APPENDIX

PUBLICATIONS

Research Article

1. Paul, N., Sengupta, M. 2013. Lead induced overactivation of phagocytes and variation in enzymatic and non-enzymatic antioxidant defenses in intestinal macrophages of *Channa punctatus*. Modern Research in Inflammation. 2(2): 28-35.
2. Begam, M., Paul, N., Sengupta, M. 2012. Effects of mercuric chloride on the activities of antioxidant enzymes in freshwater fish *Channa punctatus*. Assam University Journal of Biological and Environmental Sciences. 10(1): 1-7.

Conference Proceedings

3. Paul, N., Sengupta, M., Sharma, G.D. 2013. Toxic Potential of Lead Acetate on Non-Specific Host Defenses in Freshwater Fish *Channa punctatus* (Bloch.). Journal of Science and Technology, The IIS University (in press).

Paper communicated and under review

4. Sengupta, M., Sharma, G.D. and Paul, N. Lead toxicity on non-specific immune mechanisms of freshwater fish *Channa punctatus.* Aquatic Toxicology (under consideration).

SEMINARS/CONFERENCES ATTENDED

1. Lead toxicity on non- specific immune mechanisms of freshwater fish *Channa punctatus.* N. Paul, G. D. Sharma, M. Sengupta. XXXI Annual Conference of Society of Toxicology, at Jaipur, on 22-24 Dec, 2011.
2. Lead induced overactivation of phagocytes and variation in enzymatic and non-enzymatic oxidative stress in intestinal macrophages of *Channa punctatus*. N. Paul, G.D. Sharma, M. Sengupta. 99th Indian Science Congress, at Bhubaneswar, on 3-7 Jan, 2012.
3. Arsenic induced overactivation of phagocytes and variation in enzymatic and non-enzymatic antioxidant profiles in intestinal macrophages of freshwater fish *Channa punctatus*. N. Paul, G.D. Sharma, M. Sengupta. International Conference on Global Energy Crises, Biodiversity and Environmental Science at Assam University, Silchar, on 15-17 Feb, 2012.
4. Sodium arsenite induced immunomodulatory alterations in intestinal macrophages of *Channa punctatus*. M. Sengupta, G.D. Sharma, N. Paul. National Seminar on Biotechnoloy Research in Sustainable Development, at Dibrugarh, on 27-28 Feb, 2012.
5. Lead induced Immunomodulatory and Enzymatic Alterations in Intestinal Macrophages of *Channa punctatus.* N. Paul, G.D. Sharma, M. Sengupta. International Conference on Biodiversity Conservation and Environmental Health, at Assam University, Silchar, on 16-17 March, 2012.

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Modern Research in Inflammation

Lead induced overactivation of phagocytes and variation in enzymatic and non-enzymatic antioxidant defenses in intestinal macrophages of *Channa punctatus*

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ABSTRACT

The aim of this study is to assess the adverse effects of lead, a well-documented non-essential element that occurs naturally in the environment, on *Channa punctatus*, in relation with ROS production and oxidative stress. Fishes were sampled, acclimatized and kept treated or untreated with lead (9.43 mg/L) under observation for 4 days. At day 4, respiratory burst activity, lipid peroxidation activity and superoxide dismutase level increased significantly in treated group as compared to the control. On the contrary, catalase, glutathione S-transferase, glutathione peroxidase, glutathione reductase and reduced glutathione activity decreased on treatment with lead. These results suggest that heavy metal like lead induces oxidative stress, influences the antioxidant defense system and may lead to physiological disorders rendering the health and survival of exposed fish to a compromised state.

