APPENDICES

List of Publications

- **Moriom Begam** and Mahuya Sengupta (2015). Immunomodulation of intestinal macrophages by mercury involves oxidative damage and rise of pro-inflammatory cytokine release in the fresh water fish *Channa punctatus* Bloch. *Fish and Shellfish immunology* (In press)(Impact factor: 3.034)

- **Moriom Begam** and Mahuya Sengupta (2014). Effects of mercury on the activities of antioxidant defences in intestinal macrophages of fresh water teleost *Channa punctatus* (Bloch. 1793). *International Journal of Fisheries and aquatic studies*. 2(1):172-179. ISSN- 2347-5129**.**

- **Moriom Begam**, Nilantika Paul and Mahuya Sengupta (2012). Effects of mercuric chloride on the activities of antioxidant enzymes in the fresh water fish *Channa punctatus*. *Assam University Journal of Science and Technology* (Biological and Environmental Sciences). 10(I):1-7. ISSN 0975-2773.

SEMINARS/CONFERENCES ATTENDED

- Participated and presented a paper (poster) entitled **"Toxicity of the cadmium: effects on lipid peroxidation and activities of antioxidant enzymes in the fresh water fish *Channa punctatus*"** in the XXXI Annual Conference of Society of Toxicology (STOX), Organized by Department of Zoology, The IIS University, Jaipur, from 22nd -24th December, 2011.
- Participated and presented a paper (oral) entitled **"Effects of mercuric chloride on the activities of antioxidant enzymes in the fresh water fish *Channa punctatus*"** in the International Conference on Global Ecosystems, Biodiversity and Environmental Sustainability in the 21st Century, Organized by Department of Ecology and Environmental Science, Assam University, Silchar, from 15th -17th February, 2012.
- Participated and presented a paper (oral) entitled **"Assessment of the hazardous effects of mercury on fresh water fish *Channa punctatus* in relation to non-specific host defenses"** in the National seminar on Biotechnology research in sustainable development, Organized by Centre for Studies in Biotechnology, Dibrugarh University, Dibrugarh, from 27th -28th February, 2012.
- Participated and presented a paper (poster) entitled **"Sub lethal Toxic Effects of Cadmium on Fresh water fish *Channa punctatus* in relation to non-specific host defenses"** in the International Conference on Biodiversity Conservation and Environmental Health, Organized by Department of Life Science and Bioinformatics, Assam University, Silchar, from 16th -17th March, 2012.

PUBLICATIONS

Fish & Shellfish Immunology

Available online 30 April 2015

In Press, Accepted Manuscript — Note to users

Full length Article

Immunomodulation of intestinal macrophages by mercury involves oxidative damage and rise of pro-inflammatory cytokine release in the fresh water fish Channa punctatus Bloch

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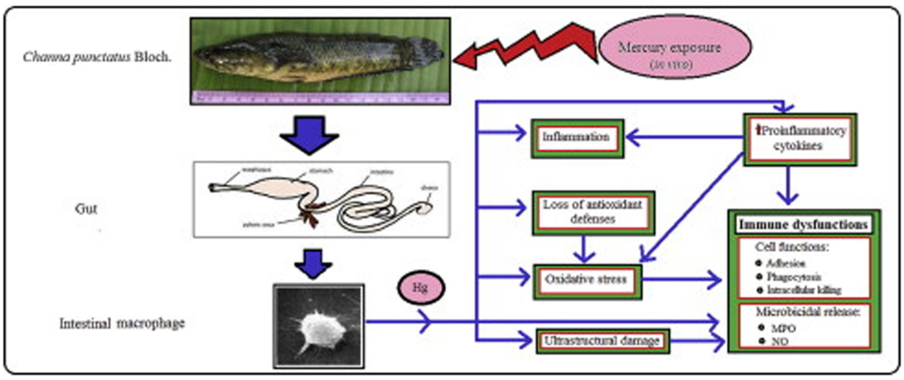
Abstract

Mercury and its compounds have been parts of widespread pollutants of the aquatic environment. The present study was designed to assess the effect of mercury on fish immune responses. Since the metal is absorbed by fish and passed up the food chain to other fish-eating species, it not only affects aquatic ecosystems but also humans through bioaccumulation. In the present study, it was found that innate immunity of the fresh water fish *Channa punctatus* Bloch. was significantly debilitated after a periods of exposure to a sub-lethal concentration of mercury (0.3mg/L). After 7 days of exposure, phagocytosis, cell adhesion and intracellular killing activity were found to decrease significantly along with significant decreases in nitric oxide (NO) and myeloperoxidase (MPO) production from macrophages as compared to the control group indicating intracellular damages. Levels of pro-inflammatory cytokines like TNF-α and IL- 6 were found to be significantly more in mercury treated groups than that of control group indicating inflammatory damage. This included significant ultrastructural changes like fragmented epithelium, lesions in mucosal foldings, degenerated mitochondria, reduction in the number of goblet cells and disoriented microvilli as evident from transmission electron micrographs.



Keywords

Mercury exposure; *Channa punctatus*; nitric oxide; myeloperoxidase; phagocytic index; intracellular killing

Immunomodulation of intestinal macrophages by mercury involves oxidative damage and rise of pro-inflammatory cytokine release in the fresh water fish *Channa punctatus* Bloch.

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ABSTRACT:

Mercury and its compounds have been parts of widespread pollutants of the aquatic environment. The present study was designed to assess the effect of mercury on fish immune responses. Since the metal is absorbed by fish and passed up the food chain to other fish-eating species, it not only affects aquatic ecosystems but also humans through bioaccumulation. In the present study, it was found that innate immunity of the fresh water fish *Channa punctatus* Bloch. was significantly debilitated after a periods of exposure to a sub-lethal concentration of mercury (0.3mg/L). After 7 days of exposure, phagocytosis, cell adhesion and intracellular killing activity were found to decrease significantly along with significant decreases in nitric oxide (NO) and myeloperoxidase (MPO) production from macrophages as compared to the control group indicating intracellular damages. Levels of pro-inflammatory cytokines like TNF-α and IL- 6 were found to be significantly more in mercury treated groups than that of control group indicating inflammatory damage. This included significant ultrastructural changes like fragmented epithelium, lesions in mucosal foldings, degenerated mitochondria, reduction in the number of goblet cells and disoriented microvilli as evident from transmission electron micrographs.

KEY WORDS: Mercury exposure, *Channa punctatus*, nitric oxide, myeloperoxidase, phagocytic index, intracellular killing.

1. INTRODUCTION:

Intense activity in industrial and agricultural sectors has inexorably increased the levels of toxic heavy metals in aquatic environment. Heavy metals are serious pollutants of the aquatic environment because of their environmental persistence and ability to be accumulated by aquatic organisms [1]. Mercury is considered a devastating environmental pollutant, mainly after the environmental disaster at Minamata and several other poisoning accidents due to the use of mercury pesticides in agriculture [2].

Mercury, a sulfhydryl-reactive heavy metal, exists in very small amounts in the nature. Moreover, fishes are also exposed to mercury due to contaminated inland water bodies that definitely is causing adverse fish health leading to decrease fish quality as well as fish production. Several reports have described the effects of mercury in fish [3] and other animals [4]. Nevertheless, the mechanism(s) of its toxicity have yet to be firmly established. It was initially considered to be an enzyme poison [5] [6]. Scientists today are concerned that people who eat fish contaminated with mercury will be put at risk – particularly pregnant mothers and children as mercury poisoning have been shown to adversely affect brain development. However it is also being linked to a higher risk of heart disease and stroke in men as well as memory loss, central nervous system damage, kidney damage, lung problem and prostate cancer. 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 methylmercury, while the source is usually inorganic mercury [7]. The occurrence of these and other pollutant in fish can cause acute effects, and the high concentration of the toxicants can lead to injury or death in a short period of time [8]. These facts were enough to pose a scientific question about eligible environmental monitoring system.

The innate immune system forms the first line of defense against antigenic insult and is also strongly implicated in adaptive immune system. Macrophages have been regarded as an important part of the cellular immune system in fish and functions to protect the host by phagocytising foreign material including disease causing agents [9]. Therefore changes in there phagocytic abilities have been of interest to ascertain the effect of environmental pollutant on the normal function of macrophages [10]. Moreover, macrophages are actively involved in the processing and presentation of phagocytosed antigens, thus affecting cell-mediated responses as well.

In the present study, we focused on mercury toxicity on fishes and demonstrated the toxicological effect of mercury on fresh water teleost *C. punctatus* Bloch. with special reference to immunological parameters. Fresh water spotted teleost *C. punctatus* Bloch. specimens were exposed to mercuric chloride (0.3 mg/L) for a period of 7 days respectively. Intestinal macrophages were studied as primary cultures because intestine is a significant site of mercury accumulation. Knowledge of the toxicity of mercury would be helpful in water quality management in fish farms; hence, this study would help evaluate the impact of the short-term exposure of mercury on survival, bioaccumulation and immunological alterations in intestine of freshwater teleost *C. punctatus* Bloch.

