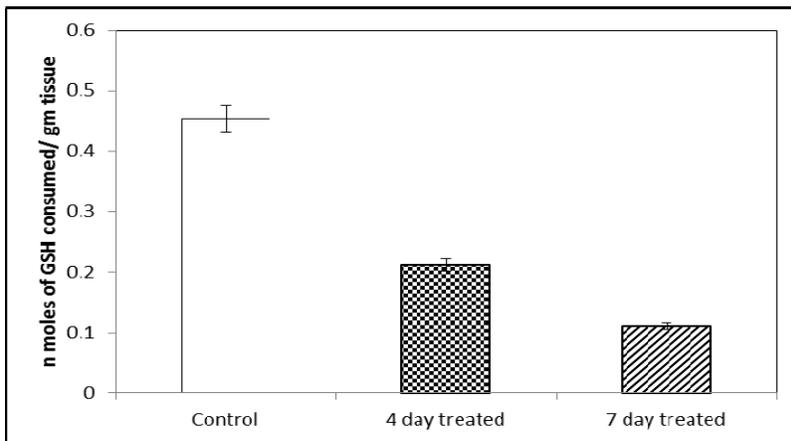


**Fig 4:** Lipid peroxidation in intestinal macrophages of *C. punctatus* treated with mercuric chloride for 4 days and 7 days. Values are expressed as mean ± SEM (P<0.05).

### 3.6 Effect of mercury on reduced glutathione activity in intestinal macrophages

The results in this figure represent the GSH consumed/g tissue. The reduced glutathione activity of the treated group was

found to be decreased as compared to the control indicating that mercury deactivates the formation of reduced glutathione which is an essential antioxidant molecule of fish defence system.

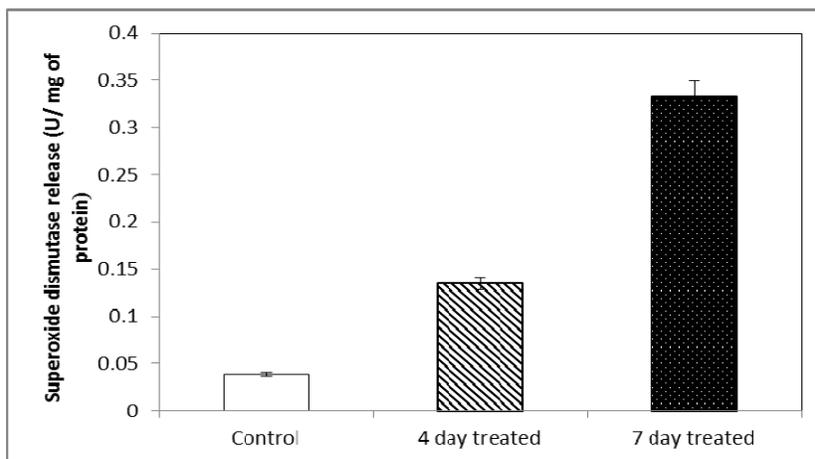


**Fig 5:** Reduced glutathione activity in intestinal macrophages of *C. punctatus* treated with mercury after 4 days and 7 days of exposure. Values are expressed as mean  $\pm$  SEM ( $P < 0.0005$ ).

**3.7 Effect of mercury on superoxide dismutase released from intestinal macrophages**

The amount of SOD released was found to be increased in the treated *C. punctatus* after 4 days and 7 days of mercury

exposure as compared to the control. This shows that mercury may probably over activate SOD activity leading to formation of hydrogen peroxide in excessive amount rendering the host cell to damage.

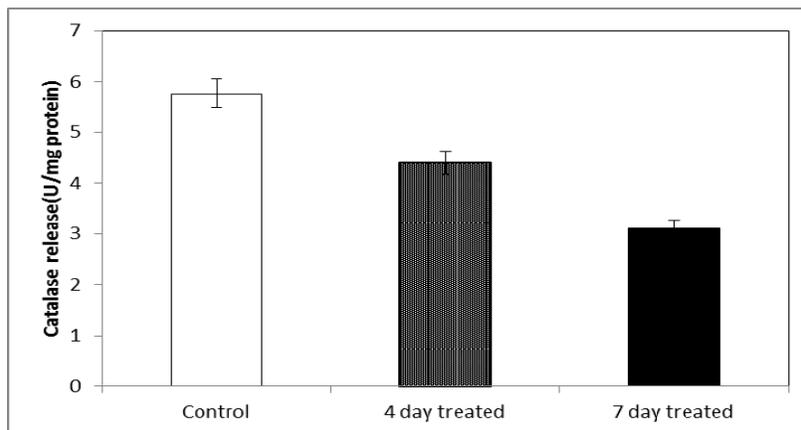


**Fig 6:** Superoxide dismutase activity in intestinal macrophages of *C. punctatus* treated with mercury after 4 days and 7 days of exposure. Values are expressed as mean  $\pm$  SEM ( $P < 0.05$ ).

**3.8 Effect of mercury on catalase released from intestinal macrophages**

Catalase (CAT) release was found be decreased in mercury

treated group which signifies that mercury suppresses the activity of catalase significantly.