Keywords: Lead; *Channa punctatus*; Antioxidant; Oxidative Stress; Lipid Peroxidation; Respiratory Burst; Glutathione; Bioaccumulation

1. INTRODUCTION

The pollution of the aquatic environment with heavy metals has become a worldwide problem during recent years, because they are indestructible and most of them have toxic effects on organisms [1]. Heavy metals occur as natural constituents of the earth crust, and are persistent environmental contaminants since they cannot be degraded or destroyed [2]. With rapid industrialization anthropogenic and geogenic activities, which are the major sources of heavy metal pollution, has increased exponentially. Lead is one of the first metals used by humans, is highly persistent, is not involved in normal metabolism and is very toxic [3,4]. Particularly in the 20th century, countless thousands of organic trace pollutants have been produced and in part released into the environment [5]. Lead intoxication is associated with several pathological conditions in children and adults and causes disturbances to the nervous and immune systems, anemia and reduced hemoglobin synthesis, cardiovascular diseases and bone metabolism, and also renal and reproductive dysfunction [6-15].

Fishes come into contact with multiple contaminants that are dissolved in the water or incorporated in the food chain, and so fishes are not only prone to endure negative toxicant-related health effects but also to bioaccumulate pollutants; fishes may therefore be used as bioindicators of environmental contamination [16-20]. Of interest to this study are reports that chemical exposure above a certain threshold may result in an integrated stress response, which can cause immunosuppression or immunoactivation [21].

Fish tissues are endowed with an antioxidant defense system to protect them from oxidative stress caused by metals [22-24]. Elevated levels of metals can induce oxidative stress by generating highly reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radical and hydroxyl radical via Haber-Weiss and Fenton reactions that can oxidize proteins, lipids and nucleic acids, often leading to damage in cell stress or even cell death [25-28]. Organisms have developed several protective mechanisms to remove ROS before the detrimental effects occur in cell. Antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPX), glutathione S transferase (GST), glutathione reductase (GR) and su-

peroxide dismutase (SOD) are of great importance in antioxidative stress to cope with free radicals leading several disturbances [29,30].

In view of the above, and considering the lack of sufficient knowledge about the toxic potential effect of lead acetate to freshwater fishes, the objective of this work was to evaluate its effect on enzymatic and non-enzymatic antioxidant profiles of *C. punctatus.*

2. MATERIALS AND METHODOLOGY

2.1. Animals

Freshwater fish *Channa punctatus*, of length 12.5 - 15.5 cm and weight 20.0 - 30.0 g, were obtained from local fish market and housed in a 60-l glass aquarium. Fish of a single lot were used throughout the investigation. Prior to exposure, fish were held for 15 days for acclimatization and evaluation of overall fish health under laboratory conditions.

2.2. Exposure

After acclimatization, fish were divided into two groups (five fishes in each group, n = 5); one served as control and the other as treated group. Sub-lethal toxicity of lead acetate solution [9.43 mg/L, 1.02% of 96 h LD50 value (925 mg/L)] on the intestinal macrophages of Channa punctatus was analysed for 4 days [31].

2.3. Isolation of Intestinal Macrophages

The fish were dissected and the whole gut of the fish were 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 [32].

2.4. Catalase (CAT) Activity

Catalase activity was measured by the method of Claiborne (1985) [33] with some modifications. One unit (U) of Catalase activity is defined as the amount of enzyme catalyzing 1 μmol of H_2O_2 per min at 25˚C.

2.5. 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 [34].

2.6. Estimation of Lipid Peroxidation

The LPO activity was determined by the procedure of Utley *et al.* (1967) [35], 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 × 105 $M^{-1}\cdot cm^{-1}$. Protein content of each sample was determined using method of Lowry *et al.* (1951).

2.7. Respiratory Burst Activity

Respiratory burst activity of intestinal phagocytes of control and treated fish was measured by the method of Fujiki and Yano (1997) [36], with some modifications. The respiratory burst activity was expressed as A630 nm per 10^6 cells.

2.8. Glutathione S-Transferase (GST) Activity

GST activity was measured by the method described by Mannervik and Guthenberg, 1981. Enzyme activity was calculated using a molar extinction coefficient of 9.6 × 10^3 $M^{-1}\cdot cm^{-1}$.

2.9. Glutathione Peroxidase (GPx) Activity

Total cellular GPx activity was determined by measuring the decrease in absorbance (340 nm) due to the decline in NADPH at 23˚C - 25˚C (Lorentzen *et al.*, 1994). The activity of GPx was expressed as mU/mg protein and 1 mU was defined as 1 nmol of NADPH consumed/min/mL of sample.

