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RESEARCH ARTICLE

Toxic Potential of Lead Acetate on Non-Specific Host Defenses in Freshwater Fish *Channa punctatus* (Bloch.)

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Abstract

This paper addresses the immunotoxicologic effects of lead acetate in intestinal macrophages of freshwater fish *Channa punctatus*. Lead has no known role in the body that is physiologically relevant, and its harmful effects are myriad. Lead from the atmosphere and soil ends up in water bodies thus affecting the aquatic organisms. This situation has thus prompted numerous investigations on the effects of this metal on the biological functions of aquatic organisms, particularly on defense mechanisms in fish.Fishes were exposed to lead acetate (9.43 mg/L) for 4 days. We reported that *in vivo* exposure of lead acetate inhibits phagocytosis, which is evident from a reduced phagocytic index of treated group from that of the control.On giving bacterial challenge (with *Staphylococcus aureus*) to intestinal macrophages of both control and lead treated groups, bacterial load showed significantly higher concentration of viable bacteria in the heavy metal treated group as compared to control. The amount of MPO and NO released by the control cell was also reduced upon *in vivo* lead treatment. The property of antigenic adherence to the macrophage cell membrane, a vital process in phagocytosis, was significantly decreased in treated group as compared to control. We thus conclude that lead affects the general immune status of *Channa punctatus* and renders the fish immunocompromised and susceptible to pathogens.

Keywords

Channa punctatus, Lead, Macrophage, Oxygen- dependent killing mechanisms

Introduction

Lead (Pb) is an immunotoxicantand has been well documented around the world (WHO, 2000). It has many uses in industry including pipes, paints, enamels, glazes, motor industry and others. The major hazard in industry arises from the inhalation of dust and fume of lead but the organic compounds may also be absorbed through the skin. Depending on the dose, lead exposure can cause a wide spectrum of health problems, including neurological, cardiovascular, renal, gastrointestinal, haematological and reproductive disorders (Mobarak and Sharaf, 2011; Theron *et al*, 2011).

Like all vertebrates, fish possess a wide array of defense systems to protect themselves against heavy metals. Moreover, teleost fish have proved to be good models to evaluate the toxicity and effects of contaminants on animals, since their biochemical responses are similar to those of mammals and of other vertebrates (Sancho *et al*,2000). *Channa punctatus* is a freshwater teleost fish and was selected as an experimental model because of its wide availability round the year, and adaptability to laboratory. With respect to interactions with cells of the non- specific immune system, lead has been reported to negatively affect the functions of both neutrophils and macrophages (Theron *et al*, 2011). Some reports are available showing immunotoxicity of lead on different fish models, such as lead nitrate in *Channa striata* and *Cyprinuscarpio* (Devi and Banerjee, 2007;Vinodhini and Narayanan, 2008) and lead acetate in *Oreochromisniloticus* and *Poecilialatipinna* (Mohammed *et al*, 2008; Mobarak and Sharaf, 2011).

However a better understanding of the mechanisms through which heavy metals influence the non-specific immune system is necessary to appreciate the many complex interactions between environmental contaminants and the system's susceptibility to infectious diseases. Thus the present study was designed to study the effect of non-lethal lead acetate exposure in *C.punctatus* focusing on immunosuppression of phagocytic as well as bactericidal responses of intestinal macrophages.

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Materials and Method

Biological Material

Experiments were performed on *C.punctatus* of almost same sizes (length 12.08±0.41 cm, weight 18.07±0.75 g). Animals were purchased from a local market; upon arrival at the laboratory, the animals were placed in aquarium filled with tap water and kept for atleast 5-6 days to allow for acclimatization. Only healthy fishes, as determined by general appearance, were used for the studies.

Exposure

After acclimatization, fish were divided into two groups; one served as control and the other as treated group. Sub-lethal toxicity of lead acetate solution [9.43 mg/L, 1.02% of 96h LD_{50} value (925 mg/L)] on the intestinal macrophages of *C. punctatus* was analysed for 4 days (Devi and Banerjee, 2007).

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. Cell suspension is then transferred to tubes and kept in ice for cell debris to settle. The supernatant is then layered over ficoll (45%) and subjected to density- gradient centrifugation (Chung and Secombes, 1998). 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 were collected and tested for viability as determined by trypan blue dye exclusion technique.