2. MATERIALS AND METHODS

2.1 Biological material

The live fresh water teleost *C. punctatus* Bloch. of average length 14.0±1.5 cm and average body weight 25±5g, were purchased from a local fishery; 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 luster, eyes and behaviour), were used for the studies. Water quality characteristics were determined and the mean values for test water qualities were as follows: temperature 27.8 ± 1.5 ^{0}C, pH 7.4 ± 0.003, dissolved oxygen 6.5 ± 0.2 mg/L, alkalinity 251 ± 2.8 mg/L as $CaCO_3$, total hardness 456± 3.5 mg/L and salinity (%) 30.8 ± 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 dark. *C. punctatus* Bloch. was considered suitable for immunomodulatory studies and was thus utilized as the model organism in this work, because it has well documented general biology, short developmental time, easy culturing and year round reproduction.

2.2 Exposure

The heavy metal mercury in the form of mercuric chloride ($HgCl_2$) was used in the present study. Stock solution of mercuric chloride was prepared by dissolving analytical grade mercuric chloride $HgCl_2$ (from Qualigens, India) in double distilled water. Desired concentration of mercuric chloride (0.3 mg/L) {a sub-lethal concentration of LD_{50} (1.8 mg/L)} [11] was prepared from the $HgCl_2$ stock solution in the double distilled water. After acclimatization, two groups of fish, one for control and the other for mercury treated for 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 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 Separation of 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 were dissected out and immediately placed in Leibovitz medium (L-15) supplemented with heparin (10 IU/ml) and fetal bovine serum (FBS) (2%), and then homogenized in ice cold condition. Cell suspension was then transferred to tubes and kept in ice for the settlement of cell debris. The supernatant was layered over Ficoll (45%) and subjected to density-gradient centrifugation [12]. The band of macrophage-enriched fraction at the interface is collected, washed and resuspended in L-15 medium containing FBS and allowed to adhere on plastic surface. The adherent cells of both exposed and unexposed group were collected and tested for viability at different time intervals as determined by trypan blue dye exclusion technique. Tissue samples showing cell viability higher than 95% were further processed for various analyses.

2.4 Heavy metal analysis by Atomic Absorption Spectrophotometer

The intestines were allowed to dry at 120ºC 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 130^0C 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 intestines were excised and rinsed in heparinized saline, rinsed in 0.1 M cacodylate buffer at pH 7.5, infiltered with 2.5% glutaraldehyde for 24 h fixation at 4^0C, rinsed in buffer, trimmed into 8.0 mm squares and subjected to post-fixation in 1% OsO_4 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 4^0C. 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 4^0C. After rinsing in cacodylate buffer, tissue block was post fixed at

4^{0}C for 1 h with 1% osmium ferrocyanide [13]. 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^{0}C. Specimens were dehydrated in a graded series of ethanol and embedded in Spurr's medium [14]. 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, Meghalaya.

2.7 In vitro cell adhesion assay

Cells were seeded separately for treated and control group in 96 well microtiter plates and allowed to adhere for different times. In time, wells were washed with Hank's balanced salt solution (HBSS), and then 100 µl of 0.5% crystal violet in 12% neutral formaldehyde, and 10% ethanol was added to each well and incubated for 4 h to fix and stain the cells. Wells were washed and air dried for 30 min. Crystal violet was extracted from the macrophage adhered in the wells by lysing with 0.1% sodium dodecyl sulphate (SDS) in HBSS. Absorbance was measured spectrophotometrically at 570 nm. Cell adhesion was expressed as increased absorbance at 570 nm [15].

2.8 Phagocytosis assay

A volume of 100 µl of cells from both control and exposed groups were allowed to adhere separately on glass slides for 1 h whereas non-adherent cells were washed out with Dulbecco's phosphate buffered saline (DPBS) (1x). To the glass slides containing the adhered macrophages 10% heat killed Staphylococcus aureus was added and incubated for 3 h at 37 ^{0} C which were then washed with DPBS (1x) and dried. The cells were at last fixed in 50% methanol, stained with Giemsa, observed under oil immersion microscope and counted for number of bacterial cells ingested [16]. Phagocytosis is expressed as Phagocytic index (P.I.):

P.I.(%)= (percentage of macrophages containing bacteria)×(mean number of bacteria per macrophage).

2.9 Preparation of bacteria (*Staphylococcus aureus* MC524) for intracellular killing assay

To obtain bacteria in the mid-logarithmic phase 100 µl of an overnight culture made in nutrient broth was added to 10 ml of nutrient broth and incubated for 2 - 5 h at 37°C with orbital shaking. The bacteria were washed in 10 mM sodium phosphate buffer (pH 7.4) and their concentration was estimated by spectrophotometry at A_{620} on the basis of the relationship: A_{620} 0.2 = 5 × 10^7/ml [17].

2.10 Intracellular killing assay

Bacteria (*Staphylococcus aureus*) were incubated with macrophages in L-15 FCS for 20 min at 37°C. After various time intervals, samples were plated on to nutrient agar to determine the number of viable intracellular bacteria [18]. Intracellular killing is expressed as the percentage decrease in the initial number of viable intracellular bacteria.

2.11 Nitric oxide release assay

Cells (10^6 cells/ml) were suspended in DPBS and were stimulated with lipopolysaccharide or LPS (100 ng/ml). The cell free supernatants were used for nitric oxide release assay using Griess reagent. Readings were taken in a UV spectrophotometer at 550 nm. The absorbance was plotted on a nitrate standard curve and NO release was expressed as µM [19].

2.12 Myeloperoxidase release assay

100 µl of cells from control and treated group were taken into microcentrifuge tubes and stimulated with LPS (100 ng/ml) for 1 h at 37^0C and centrifuged at 13,000 rpm for 10 min. The supernatants thus obtained from both the sets was recovered separately and kept at -20^0 C until further use. The cell free supernatant was used for assay of the partial MPO release for both groups. The pellet that recovered from the two groups were lysed in 0.01% SDS and then centrifuged again. The supernatant was recovered as before for total MPO release assay. Subsequently 100 µl of cell free supernatant as well as from lysis of cells were reacted with 100 µl of substrate buffer and kept at 37^0C for 20 min, then the reaction was stopped by adding 100 µl of 2(N) H_2SO_4 and absorbance was measured at 492 nm [20].

2.13 Preparation of samples for cytokine analysis

2.13.1. From cell supernatant (in vitro)

Isolated cells were treated with LPS (5 ng/ml) with or without metal treatment. The cells were lysed and any particulate material was removed by centrifugation, supernatant collected and assayed immediately.

2.13.2. From serum

Peripheral blood was obtained from treated and untreated fish by heart puncture into 1.5 ml microcentrifuge tubes. After cooling overnight at 4^0C, serum was collected after centrifugation and used for cytokine analysis.

2.14 Cytokine analysis

The 96 well microtiter plates (Iwaki Glass, Japan) were coated with 100 µl of cell supernatant diluted 10 times in phosphate buffer saline (PBS) and incubated in a moist chamber overnight at 4^0C. TNF-α rabbit polyclonal antibody was used as the primary antibody (Sigma, USA). Vectastain ABC-PO kit (Vector Lab., USA) was applied for ELISA and peroxidase ABTS substrate kit (Vec-tor Lab., USA) was used for coloration. The plate was read at 490 nm on a microplate reader (BioRad). For the blank, physiological saline was used instead of serum. Fish sera from untreated groups served as control. All assays were performed in triplicate.

2.15 Statistical Analysis

All the values were expressed as mean ± standard error of mean (SEM). The data were compared by using the 'Student t' test (two-sample assuming unequal variances). All the differences were considered significant at $P<0.05$. All treatments were assayed in triplicate for each fish. Statistical comparisons were done between control and exposure data from same species. P* = P<.05, P**= P<.001, P***= P<.0001 comparing with control.

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.

Sl. No.	Group	Mercury accumulation in the intestinal

		tissue
1	**Control**	**0.072±0.001**
2	**7 day treated**	**1.065±0.06**

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

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

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.