2.10. Glutathione Reductase (GR) Activity

The principle of the method is the reduction of oxidized glutathione by glutathione reductase in the presence of NADPH (Carlberg and Mannervik 1975). One unit was defined as an amount of the enzyme which will reduce 1 μM of oxidized glutathione per minute at pH 7.6 at 25˚C, using a molar extinction coefficient of 6.22 × 10^3 for NADPH.

2.11. Reduced Glutathione (GSH) Assay

Non-enzymatic antioxidant, reduced glutathione, was assayed by the method previously described by Ellman (1959) [37]. The amount of glutathione was calculated using a GSH standard curve and expressed as micrograms of GSH formed/mg protein.

2.12. Analysis of Lead Bioaccumulation by Atomic Absorption Spectrophotometer

The intestine from treated and untreated group were 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 1300˚C until all the materials were dis-

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solved and diluted with double distilled water appropriately. The element lead was assayed using Shimadzu AA 6200 Atomic Absorption Spectrophotometer at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya. The results were expressed as ppb/g tissue.

2.13. Statistical Analysis

The data were expressed as mean ± standard deviation. Data were analyzed using Student's t-test (two-sample assuming unequal variances) for determining the significant change over control values. The significance level was set at $P < 0.05$.

3. RESULTS

3.1. Effect of Lead on Catalase Released from Intestinal Macrophages

Catalase (CAT) release was found to decrease from 5.764 ± 0.127 U/mg protein in control to 4.496 ± 0.112 U/mg protein ($P < 0.045$) in lead treated group (**Figure 1**) which signifies that lead suppresses the activity of catalase significantly.

3.2. Effect of Lead on Superoxide Dismutase Released from Intestinal Macrophages

Superoxide dismutase (SOD) release was found to increase from 0.015 ± 0.01 U/mg protein in control group to 0.1835 ± 0.01 U/mg protein ($P < 0.05$) in lead treated-group (**Figure 2**). This shows that lead may probably overactivate SOD activity leading to formation of hy drogen peroxide in excessive amount rendering the host cell to damage.

3.3. Effect of Lead on Lipid Peroxidation in Intestinal Macrophages

There was a significant increase in lipid peroxidation activity as it is evident from the results that showed increase of TBARS from 0.385 ± 0.145 nmoles/hr in control group to 0.87 ± 0.112 nmoles/hr ($P < 0.05$) in lead treated group (**Figure 3**). Increased production of lipid peroxides signifies that lead can indeed disturb the integrity of plasma membrane, which is essential for cell viability, making cells prone to damage.

3.4. Effect of Lead on Respiratory Burst Activity in Intestinal Macrophages

There was a significant increase in respiratory burst activity from 0.521 ± 0.02 in control group to 1.148 ± 1.134 ($P < 0.007$) in lead treated group (**Figure 4**) which depicts the capability of lead in stimulating cells to produce large amount of ROS leading to cell damage.

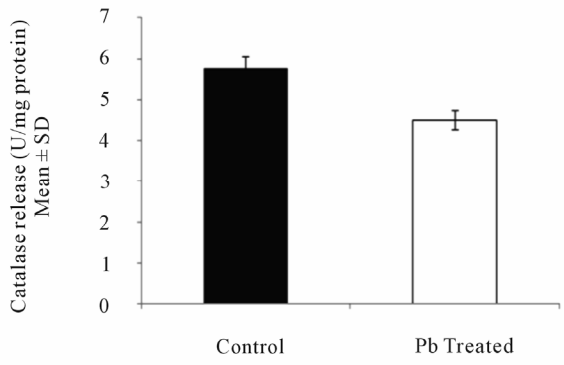

Figure 1. Catalase activity in intestinal macrophages of fish treated with lead. Values are expressed as mean ± S.D. Significant difference from control value is $P < 0.045$.