Phagocytosis Assay

The cells from both control and exposed groups were allowed to adhere separately on glass slides for one hour. Phagocytosis assay were performed with heat killed *Staphylococcus aureus* and phagocytic index calculated (Czuprynski *et al*,1984).

Intracellular Killing Assay

Bacteria were incubated with macrophages in L-15 FBS for 20 min at 37°C. After various time intervals, samples were plated onto nutrient agar to determine the number of viable intracellular bacteria (Leigh *et al*,1986). Intracellular killing is expressed as the percentage decrease in the initial number of viable intracellular bacteria.

In vitro Cell Adhesion Assay

Cells were seeded separately for treated and control group in 96 well microtitre plates and allowed to adhere differentially for different time intervals. The cells were stained with crystal violet and the dye extracted from the adhered macrophage in the wells by lysing with 0.1% SDS in HBSS. Cell adhesion was expressed as the increased absorbance measured spectrophotometrically at 570nm (Lin *et al*,1997).

Nitric Oxide (NO) Release Assay

Cells (10^6 cells/ml) were suspended in DPBS and were stimulated with 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 550nm (Saggers and Gould, 1989).

Myeloperoxidase (MPO) Release Assay

Cell suspension were taken, stimulated with LPS and centrifuged. The supernatants were collected in separate microcentrifuge tubes. Supernatants and cell lysate were allowed to react with orthophenylenediamine (OPD) substrate and readings were taking at 492 nm in a spectrophotometer (Bos *et al*,1990).

Statistical Analysis

The data were expressed as mean±S.D. 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 $P<0.05$.

Results and Discussion

Effect of Lead Acetate on Phagocytic Capacity of Fish Intestinal Macrophages Against *S. aureus*

Phagocytic index of the control group was found to be 21666.66 ± 522.66 while that of lead treated group was found to be 10667.62 ± 428.17 ($P<0.01$) (Fig.1).Chronic lead exposure can affect proliferation of white blood lymphocytes. Macrophages play a critical role in body's defense system by eliminating microorganisms from infected tissues (Gonsebatt *et al*, 1992). Macrophages recognize and engulf bacteria into phagosomes, which subsequently acidify. These phagosomes mature into phagolysosomes upon vesicle-mediated delivery of various antimicrobial effectors (Garin *et al*, 2001). The most active promoter of phagocytosis is the C3 component of complement, which is bound to the bacterial surface LPS directly via the alternative complement pathway (ACP)

or indirectly via lectins or CRP (Secombes, 1996). Reduction of phagocytic capacity by lead exposed group may suggest that the biological activity of ACP, which is an important pathway in the defense mechanism of fish (Ellis, 2001), and that of the C3 component is somehow inhibited making the host cells prone to infection and diseases.

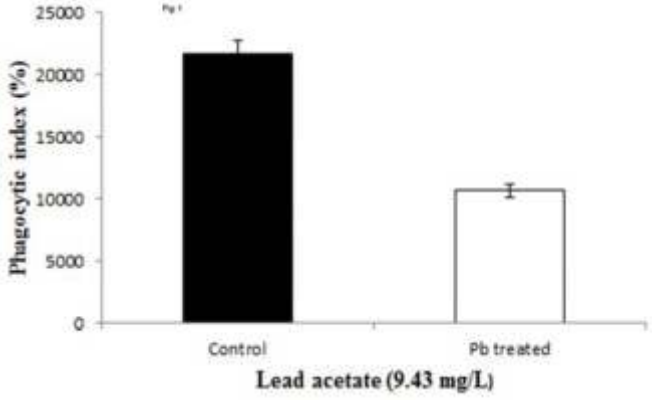

Fig.1. Phagocytic activity in intestinal macrophages of fish treated with lead. Values are expressed as mean±S.D, significant difference with the control group (P<0.01)

Effect of Lead Acetate on Intracellular Killing Capacity of Fish Intestinal Macrophages