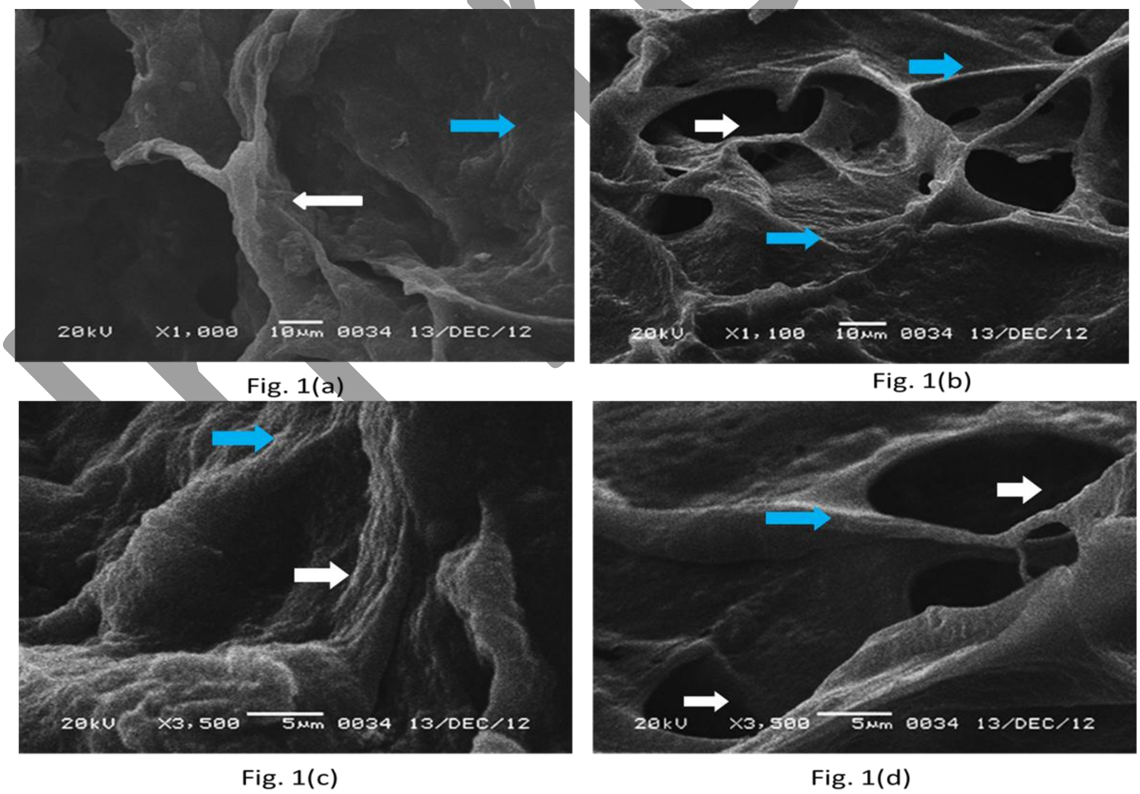

Fig. 1(a) & 1(c): Ultramicroscopic photographs of control fish *C. punctatus* Bloch. showing normal epithelium (light blue arrow) and prominent mucosal folding's (white arrow).
Fig. 1(b), 1(d): Ultramicroscopic photographs of mercury treated *C. punctatus* Bloch. showing damaged and degenerated epithelium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

3.3 Ultrastructural analysis of tissue by Transmission Electron Microscope

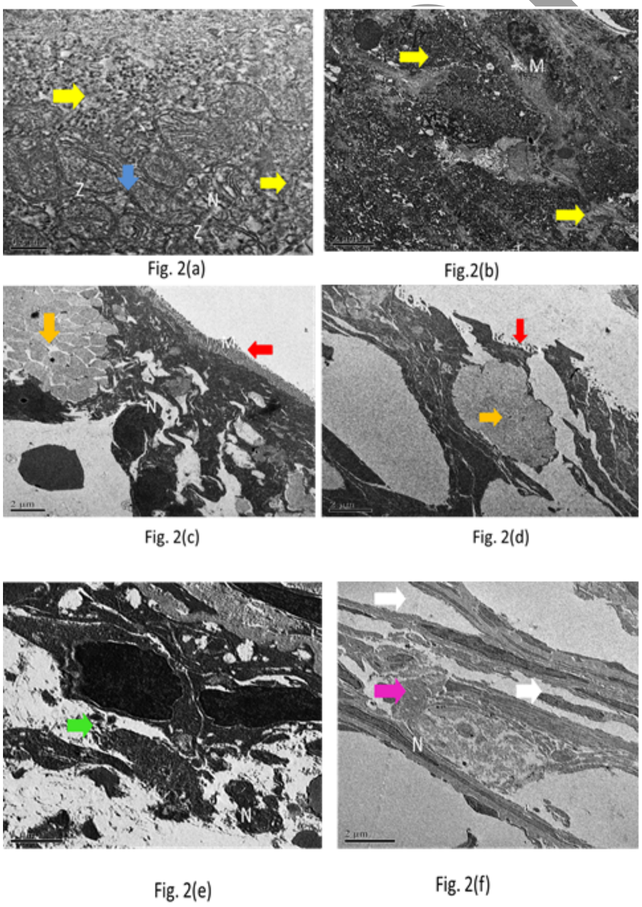

Fig. 2(a): Transmission electron micrograph (TEM) of the control fish showing intact rough endoplasmic reticulam (RER) of epithelial cells (yellow arrow), zona occludens (Z), gap junction (blue arrow) and elongated nucleus with well-developed nucleolus (N). Fig. 2(b): Transmission electron micrograph (TEM) of *C. punctatus* Bloch. after 7 days of mercury treatment showing disrupted RER (yellow arrow), degeneration in mitochondria (M) and lack of zona occludens and gap junction as compared to the control group. Fig. 2(c): Transmission electron micrograph (TEM) of the intestinal tissue of control fish showing goblet cells (orange arrow), regularly arranged microvilli (red arrow). Fig. 2(d): Transmission electron micrograph (TEM) of *C. punctatus* Bloch. after 7 days of mercury exposure showing denudation of goblet cells (orange arrow), disrupted microvilli (red arrow) as compared to the control fish. Fig. 2(e): Transmission electron micrograph (TEM) of *C. punctatus* Bloch. showing degenerated nucleus (N) lack of cell cluster (green arrow), after exposure. Fig. 2(f): TEM of *C. punctatus* Bloch. Showing less electron dense material (white arrow) after 7 days of exposure. The apical cell-cell junction area was found to be narrowed (pink arrow) in treated group after 7 days of exposure..

3.4 Effect of mercury on cell adherence activity of intestinal macrophages

Cell adherence was expressed as the decreased absorbance at 570 nm. After 30 min and 60 min incubation, absorbance in mercury treated group is found to be significantly decreased after 7 days of exposure when compared to control group.

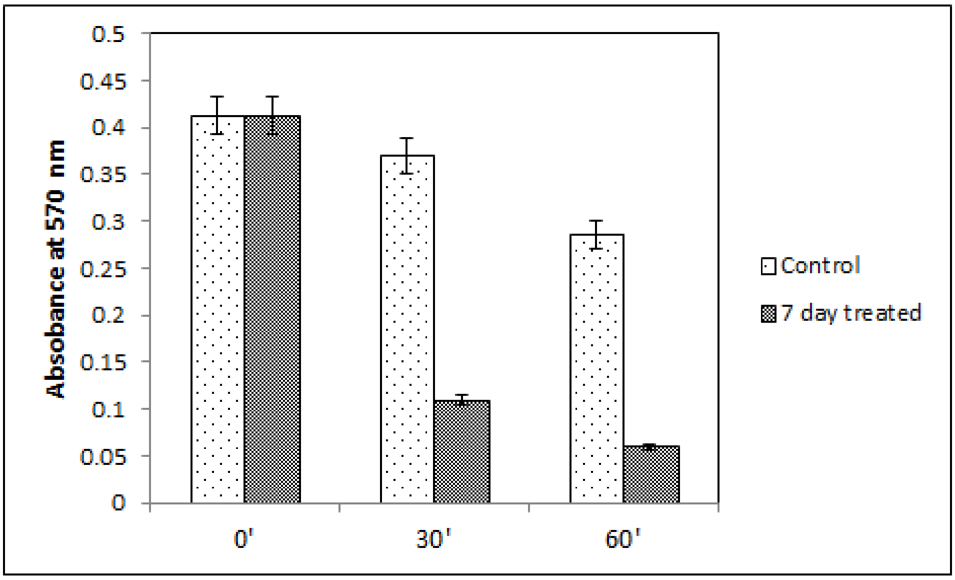

Fig. 3: Cell adhered after exposure of mercuric chloride from the fish *C. punctatus* Bloch. at 570nm. Values are expressed as mean ± SEM, P**, P***).

3.5 Effect of mercury on phagocytosis of intestinal macrophages

The results in this figure represent the phagocytic index. Phagocytic index of the mercury treated group was found to be decreased after 7 days of exposure.

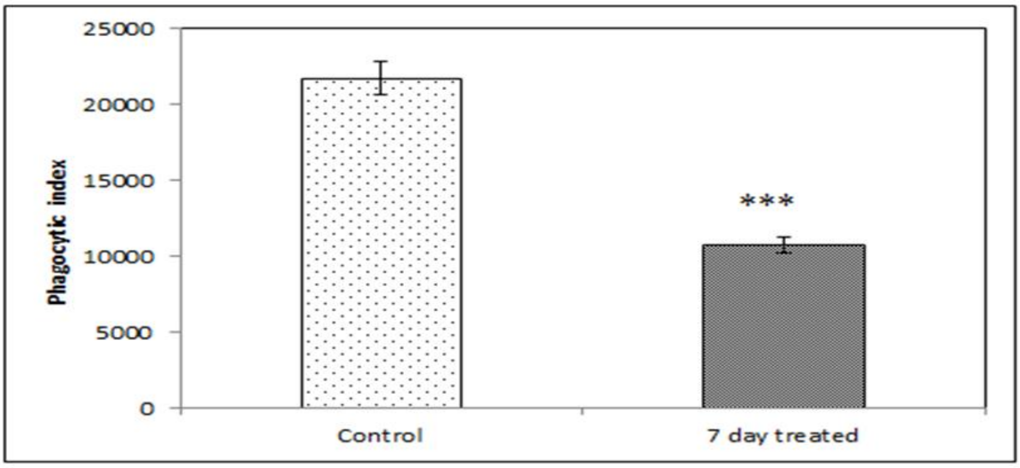

Fig. 4: The phagocytic index of macrophages exposed to mercuric chloride isolated from *C. punctatus* Bloch. Values are expressed as mean ± SEM (P***).