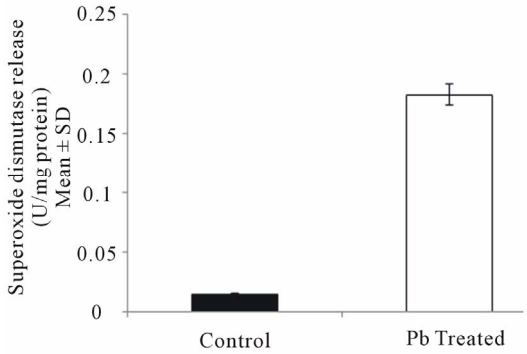

Figure 2. Superoxide dismutase activity in intestinal macrophages of fish treated with lead. Values are expressed as mean ± S.D. Significant difference from control value is $P < 0.05$.

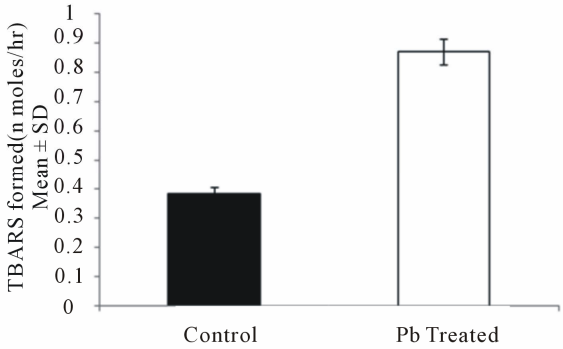

Figure 3. Lipid peroxidation activity in intestinal macrophages of fish treated with lead. Values are expressed as mean ± S.D. Significant difference from control value is $P < 0.05$.

3.5. Effect of Lead on Glutathione S-Transferase Activity in Intestinal Macrophages

Glutathione S-transferase activity was found to decrease from 28.13 ± 0.15 in control group to 24.66 ± 0.01

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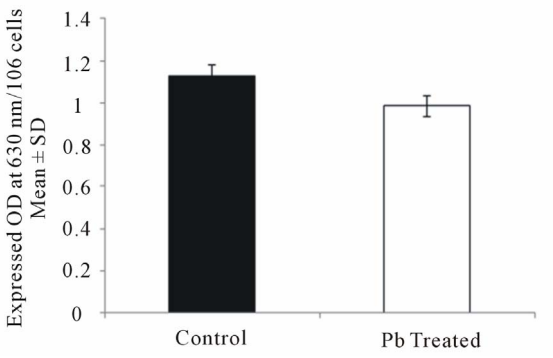

Figure 4. Respiratory burst activity in intestinal macrophages of fish treated with lead. Values are expressed as mean ± S.D. Significant difference from control value is P < 0.007.

(P < 0.05) in lead treated group (**Figure 5**) which may indicates lead induced impaired detoxification mechanism in fish.

3.6. Effect of Lead on Glutathione Peroxidase Activity in Intestinal Macrophages

Glutathione peroxidase activity was found to decrease from 5.8 ± 0.15 in control group to 4.7 ± 0.01 (P < 0.001) in lead treated group (**Figure 6**) which indicates probable persistence of free radicals like hydrogen peroxide leading to severe cell damage.

3.7. Effect of Lead on Glutathione Reductase Activity in Intestinal Macrophages

Glutathione reductase activity was found to decrease from 3.96 ± 0.15 in control group to 3.6 ± 0.01 (P < 0.02) in lead treated group (**Figure 7**) indicating lead induced compromised antioxidant defence system in fish.

3.8. Effect of Lead on Reduced Glutathione Activity in Intestinal Macrophages

Reduced glutathione activity was found to decrease from 0.823 ± 0.15 in control group to 0.555 ± 0.01 (P < 0.005) in lead treated group (**Figure 8**) indicating that lead deactivates the formation of reduced glutathione which is an essential antioxidant molecule of fish defence system.

3.9. Concentration of Lead (ppb) in Intestinal Tissues of Treated and Untreated Group

Marked increase in lead accumulation was observed in intestine of treated group as compared to that of the control group (**Table 1**).