On giving bacterial challenge (with *S. aureus*) to intestinal macrophages of both control and lead treated groups, bacterial load showed significantly higher concentration of viable bacteria (P<0.004) in the heavy metal treated group as compared to control (Fig.2). Normally, bacteria within macrophage are rapidly killed and degraded in the phagolysosome with the secretion of enzymes and toxic peroxides, making it difficult to dissect the mechanism of death (Slauch, 2011). Furthermore, the host cell shows a hypoferraemic response by restricting the availability of iron, which is required by bacterial cells as a co-factor for many enzyme systems, by being bound to the high-affinity iron binding protein, transferrin, in the plasma (Ellis, 2001). The ability of fish to express the hypoferraemic response following injection of LPS has been clearly demonstrated (Congleton and Wagner, 1991; Langston *et al*, 2001). Most bacteria are thereby unable to grow in the host tissues. Prolonged exposure to lead tends to inhibit the killing capacity of intestinal macrophages indicating that lead exposed leucocytes are either somehow less potent to kill the bacteria efficiently or fails to express the hypoferraemic response that allows pathogens to easily gain access to the host tissues.

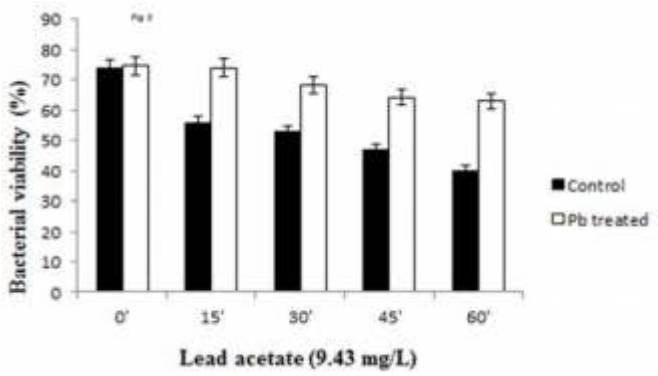

Fig.2. Intracellular killing capacity of control and lead exposed fish intestinal macrophages. Values are expressed as mean±S.D, significant difference with the control group (P<0.004)

Effect of Lead Acetate on Cell Adhesion Property of Fish Intestinal Macrophages

Cell adherence was expressed as the decreased absorbance at 570 nm. After 30 min incubation, absorbance in lead-treated group was found to be 0.07±0.002 from 0.15±0.002 of control group(P<0.04). After 60 min incubation, absorbance in lead-treated group was found to be 0.13±0.002 from 0.21±0.002 of control group (P<0.02) (Fig.3).Adherence of antigen to the macrophage cell membrane is a vital step in phagocytosis. Phagocytosis first requires attachment of the bacteria to the surface of the phagocyte, which may involve hydrophobic interactions or sugar/lectin interactions (Secombes, 1996). It is well established that lectins isolated from fish serum have opsonising activity for microorganisms (Ellis, 2001). Lectin-coated bacteria were more susceptible to being killed by macrophages (Ottinger *et al*, 1999). Inhibition of cell adherence shows that the heavy metal may either suppress enough production of lectins or may reduce its opsonising activity, which may have obvious effect on the host defense mechanism.

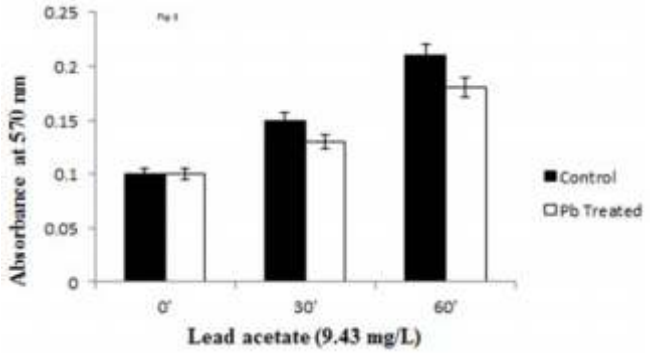

Fig.3. Cell adhesion capacity of control and lead exposed fish intestinal macrophages. Values are expressed as mean±SD, significant difference with the control group at 30 minutes (P<0.04) and 60 minutes (P<0.02)