3.6 Effect of mercury on intracellular killing activity of intestinal macrophages

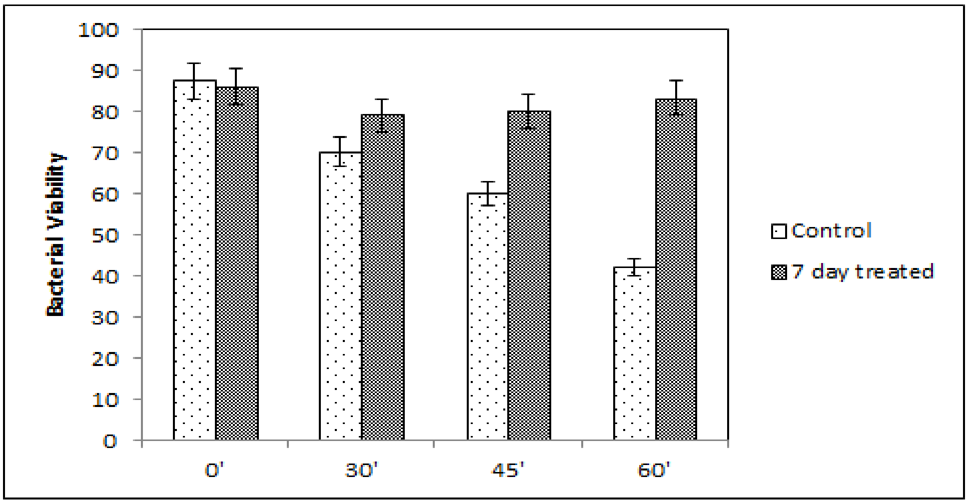

Fig 5: Intracellular killing capacity after exposure of mercuric chloride for 7 days (mean ± SEM, P**).

3.7 Effect of mercury on nitric oxide released from intestinal macrophages

The results represents NO released(in µM) and values are expressed as mean± SEM. NO released with stimulation from the mercury treated group was found to decreased significantly after 7 day treatment when compared with control group.

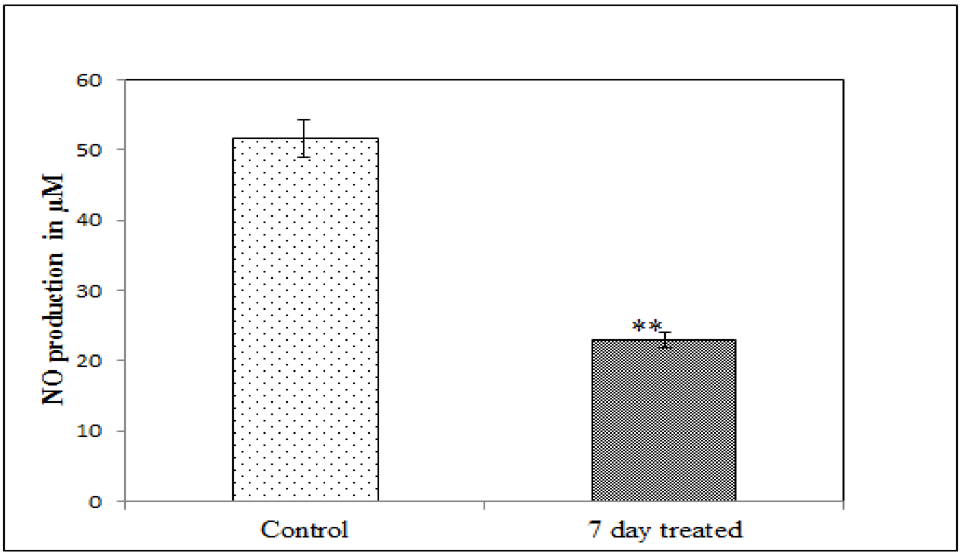

Fig 6: Nitric oxide (NO) released after 7 day treatment with mercuric chloride. (mean ± SEM, P**).

3.8 Effect of mercury on myeloperoxidase released from intestinal macrophages

Myeloperoxidase (MPO) release was studied and absorbance was found to decrease significantly in the treated group.

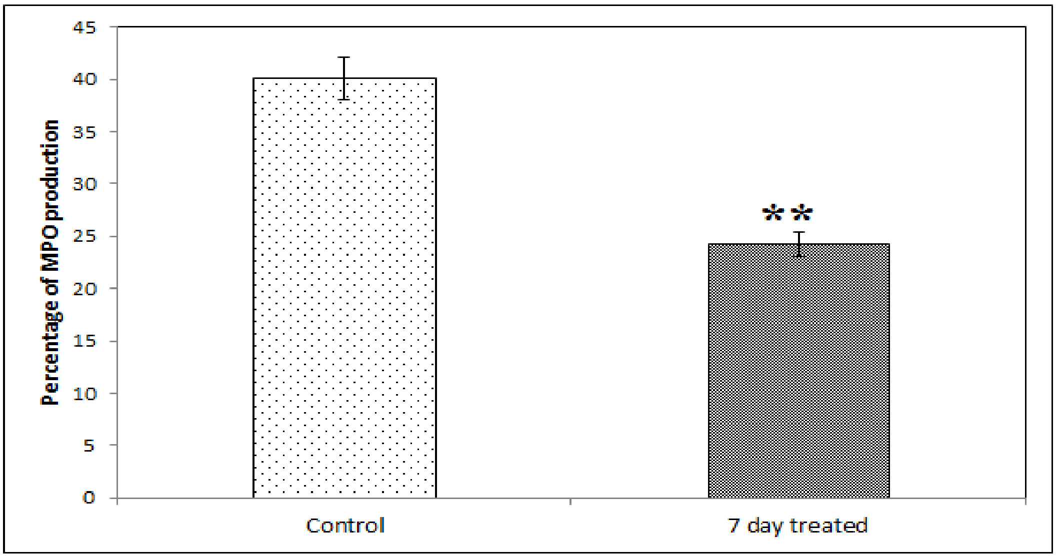

Fig 7: MPO released after exposure of mecuric chloride for 7 days. (mean ± SEM, P**).

3.9 Effect of mercury on cytokines released in macrophages

The cytokines TNF-α, IL-6 of the mercury treated group were found to be higher when compared to the control group after 7 days of exposure.

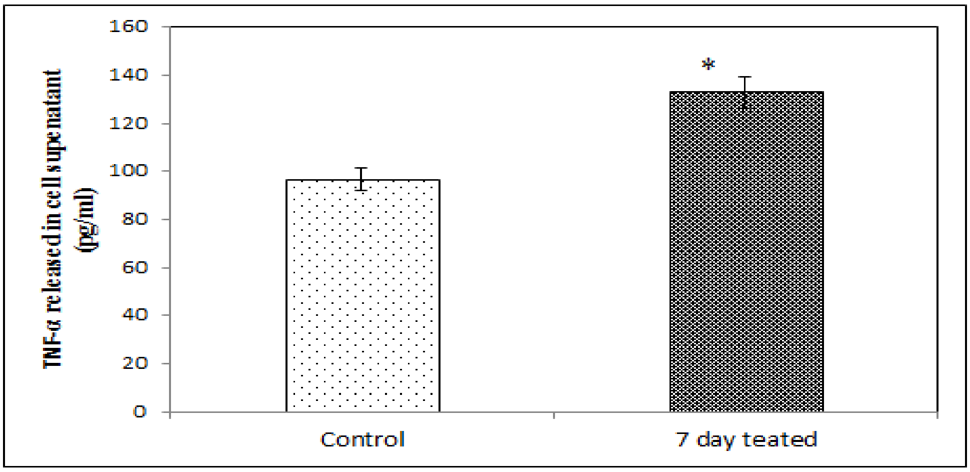

Fig. 8(a): TNF-α released in cell supernatant after exposure of mercuric chloride for 7 days. (mean ± SEM, P*).

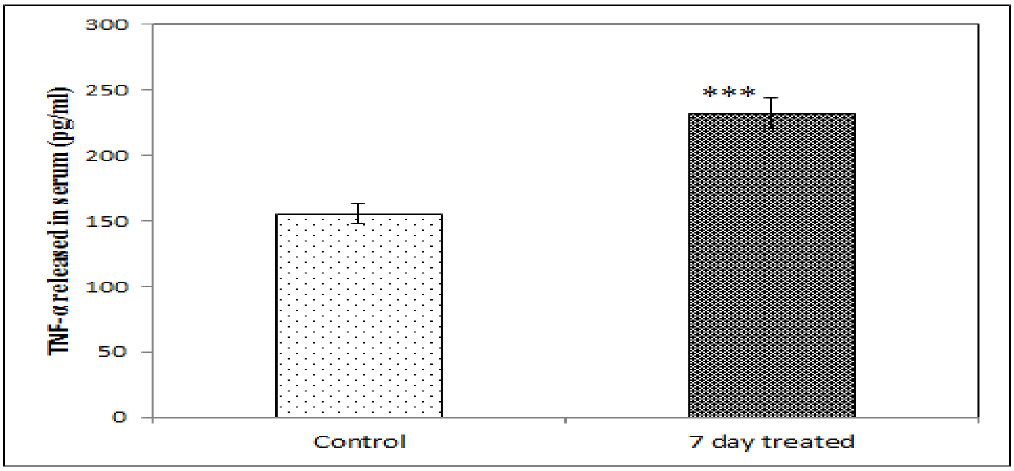

Fig. 8(b): Serum levels of TNF-α after 7 days of mecury exposure. (mean ± SEM, P***).