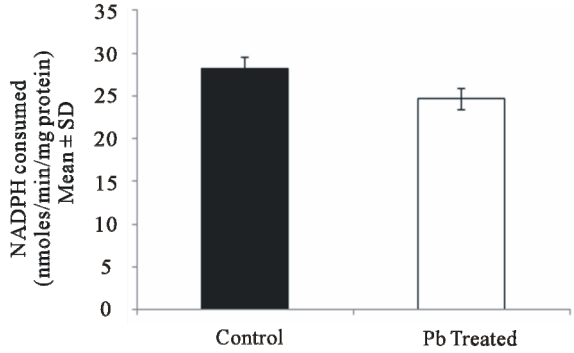

Figure 5. Glutathione S-transferase activity in intestinal macrophages of fish treated with lead. Values are expressed as mean ± S.D. Significant difference from control value is P < 0.05.

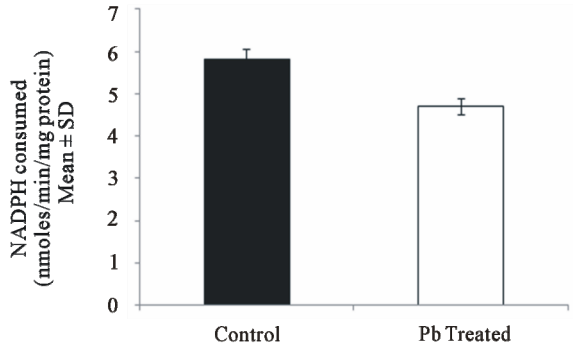

Figure 6. Glutathione peroxidase activity in intestinal macrophages of fish treated with lead. Values are expressed as mean ± S.D. Significant difference from control value is P < 0.001.

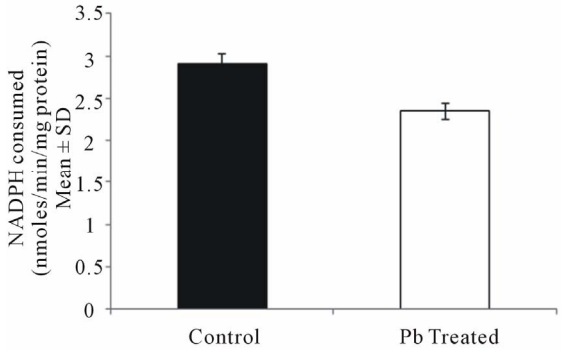

Figure 7. Glutathione reductase activity in intestinal macrophages of fish treated with lead. Values are expressed as mean ± S.D. Significant difference from control value is P < 0.02.

4. DISCUSSION

Fishes possess different defensive mechanisms to counteract the impact of toxicants. Heavy metals accumulated in fish tissues may trigger redox reactions that generate free radicals especially reactive oxygen species (ROS). These highly reactive compounds may damage lipids,

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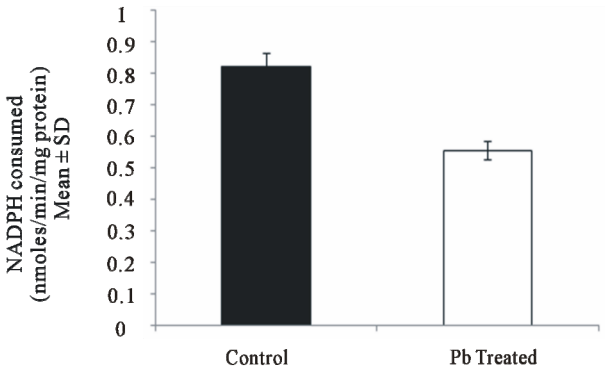

Figure 8. Reduced glutathione activity in intestinal macrophages of fish treated with lead. Values are expressed as mean ± S.D. Significant difference from control value is $P < 0.005$.

Table 1. Concentration of lead accumulated in intestinal tissues of treated and untreated group.