Effect of Lead Acetate on Nitric Oxide Release in Fish Intestinal Macrophages

NO released from lead-treated+ LPS stimulated intestinal macrophages was found to be 4.61±0.197µg/100 ml whereas that in control group was 50.94±0.914µg/100 ml (P<0.00005) (Fig.4). In recent years, NO has been shown to be a very important molecule in regulating immune functions as well as having a direct antimicrobial effect (Gunasegaran *et al*,1993;Liew*et al*, 1990). It exhibits a wide range of important functions in vivo, acting as a releasing factor mediating vasodilation, as a neuronal messenger molecule, and as a major regulatory molecule and principal cytotoxic mediator of the immune system (Beckman and Koppenol, 1996; Dimmeler and Zeiher, 1997; Langston *et al*, 2001). Reactive nitrogen species (RNS), a family of antimicrobial molecules derived from nitric oxide (NO), are produced via the enzymatic activity of inducible nitric oxide synthase 2 (NOS2) (Fang, 2004;Nathan and Shiloh,2000). Decreased production of NO may probably signify lead induced suppression of NOS2 expression and subsequent decrease in antimicrobial activity of macrophage.

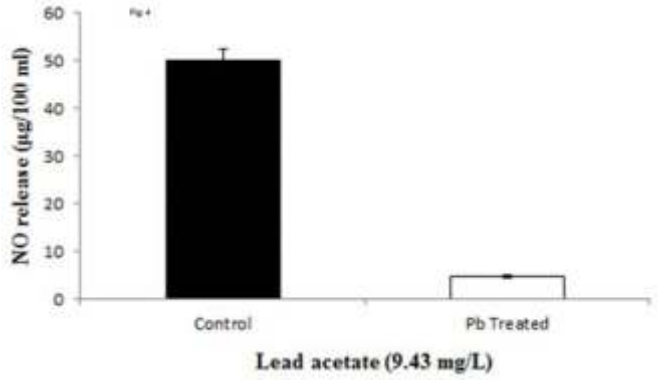

Fig. 4. NO released from control and lead exposed fish intestinal macrophages. Values are expressed as mean±SD, significant difference with the control group (P<0.00005)

Effect of Lead Acetate on Myeloperoxidase Release in Fish Intestinal Macrophages

Myeloperoxidase (MPO) release was studied and absorbance was found to decrease from 43.51±1.66 % in control to 24.42±1.32 % (P<0.02) in lead-treated group (Fig.5). MPO produced by macrophages is known to play an important role in cellular defenses against various bacterial infections. MPO can generate oxidants from hydrogen peroxide (H_2O_2) and a range of co-substrates, most notably chlorine (Foote *et al*, 1983) and nitrite (Eiserich, 1998). Hypochlorous acid (HOCl), which is produced by MPO, is strongly bactericidal (Klebanoff, 1967) and markedly increases the antibacterial potency of ROS (Rosen and Klebanoff, 1979; Rosen *et al*, 1990), which might indicate that MPO is important in host defense. Insufficient release of MPO from lead treated macrophages may indicate that its bactericidal potency has been suppressed resulting in poor host defense mechanism.

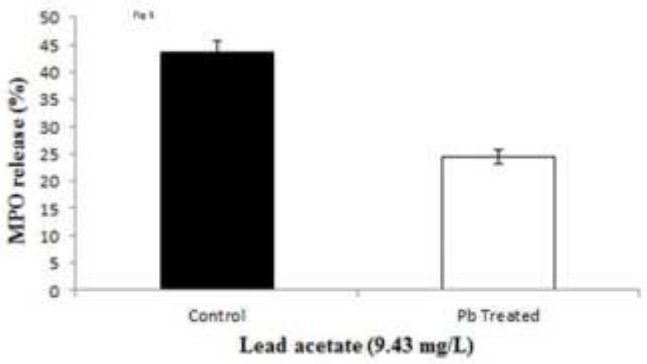

Fig.5. MPO released from control and lead treated fish intestinal macrophages. Values are expressed as mean±SD, significant difference with the control group (P<0.02)

As a conclusion,lead has a deleterious effect on the immune functions of fish, specifically on its digestive organs. The exposure to sub-lethal concentration of lead acetate caused suppression of the parameters (phagocytic activity, intracellular killing, cell adhesion, nitric oxide release and myeloperoxidase release) measured in the intestinal macrophage of *C. punctatus*. These results suggest that the alterations in the phagocytic activity and the oxygen dependent killing mechanisms can be used as potential biomarkers for risk assessment in aquatic ecosystem.

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