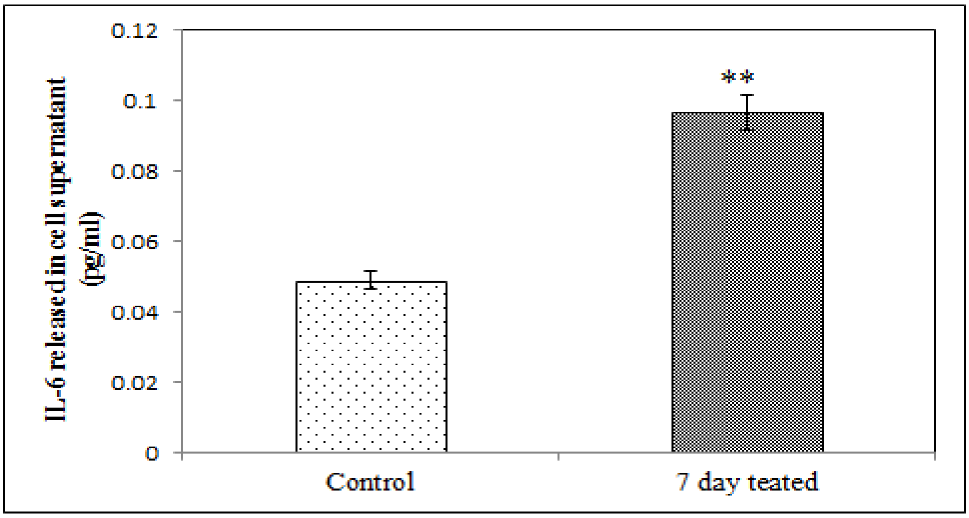

Fig. 8(c): IL- 6 released in cell supernatant after 7 days of mercury exposure (mean ± SEM, P**).

4. DISCUSSION

The mercuric compounds were found to cause serious impairment in immune and physiologic functions and suppressive non-specific and specific immune system of fishes [21] [22]. The non-specific immune parameters are useful to determine the health status of fish and to evaluate the immunomodulatory substances for fish farming as markers for pollution and diseases resistances [23]. In the present study, the immunotoxicological effects of mercury on immune system were documented by evidence of impairment in cell adhesion, phagocytic index, intracellular killing. Mercury is known for its high toxicity and its bioaccumulation in various organisms which is a significant cause of mortality of aquatic species. Methylmercury is supposed to be the most toxic form of mercury: the methyl group facilitate penetration into cells and binding to proteins.

Mercuric chloride is reported to be less toxic than methylmercury. Some cellular activities were investigated in the presence of mercuric chloride. Exposure of organisms to bacterial infection results in the activation of a variety of host defense mechanisms which include phagocytosis, degradative enzyme release and respiratory burst response. The first step in macrophage function is the activation or differentiation of macrophages. In order for circulating macrophages to enter inflamed tissue or peripheral lymphoid organs, the cell must adhere to and pass between the endothelial cells, lining the wall of blood vessels, a process called extravasation. Endothelial cells express leukocyte-specific cell adhesion molecules (CAM). Some of these membrane proteins are expressed constitutively; others are expressed only in response to local concentrations of cytokines produced during an inflammatory response. Recirculating macrophage bears receptors that bind to CAMs on the vascular endothelium, enabling these cells to extravasate into the tissues. The decrease in cell adherence in mercury treated group might be primarily caused by a decreased expression of cell adhesion molecules on the surface of macrophage cells, as envisaged from the loss of intact gap junctions and zona occludens in the TEM images of mercury-treated group {Fig. 2(b)}.

Phagocytosis is a primitive defense mechanism in all multicellular animals. Phagocytes such as macrophages and neutrophils play an important role in limiting the dissemination of infectious agents, and are responsible for the eventual destruction of phagocytosed pathogens. These cells have evolved elaborate killing mechanisms for destroying pathogens. In addition to their repertoire of degradative enzymes and antimicrobial peptides, macrophages and neutrophils can be activated to produce a number of highly toxic molecules. Production of reactive oxygen and nitrogen intermediates by these cells are potent cytotoxic mechanisms against bacteria and other pathogen [24]. Macrophages recognize and engulf bacteria into phagosomes, which subsequently acidify. These phagosomes mature into phagolysosomes upon vesicle-mediated delivery of various antimicrobial effectors [25]. From the phagocytic index of the present findings it is observed that intestinal macrophages from the control group phagocytose *S. aureus* at a higher rate, which is reduced upon mercury exposure. This suggests that mercury exposed macrophages fail to perform their normal functions, thereby allowing foreign particles or pathogens to gain access to the body and which may leads to a diseased state upon bacterial invasion.

The immune-mediator NO is an important cellular messenger molecule involved in many physiological and pathological processes [26]. At the interface between the innate and adaptive immune systems lies the high output isoform of nitric oxide synthase (NOS2 or iNOS). This remarkable molecular machine requires at least 17 binding reactions to assemble a functional dimer. The antimicrobial and cytotoxic actions of NO are enhanced by other macrophage products such as acid, glutathione, cysteine, hydrogen peroxide, or superoxide. Although the high-output NO pathway probably evolved to protect the host from infection, suppressive effects on lymphocyte proliferation and damage to other normal host cells confer upon NOS2 the same protective/destructive duality inherent in every other major component of the immune response [27]. The effects of sub-lethal concentration (0.3mg/L) of mercuric chloride in the macrophages of *C. punctatus* are estimated during the exposure of 7 days. A significant decrease ($p<0.05$) in macrophage NO production was observed as compared to the control group. A significant decrease in the total MPO production has been observed as compared to the control group of fish. Macrophage secretes lysosomal proteolytic enzymes active at tissue pH that may be important in their ability to kill bacterial cells. As macprophage mature, there is a progressive rise in lysosomes and hydrolytic content. The mercuric chloride may lead to immaturation of macrophages in vivo. And it can be assumed that due to this exposure they have lesser lysosomal content and hence are less capable of secreting myeloperoxidase (MPO) and nitric oxide (NO). Due to these lesser lysosomal enzyme, the mercuric chloride treated intestinal macrophages could not able to kill the intracellular *S. aureus* and this was evident from our finding increased viability of *S. aureus* in mercury treated group.

Fish are often at the top of aquatic food chain and accumulate large amounts of different heavy metals from the water [28]. Unlike organic contaminants that lose toxicity with time by biodegradation, heavy metals cannot be degraded; their concentration can be increased by bioaccumulation [29]. In the present study, the amount of mercury accumulated in the intestine of *C. punctatus* Bloch. was found to be higher in treated group as compared to the control after 7 days of mercury exposure and it exceeds the permissible limits set for heavy metals by ATSDR 2013 [30].

The apical surface of the normal epithelial cell is characterized by the presence of regular microvilli {Fig. 2(c)} and intact RER, frequent invaginations of the plasma membrane at the base of microvilli {Fig. 2(a)}. It is well known that goblet cells secrete mucous which is used for lubrication and for protection of the mucosa against chemical and physical damage. But after 7 days of mercury exposure it was found from transmission electron micrograph (TEM) of fish intestine that there is a abnormalities in mitochondria {Fig.2(b)} reduction in the number of goblet cells in the stomach{Fig. 2(d)}. The cells were irregularly shaped with electron-dense nuclei and the nuclei were also irregular in shape with deep indented margins {Fig. 2(f)}. This may lead to damage of the epithelial cells and other tissues [31]. Hallmarks of oxidative damage such as lack of apical cytoplasm with unifom microvilli, junctional complexes like zona occludens, desmosomes and gap junctions between epithelial cells were found in the mercury treated group as compared to the control group. Further, the presence of excess number of granular cells (eosinophil?) in the intestinal tissue in treated group as compared to the control showed the characteristics of infection and inflammation.

Macrophages play an essential role for the initiation and activation of the innate immune system, by recognizing and releasing a large number of cytokines such as tumor necrosis factor-α (TNF- α), interleukin-1 (IL-1) and IL-6 [32]. Cytokines as modulators of the immune response have been little studied in fish. A significant number of cytokines are functionally active in teleost [33]. Levels of TNF- α and IL-6 was found to be significantly more in mercury treated groups than that of control group. Cytokine expression becomes up regulated, probably by the action of mercury at multiple levels viz., PAMP recognition, receptor dysfunction and/or by affecting the cell-signalling network.

Thus, it can be concluded from this study that the water born mercury pollution is highly toxic to fresh water teleost *C. punctatus* Bloch. as well as causes immune suppression while decreasing disease resistance.