Lead accumulation in intestinal tissue of untreated fish (ppb)	Lead accumulation in intestinal tissue of treated fish (ppb)
0.02 ± 0.025	0.33 ± 0.021

Values are expressed as mean ± SD ($P < 0.05$).

proteins, carbohydrates and nucleic acids and may induce morphological and physiological alterations in fish tissues [38,39]. Many aquatic organisms have been shown to possess defense pathway to protect them against damages induced by oxyradical production [40]. An earlier study report on enzymatic and non-enzymatic antioxidant processes contributes to reducing the impact of ROS in fishes [41]. Therefore, both the activity of antioxidant enzymes and the occurrence of oxidative damage have been proposed as indicators of pollutant-mediated oxidative stress [42].

Oxidative stress occurs when the critical balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants or excessive accumulation of ROS, or both, leading to cellular damage [43]. The SOD-CAT system provides the first defense against oxygen toxicity and represents a cellular defense mechanism to counteract toxicity of ROS. SOD catalyzes the dismutation of the superoxide anion radical to water and hydrogen peroxide, which is detoxified by the CAT activity [44]. Palace *et al.* (1992) [45] have stated that SOD is the most responsive indicator of exposure to contaminants eliciting oxidative stress. Low levels of CAT in lead treated fishes could be attributed to high production of superoxide anion radical, which has been reported to inhibit CAT activity [46]. The reduction of superoxide radicals to H_2O_2 is catalyzed by SOD. The increase in SOD activity in lead treated fishes may be due to increased generation of reactive oxygen species.

Phagocytes, upon stimulation with various agents, produce ROS through activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase [47]. NADPH oxidase is a superoxide-producing enzyme consisting of the membrane (gp91PHOX and p22PHOX) and the cytosolic (p47PHOX, p67PHOX, and p40PHOX) components [48,49]. In addition, small G-proteins such as rac1, as well as kinases including PKC, regulate its activity [50]. Our observations reveal 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 the reactive intermediates released during phagocytic respiratory burst activity possess bactericidal activity [51,52]. Elevation in the respiratory burst activity on lead exposure may suggest over activation of the superoxide-producing enzyme NADPH oxidase and generation of large amount of ROS. Further, it could also be assumed that lead acetate in macrophage might have suppressed the activity of the regulatory proteins leading to uncontrolled enzyme activity which is destined to cell damage.

Heavy metal induced lipid peroxidation has already been described in various fish species [53-55]. LPO estimation in particular has also been found to have a high predictive importance as revealed from a credible number of research papers describing its suitability as a biomarker of effect [56-60].When the animal's defenses are insufficient to neutralize ROS, oxidative damage may occur, and one of the most serious damages is membrane lipid peroxidation [44]. The integrity of plasma membrane is essential for cell viability, and as a consequence of over activation of phagocytes, its fluidity seems to be effected in fish [61]. Our results demonstrate that lead acetate exposure induces production of high levels of lipid peroxides thus causing deleterious membrane damaging effect. Thus it may be hypothesized that lead may stimulate the peroxidation of lipids by acting as catalysts in the formation of oxygen radicals.

Antioxidant enzymes that have often been studied as oxidative stress biomarkers link detoxification of ROS with the metabolism of reduced glutathione [62,63]. These include glutathione peroxidase (GPx), an enzyme removing hydrogen peroxide by the simultaneous oxidation of reduced GSH to its oxidized form glutathione disulfide (GSSG) and glutathione reductase (GR), an enzyme catalyzing the conversion of GSSG back to its reduced bioactive form maintaining thus GSH/GSSG equilibrium [64]. Glutathione S-transferase (GST), an enzyme involved in the detoxification process and in protecting against peroxidative damage, is ubiquitous in the cytosol and microsomes of eukaryotes [65]. Our results reveal significant depletion of GPx, GR and GST activity indicates an impaired detoxification mechanism of the fish upon low concentration of lead exposure.

One of the most remarkable effects of lead exposure

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on intestinal macrophage is a time-dependent decrease in GSH. GSH is the most well studied antioxidant molecule in fish. Heavy metal cations are characterized by an extremely high affinity to –SH residues [63] resulting in decrease of GSH level. Sandhir *et al.*, [66] establish 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. Our results clearly show depleted levels of GSH in lead treated group of fish as compared to control, and may contribute to the above facts.

