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Effect of Lead on Oxidative Stress, Na^+K^+ATPase Activity and Mitochondrial Electron Transport Chain Activity of the Brain of *Clarias batrachus* L.

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Abstract The present invivo study was designed to elucidate the toxic effect of lead on oxidative stress, Na^+K^+ATPase and mitochondrial electron transport chain activity of the brain of *Clarias batrachus.* The fish were exposed to 10 and 20% of the derived 96 h LC_{50} value, 37.8 and 75.6 mg L^{-1}, respectively, and sampled on 20, 40 and 60 days. Exposure of fish brain to lead demonstrated an increased production of reactive oxygen species, increased lipid peroxidation, loss of protein thiol groups in synaptosomal fraction with the decreased activity of Na^+K^+ATPase, partial inactivation of mitochondrial electron transport chain activity and energy depletion. However, no change in protein carbonyl content in synaptosomal fraction was observed due to lead exposure. Concluding the results of our investigation we suggest that lead exposure induces oxidative stress in the brain of *Clarias batrachus* and the decline in Na^+K^+ATPase activity was presumeably mediated by the combined action of lipid peroxidation and deficient mitochondrial electron transport chain activity.

Keywords Lipid peroxidation · Mitochondrial electron transport chain activity

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Lead is an environmentally reactive metal that exhibits a high degree of toxicity to living organisms. Recently, oxidative stress has been proposed as a possible mechanism involved in lead toxicity (Ercal et al. 2001; Pande and Flora 2002; Patrick 2006). The ill effects of Pb^{2+} exposure have also been noted in brain (Bennet et al. 2007; Flora and Seth 2000) since lead can pass the blood-brain barrier (Skerfving 1988). Previous studies on brain have revealed the propensity for lead to catalyse oxidative reactions generating the reactive oxygen species (ROS) that inhibits the production of sulphydryl antioxidants (Flora et al. 2003), induces genotoxic effects (Valverde et al. 2002) and initiates lipid peroxidation in cellular membranes (Flora and Seth 2000). Though lead toxicity seems to be apparently simple, its mechanism or the exact site of action for the effects of lead is still unclear.

The Na^+K^+ATPase, an energy dependent transmembrane enzyme composed of α and β subunits, is severely affected by lead induced oxidative stress through propagation of lipid peroxidation in cell membranes of various organ systems (Rogers et al. 2003). Among the various cell organelles, mitochondria has been identified as a prime target for neurotoxic action of lead possibly through increased generation of reactive oxygen species (ROS) and their subsequent oxidative injuries (Chen et al. 2003). Because of the central importance of Na^+K^+ATPase and mitochondrial electron transport chain in cellular functions, the present study has tried to explore the effect of lead on oxidative stress, Na^+K^+ATPase and mitochondrial electron transport chain activity of piscine brain. Further it has been ascertained whether the decline in cellular Na^+K^+ATPase activity induced by lipid peroxidation due to lead exposure was further aggravated by mitochondrial dysfunction wherein individual enzyme complexes of mitochondrial electron transport chain gets partially inactivated leading to energy depletion in the cell.

The piscine model of *Clarias batrachus* was chosen for this study because *Clarias batrachus* is naturally cultivated in India in the marshy lands and aquatic bodies adjacent to the industrial settlements. *Clarias batrachus* being bottom dwellers become exposed to metals (like lead) that are present in sediments of fresh water bodies as a result of industrial discharge. The concentration of lead in polluted water bodies often varies from 5 to 40 mg L^{-1} and acts like a toxicant in water.

Materials and Methods

Animal use protocols have been approved by the University of Kalyani Animal Care Committee in accordance with national guidelines. Healthy adult specimens of *Clarias batrachus* L. (56 ± 2.20 g body weight, 14.1 ± 1.51 cm total length) were collected from a local hatchery and were acclimatized to laboratory experimental condition for 2 weeks in dechlorinated tap water in large glass aquaria in the laboratory. They were fed *ad libitum* on alternate days and the water with requisite Pb^{2+} salt was renewed after every 48 h, leaving no faecal matter, unconsumed food or dead fish, if any. Prior to the commencement of the experiment, 96 h median lethal concentration (96 h LC_{50}) of lead nitrate (E. Merck, India) was estimated by probit analysis (Finney 1971).

Adult *Clarias batrachus* were exposed to $Pb(NO_3)_2$ treated water at 10% (37.8 mg L^{-1} Pb) and 20% (75.6 mg L^{-1} Pb) of the 96 h LC_{50} value (378 mg L^{-1} Pb). Eight fish were randomly assigned for each aquaria containing 30 l of $Pb(NO_3)_2$ treated water, prepared in tap water (having dissolved O_2 6.2 mg L^{-1}, pH 7.12, water hardness 24.2 mg L^{-1} and water temperature 25 ± 3°C). Identical groups of eight fishes each were kept in separate aquaria containing 30 L of plain dechlorinated tap water (without lead salt) as controls. After each of the exposure periods of 20, 40 and 60 days, fishes from the respective experimental, as well as control aquaria were sacrificed and the brain tissues were utilised for various biochemical experiments. Atomic absorption spectrometry was used to measure the actual concentration of lead in experimental water during the exposure periods of 20, 40 and 60 days and was found very near to the desired concentration levels.