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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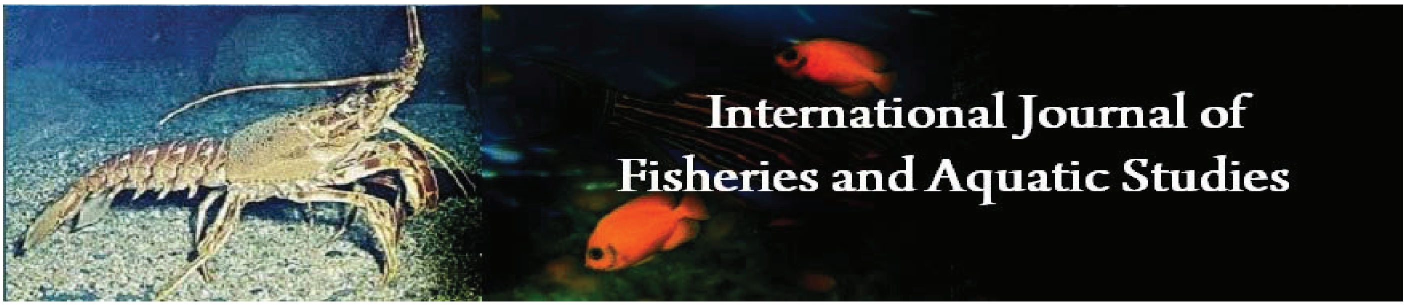

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Effects of mercury on the activities of antioxidant defences in intestinal macrophages of fresh water teleost *Channa punctatus* (Bloch 1793)

Moriom Begam, Mahuya Sengupta

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 [1], 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 [2], with metals being accumulated mainly in metabolically active tissues [3], such as the kidney, liver, gills and digestive tract [4]. In fact, several studies have reported that some fish species are far more sensitive to heavy metals toxic effects as compared to mammals [1]. 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-alkenals and ketones [5]. 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

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 sulfydryl (-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 ± 1.5 ºC, pH 7.4 ± 0.003, dissolved oxygen 6.5 ± 0.2 mg/L, alkalinity 251 ± 2.8 mg/L as CaCO3, total hardness 456 ± 3.5 mg/L and salinity (%) 30.8 ± 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 ($HgCl_2$) was used in the present study. Stock solution of mercuric chloride was prepared by dissolving analytical grade mercuric chloride $HgCl_2$ 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 $HgCl_2$ 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 ºC 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 ^{0}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, infiltered with 2.5% glutaraldehyde for 24 h fixation at 4ºC, rinsed in buffer, trimmed into 8.0 mm squares and subjected to post-fixation in 1% OsO_4 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 4ºC. 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 4ºC. After rinsing in cacodylate buffer, tissue block was post fixed at 4ºC 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 ^{0}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_{630} nm per 10 [6] 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 1.56×10^5 $M^{-1}cm^{-1.}$ 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_2O_2 per minute at 25 ^{0}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.

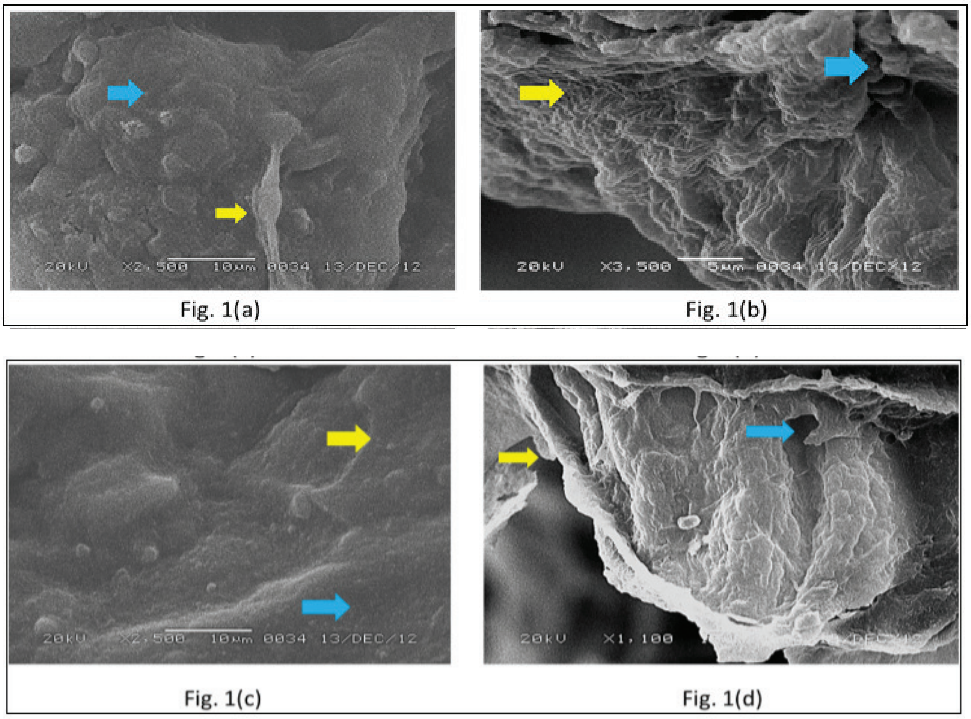

Fig 1(a) & Fig 1(c): Ultramicroscopic photographs of control *C. punctatus* (Bloch 1793) showing normal epithelium (light blue arrow) and prominent mucosal folding's (yellow arrow). Fig. 1(b), 1(d): Ultramicroscopic photographs of mercury treated *C. punctatus* (Bloch 1793) showing damaged and degenerated 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

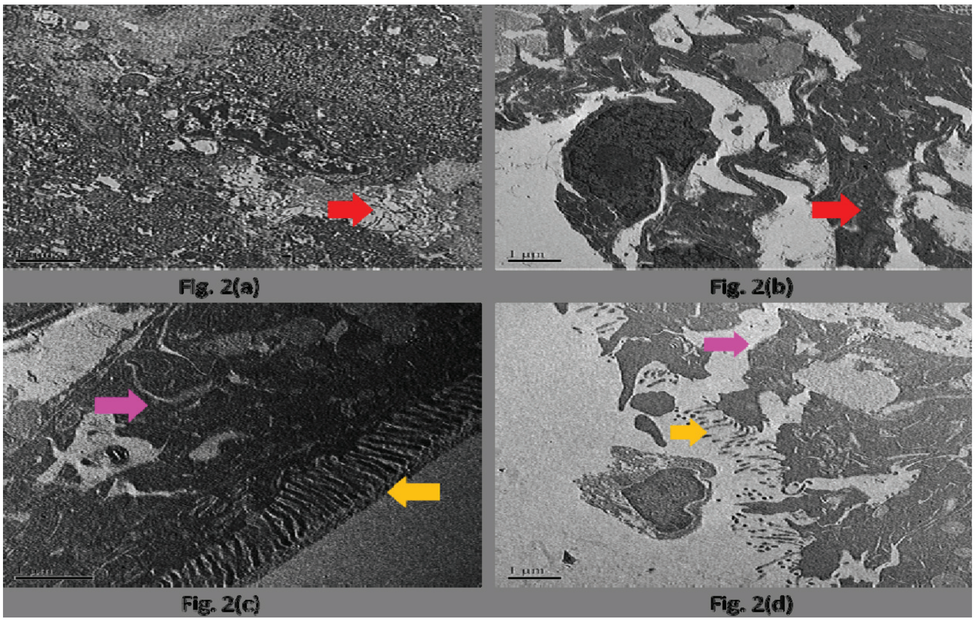

Fig 2: (a) Transmission electron micrograph (TEM) of the control fish showing intact rough endoplasmic reticuum (RER) of epithelial cells (red arrow), Fig. 2(b): Transmission electron micrograph (TEM) of *C. punctatus* (Bloch 1793) after 4 days of mercury treatment showing disrupted RER (red arrow) as 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.

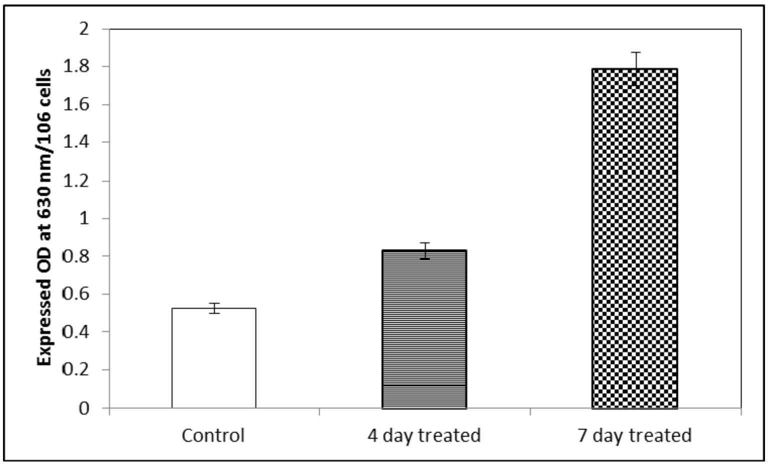

Fig 3: Respiratory burst activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± SEM. Significant difference from control value is $P < 0.001$.

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.

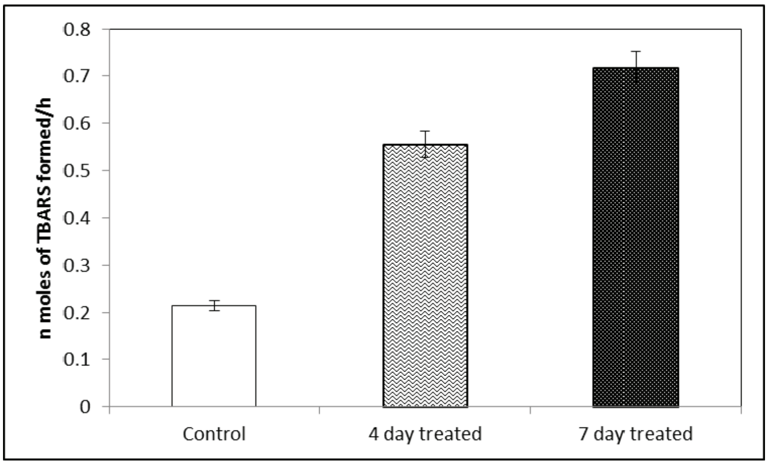

Fig 4: Lipid peroxidation in intestinal macrophages of *C. punctatus* treated with mercuric chloride for 4 days and 7 days. Values are expressed as mean ± SEM ($P<0.05$).