The presence of metal pollutant in fresh water is known to disturb the delicate balance of the aquatic ecosystem. Among the various toxic pollutants, heavy metals are particularly severe in their action due to tendency of bio-magnification in the food chain. They readily tend to concentrate in different organs of fishes resulting in bioaccumulation and biomagnification of these metals to a toxic level even when the exposure is low [67,68]. Studies carried out on fish species have shown that heavy metal bioaccumulation may alter the physiological activities and biochemical parameters both in tissues and blood [22]. Our results show significant level of lead accumulation in intestinal tissues when compared with the untreated group and consequently correlates with the induced oxidative stress as well as altered enzyme activities in the intestinal macrophages.

In conclusion, the present study indicates that sub-lethal concentration of lead acetate has the capacity to bioaccumulate, thereby altering the normal functional activities of freshwater fish *C. punctata*. Further, the association of oxidative stress including variation in its antioxidant profile suggests that the defense system of *C. punctata* is significantly compromised upon metal exposure at low concentrations.

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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 1 μM 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).

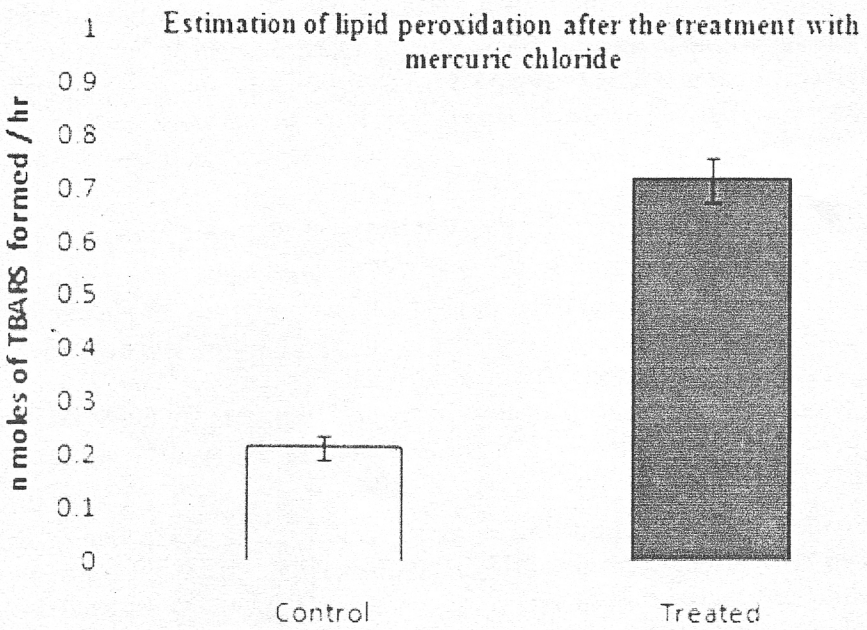

Fig: 1.1 Lipid peroxidation activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± S.D. (P<0.05)

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).