**Fig 7:** Catalase activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean  $\pm$  SEM ( $P < 0.01$ ).

#### 4. Discussion

Oxidative stress is as an imbalance between pro- and antioxidants in favour of the former, which implicates a loss of redox signalling. It can be triggered by excessive reactive oxygen species (ROS) production as well as by low antioxidant enzyme activities. One of the important features of antioxidant enzymes is their altered activities under condition of oxidative stress, and such a change can be an important adaptation to pollutant-induced stress. An earlier study report on enzymatic and non-enzymatic antioxidant processes contributes to reducing the impact of ROS in fish [22]. Therefore, both the activity of antioxidant enzymes and the occurrence of oxidative damage have been proposed as indicators of pollutant-mediated oxidative stress [23].

Under normal conditions, activation of cells of nonspecific immunity may be favourable to the host, particularly the reactive intermediates released during phagocytic respiratory burst activity possess bactericidal activity [24, 25]. Elevation in the respiratory burst activity and lipid peroxidation on mercury exposure may suggest over activation of the superoxide-producing enzyme NADPH oxidase and generation of large amount of ROS. ROS and oxidative stress have been demonstrated to be triggers of apoptosis [26]. Further, it could also be assumed that mercuric chloride in macrophage might have suppressed the activity of the regulatory proteins leading to abandoned enzyme activity which is destined to cell damage. Oxidative stress may also be due to the depletion of cellular GSH content below the critical level which prevents the conjugation of mercury to GSH and thus enables them to freely combine covalently with cell proteins [27]. However, organisms are equipped with interdependent cascades of enzymes to alleviate oxidative stress and repair damaged macromolecules, produced during normal metabolism or due to exposure to heavy metals. GSH is the most well studied antioxidant molecule in fish. Heavy metal cations are characterized by an extremely high affinity to -SH residues [28] resulting in decrease of GSH level. Previous studies have established that glutathione reductase, the enzyme responsible for recycling of glutathione from the oxidized form (glutathione disulfide; GSSG) to the reduced form (reduced glutathione; GSH) is deactivated by heavy metals, resulting in low levels of GSH [29]. Our results clearly show depleted levels of GSH in mercury treated group of fish as compared to control, and may contribute to the above phenomenon. SOD plays an important role in the body's antioxidant system, intervening in the first transformation by converting the superoxide free radicals ( $O_2^-$ ) into most reactive forms of oxygen ( $H_2O_2$ ) [20]. SOD induction was observed in the gut associated lymphoid tissue (GALT) of the fish compared to control group after exposure to mercuric chloride for a period of 4 days and 7 days respectively. The increase in superoxide formation in the electron transport chain is associated with a high (inner) mitochondrial membrane potential. This causes a decrease in the electron flow through the respiratory chain, increasing the probability of superoxide formation by the retained electrons at various sites in the mitochondrial respiratory chain. Thus the increase in SOD level in the treated group could be due to free radical generation in the tissues. CAT is mainly located in the peroxisomes and is responsible for the reduction in hydrogen peroxide produced from the metabolism of long-chain fatty acids in peroxisomes to water and oxygen. After treatment with mercuric chloride for 4 days and 7 days, CAT was found to be decreased in the gut associated tissues than that of the control group. The reduction

may be associated with the direct binding of metal to -SH groups on the enzyme molecule. The inhibition of CAT level could be due to the flux of superoxide radicals, resulting in  $H_2O_2$  increase in the cell [21].

Fish are often at the top of the aquatic food chain and can accumulate different heavy metals from the aquatic environment. Heavy metals cannot be degraded or lose toxicity with time by biodegradation, but their concentration can be increased by bioaccumulation [30]. In the present study, the amount of mercury accumulated in the intestine of *C. punctatus* was found to be higher in treated group as compared to the control after 4 days and 7 days of  $HgCl_2$  exposure and it exceeds the permissible limits set for heavy metals by ATSDR 2013 [31]. The apical surface of the normal epithelial cell is characterized by the presence of regular microvilli {Fig. 2(c)} and intact RER {Fig 2(a), frequent invaginations of the plasma membrane at the base of microvilli. But after 4 days and 7 days of mercury exposure it was found from transmission electron micrograph (TEM) of fish intestine that there is a abnormalities in the RER {Fig. 2(b)}, and the cells were irregularly shaped with electron-dense nuclei and the nuclei were also irregular in shape with distorted microvilli deep indented margins {Fig. 2(d)}. This may lead to damage of the epithelial cells and other tissues [32].

#### 5. Conclusion

Sub-lethal concentration of mercuric chloride has the capacity to bio-accumulate, thereby altering the normal functional activities of freshwater teleost *C. punctatus*. Furthermore, the association of oxidative stress including dissimilarity in its antioxidant profile suggests that the defense system of *C. punctatus* is significantly compromised upon metal exposure at low concentrations. Thus the antioxidant enzymes such as SOD, CAT and LPO in fish could be effectively used as biomarkers of heavy metal toxicity in both natural and aquatic bio-systems.

#### 6. Acknowledgement

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#### 7. Conflict of interest

The authors declare that there are no conflicts of interest.

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