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.

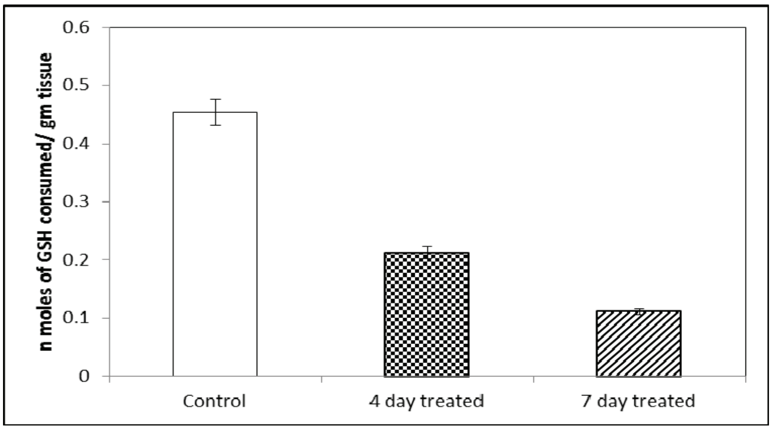

Fig 5: Reduced glutathione activity in intestinal macrophages of *C. punctatus* treated with mercury after 4 days and 7 days of exposure. Values are expressed as mean ± SEM (P<0.0005).

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.

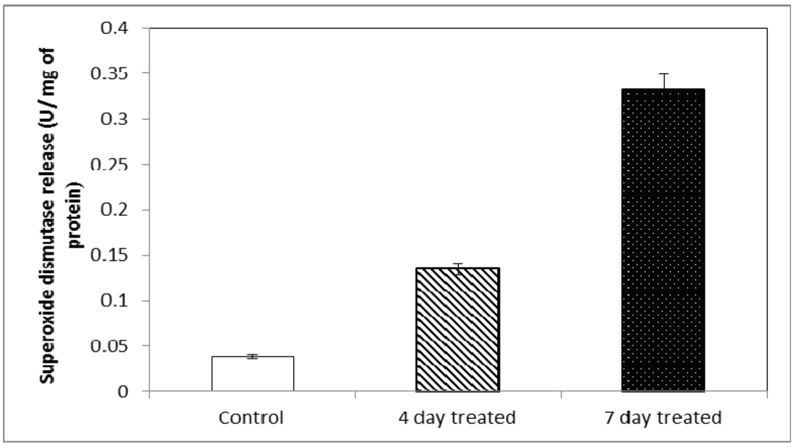

Fig 6: Superoxide dismutase activity in intestinal macrophages of *C. punctatus* treated with mercury after 4 days and 7 days of exposure. Values are expressed as mean ± SEM (P<0.05).

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.

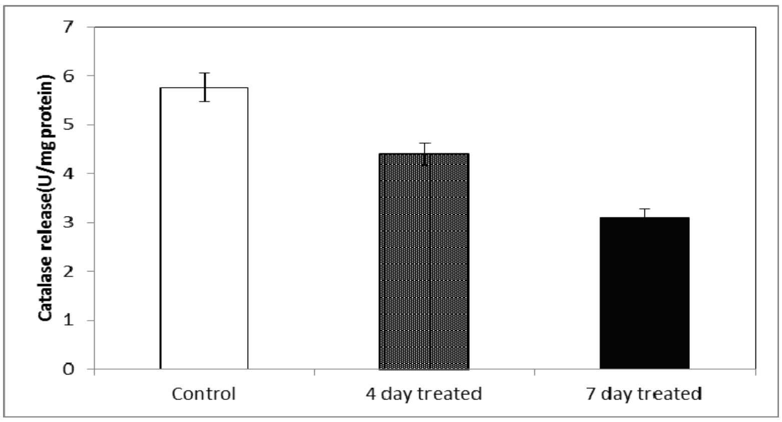

Fig 7: Catalase activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± SEM (P<0.01).

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.

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7. Conflict of interest

The authors declare that there are no conflicts of interest.

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Effects of mercuric chloride on the activities of antioxidant enzymes in the fresh water fish *Channa punctatus.*

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Abstract

This study aims to investigate the role of mercury on the antioxidant enzyme system of fresh water fish Channa punctatus. The aquatic ecosystem may extensively be contaminated with heavy metals released from domestic, industrial and other man made activities 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. The purpose of the study is to analyse the oxidative stress caused by short term exposure to mercuric chloride in the freshwater fish Channa punctatus. In present study, Channa punctatus were exposed to a sublethal concentration of mercury (0.3mg/L). After 7 days of exposure, in the treated fish induction of oxidative stress in the liver was evident from increased lipid peroxidation levels. The antioxidants superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) responded positively in a time dependent pattern, thus, suggesting the use of these antioxidants as potential biomarkers of toxicity associated with exposure to contaminants in freshwater fishes.

Key words: mercury toxicity, lipid peroxidation, superoxide dismutase, catalase, reduced glutathione.

Introduction

Chronic contamination by heavy metals in the aquatic environment is a severe problem particularly in fishes. Heavy metals may alter the structure of the cell membranes by stimulating the lipid peroxidation process with consequent complex sequences of biochemical reactions (Viarengo *et al.*,1989). Biological membranes are particularly susceptible to ROS effects. This process is broadly defined as oxidative deterioration of polyunsaturated fatty acids. The peroxidation of unsaturated fatty acids in biological membranes produces: first a decrease in fluidity, then 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 malonyldialdehyde and other aldehydes such as alkanals, hydroxyalkenals and ketones (Viarengo *et al.*, 1989). Most biological molecules have more than one function. In particular, many molecules have the ability to directly/indirectly scavenge free radicals and thus act as antioxidants in living organisms. The increased levels of these molecules during oxidative stress seems to be a biological response that may protect cells from oxidation, in synergy with other antioxidant defence systems. Oxidants fulfill signaling 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 ($2O_2^{-} + 2H^{2}+$ 6 H O + O) whereas catalase eliminates hydrogen peroxide (2H O 6 2H O + O) (Viarengo *et al.*,1989). Several reports have descrived the effects of mercury in fish (Alexander et al., 1973) and other animals (Miller *et al.*,1972) . Nevertheless, the mechanism(s) of its toxicity have yet to be firmly established. It was initially considered to be an enzyme poison (Rana *et al.*,1982)(Stacy *et al.*, 1982). 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 methylmercury, while the source is usually inorganic mercury (Hall *et al.*, 1997). Several hypotheses of how and where methylation occurs have been proposed. The main hypotheses are: biological methylation, bacterial in origin, which produces methylmercury in the environment (methylmercury 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 (Tabellini *et al.*, 2005).

This study aims to investigate the role mercury on the antioxidant enzyme system of fish. Mercury has a high affinity for sulfydryl (-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 (Hall *et al.*,1997). In the present research, the fresh water fish *Channa punctatus* was selected due to its storng immune system and its adoption to a polluted environment.

Materials and methods

The live fish *Channa punctatus* of average length 12.5–15.5 cm and average weight 20.0–30.0 g, caught from local ponds were brought to the laboratory and kept in aquaria for acclamatization to laboratory condition for one week, before starting experimental studies. The fish were fed twice daily with pelleted diet (prawn powder, fish powder and minced liver in 2 : 2 :1).

Exposure

After acclimatization thirty fish were randomly divided into two groups of ten each, one group was exposed to a sub-lethal concentration of mercuric chloride (0.3mg/L) (Sastry *et al.*, 2004) for 7 days. One group of fish, maintained in placebo, served as control. After 7 days, from each of the experimental and control groups surviving fishes were sacrificed for the estimation of mercury in the intestine.

Isolation of intestinal macrophages

The fish were dissected and the whole gut of the fish was isolated, immediately placed in Leibovitz medium (L-15) supplemented with heparin (10 IU/ml) and fetal bovine serum (2%), and then homogenised in ice cold condition. Macrophage was then isolated from the cell suspension by the method of Secombes(1990). Macrophage cells were collected, then checked for viability and counted using trypan blue exclusion test.95% cells were found to be viable.

Estimation of lipid peroxidation (LPO)

The LPO activity was determined by the procedure of Utley et al (1967), 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 1.56×10^5 $M^{-1}cm^{-1}$ Protein content of each sample was determined using method of Lowry et al. (1951).

Reduced glutathione (GSH) assay

Non-enzymatic antioxidant, reduced glutathione, was assayed by the method previously described by Ellman(1959). The amount of glutathione was calculated using a GSH standard curve and expressed as micrograms of GSH formed/mg protein.

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 (Marklund *et al.*, 1974).

Catalase (CAT) activity

Catalase activity was measured by the method of Claiborne (1985) with some modifications. . One unit (U) of Catalase activity is defined as the amount of enzyme catalyzing 1µM of H_2O_2 per minute at 25°C.

Statistical Analysis

All the values were expressed as mean ± standard deviation (SD). 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.

Results

Effects of mercury on lipid peroxidation in intestinal macrophages

The results in this figure show the lipid peroxidation which is expressed as nano moles of TBARS formed per hour (S.D ± mean). The lipid peroxidation of the control group was found to be 0.215±0.023 while that of mercury treated group was found to be 0.717±0.04 after 7th day ($P<0.05$) (Fig: 1.1).