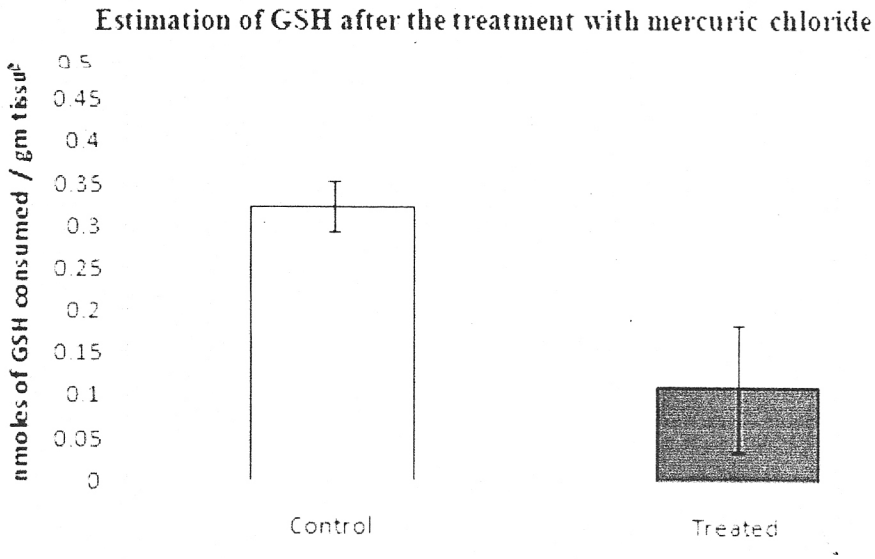

Fig: 1.2 Reduced glutathione activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± S.D. (P<0.0005)

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).

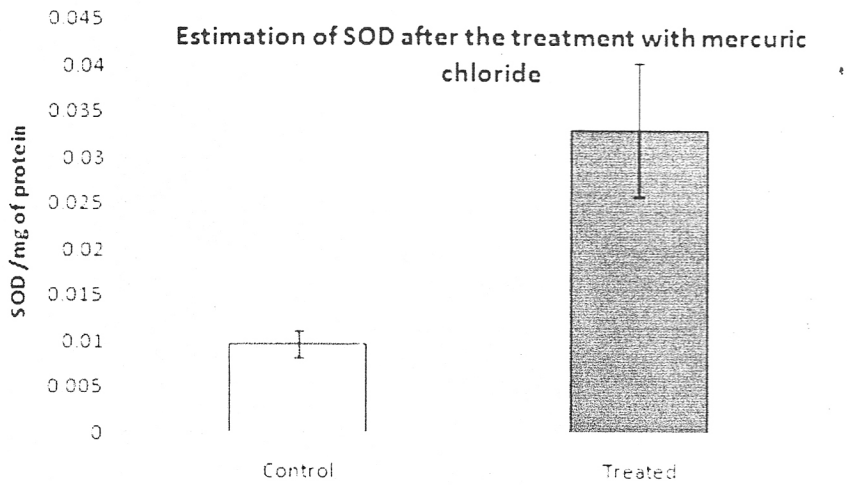

Fig: 1.3 Superoxide dismutase activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± S.D. (P<0.05)

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)

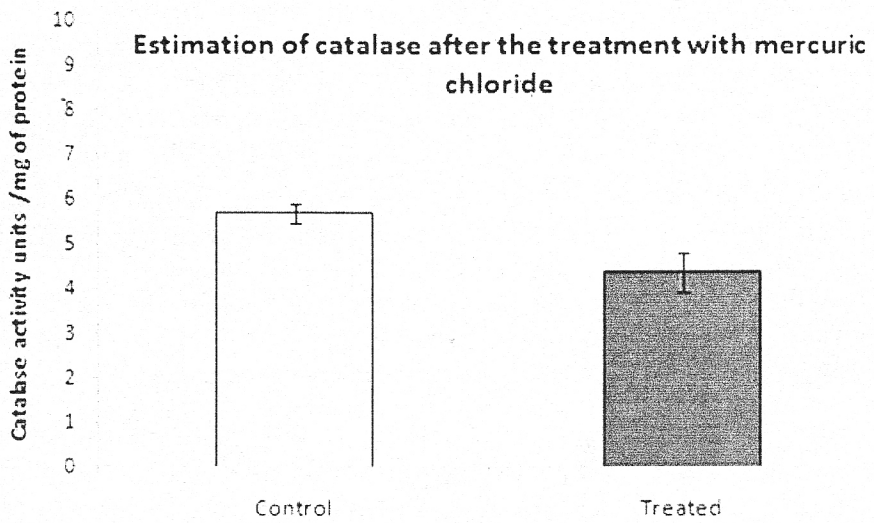

Fig: 1.4 Catalase activity in intestinal macrophages of fish treated with mercury. Values are expressed as mean ± S.D. (P<0.01)

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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