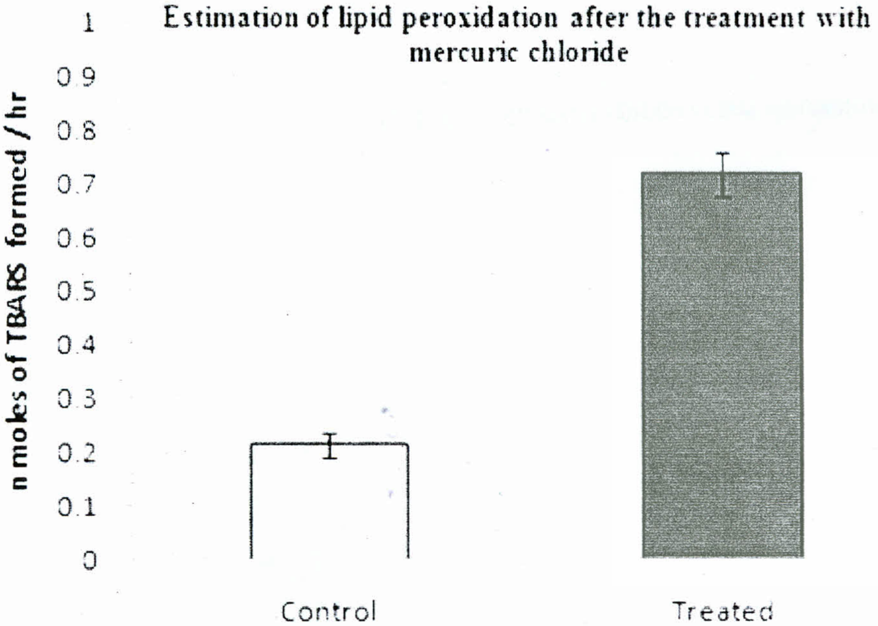

Fig: 1.1 Lipid peroxidation activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± S.D. (P<0.05)

Effect of mercury on reduced glutathione activity in intestinal macrophages

The results in this figure represents the GSH consumed/g tissue (mean ±SD).The GSH release of the control group was found to be 0.323 ± 0.030 while that of mercury treated group was found to be 0.111±0.074 ($P<0.0005$)(Fig:1.2).

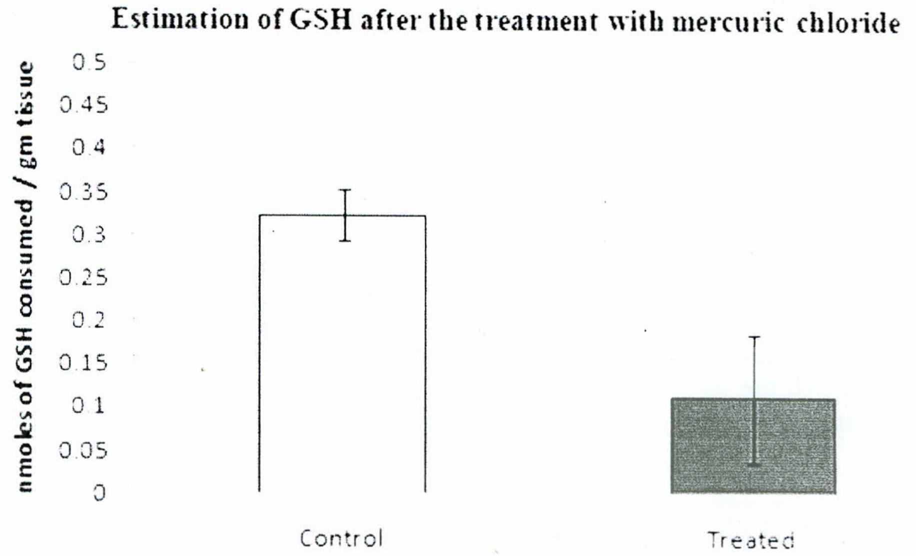

Fig: 1.2 Reduced glutathione activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± S.D. (P<0.0005)

Effect of mercury on superoxide dismutase released from intestinal macrophages

The results in this figure represents the SOD released per mg of protein (mean ±SD).The SOD release of the control group was found to be 0.009±0.001 while that of mercury treated group was found to be increased to 0.033±0.007 (P<0.05)(Fig :1.3).

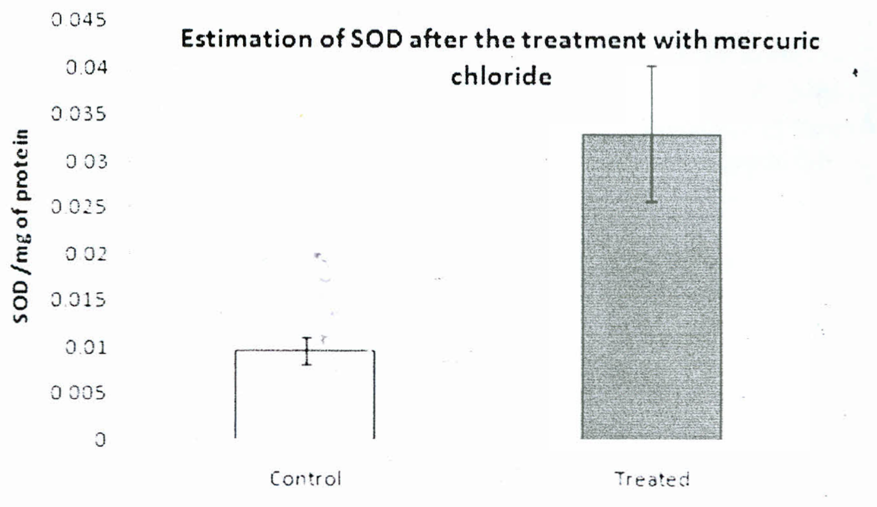

Fig: 1.3 Superoxide dismutase activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± S.D. (P<0.05)

Effect of mercury on catalase released from intestinal macrophages

The results in this figure show the catalase activity/mg of protein (S.D ±mean). The catalase release of the control group was found to be 5.69±0.23while that of mercury treated group was found to be 4.39±0.43 (P<0.01)(Fig:1.4)

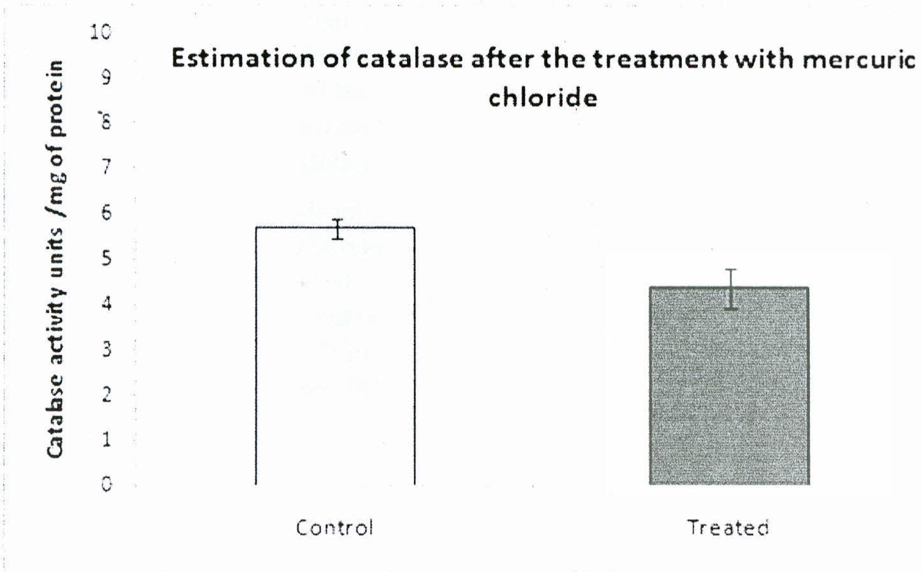

Fig: 1.4 Catalase activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± S.D. (P<0.01)

Discussion

Oxidative stress occurs when the critical balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants or excessive accumulation of reactive oxygen species (ROS), or both, leading to damage (Simpson *et al.*,1961). 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.

The elevated level of lipid peroxidation (LPO) in the liver of *C. punctatus* in response to the exposure to mercury as observed in the present investigation suggests that there is increased production of ROS. ROS and oxidative stress have been demonstrated to be triggers of apoptosis (Shen *et al.*, 2008). The observed LPO resulting from ROS generated by mercury may lead to cell apoptosis. Our observations revealed that the respiratory burst activity of the treated group increased significantly as compared to that of the control group. Under normal circumstances, activation of cells of nonspecific immunity may be beneficial to the host, particularly because the reactive intermediates released during phagocytic respiratory burst activity possess bactericidal activity (Baggiolini *et al.*, 1984) (Secombes *et al.*, 1990).

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 (Yamono *et al.*, 1995). 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 (Viarengo et al., 1993) 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 lead, resulting in low levels of GSH (Sandhir *et al.,* 1994). Our results clearly show depleted levels of GSH in lead 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 dismuting the superoxide free radicles (O^{2-}) in to most reactive forms of oxygen (H_2O_2)(Marklund *et al.,* 1974). 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 7 days. The cause of increase in SOD level 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 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 (Claiborne *et al.*, 1974).

Conclusion

The present investigation substantiated earlier findings that the mercury is toxic to fish and further indicated that 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 biosystems.

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