The brain synaptosomal and mitochondrial fractions were prepared and were utilized for biochemical assays. The protein carbonyl content of synaptosomal fraction was estimated by 2,4-dinitrophenylhydrazine assay following the method of Levine et al. (1990). The sulphydryl content of protein of the synaptosomal fraction was estimated using Ellman's reagent following the method of Habeeb (1972). Brain synaptosomal fraction was used for the assay of lipid peroxidation according to the method of Ohkawa et al. (1979) followed by measurement of Na^+K^+ATPase activity as adapted from the method of Mallik et al. (2000). The activity of complex I was assayed by using ferricyanide as the electron acceptor as adapted from Hatefi (1978). The MTT reduction assay was carried out to measure complex II–III activity with freshly prepared mitochondria following the method of Cohen et al. 1997. The activity of complex IV was assayed by noticing the oxidation rate of reduced cytochrome c (ferrocytochrome c) at 550 nm as mentioned in Wharton and Tzagoloff (1967). Mitochondrial ATP production was carried out using luciferin-luciferase bioluminescent assay following the method of Hays et al. (2003). DCFH-DA dye was used for measurement of ROS production as mentioned in the method of Dreiem et al. (2005). For all parameters mean ± SE were calculated. All data were subjected to analysis of variance and Duncan's multiple range test (DMRT) was used to determine the significant differences among means at 5% level of significance. The control values of the biochemical variables of 20, 40 and 60 days duration are nearly the same, within 5% variation.

Results and Discussion

The extent of lipid peroxidation in the synaptosomal fraction was estimated by the amount of malondialdehyde (MDA), an important byproduct of lipid peroxidation formed. Among all the combinations of exposure of Pb^{2+} ions with respect to duration and concentrations of treatment, the combination of 60 days treatment with Pb^{2+} 20% (of 96 h LC_{50}) exposure recorded the highest increase in MDA formation (Fig. 1a). A general tendency of increase in MDA formation was observed in the synaptosomal fraction with successive increase in duration and concentration of Pb^{2+} exposure. The brain is prone to lipid peroxidation because of its high rate of oxygen utilization, availability of oxidizable substrates like polyunsaturated fatty acids and catecholamines and a deficient antioxidant defense system (Halliwell and Gutteridge 1989). However, no incorporation of protein carbonyl, an important marker of protein damage induced by oxidative stress was noticed in the synaptosomal fraction of fish brain (Fig. 1b). But under our experimental conditions, the oxidative stress induced by Pb^{2+} caused a gradual loss of –SH groups in fish synaptosomal fraction with increase in time and concentration of Pb^{2+} exposure (Fig. 1c). In this regard it may be noted that Na^+K^+ATPase enzyme, also being a –SH group containing enzyme gets affected due to Pb^{2+} exposure suggesting that –SH groups are essential for the enzyme activity. Maximum loss of Na^+K^+ATPase activity was observed at the 60 days duration at 20% of 96 h LC_{50}

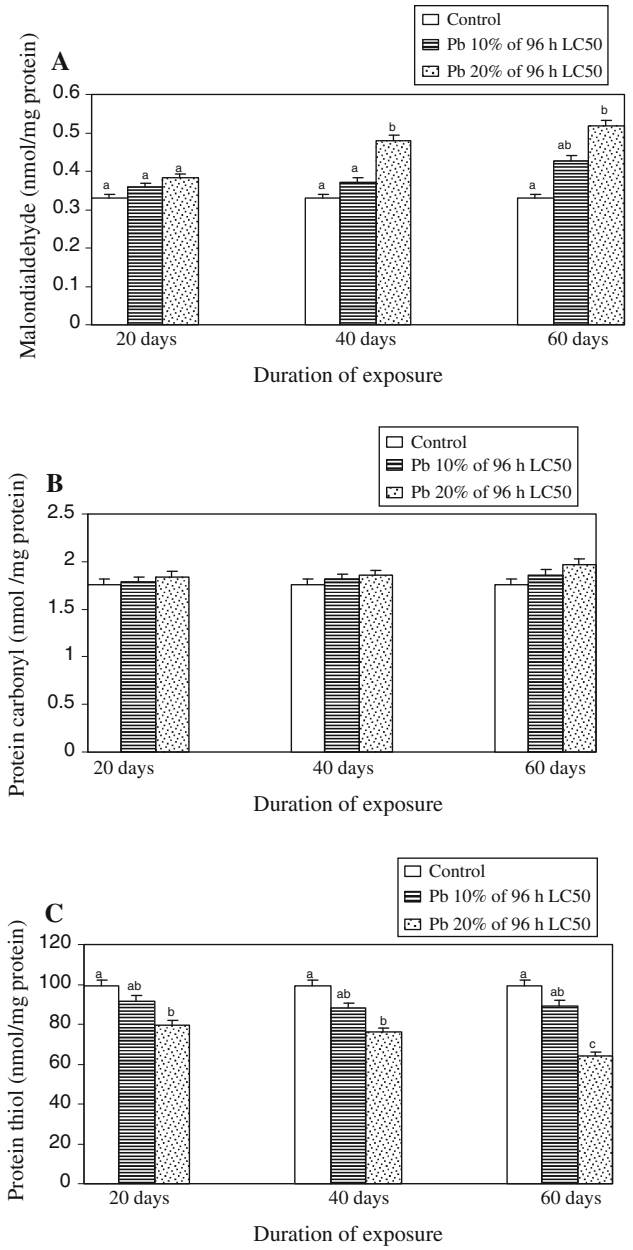

Fig. 1 Variation of malondialdehyde (**a**), protein carbonyl (**b**) and protein thiol (**c**) of fish synaptosomal fraction exposed to 0% (0.00 mg L^{-1}), 10% (37.8 mg L^{-1}) and 20% (75.6 mg L^{-1}) of 96 h LC_{50} of Pb^{2+} at different duration (20, 40, 60 days) of exposure. Data are mean ± SEM of eight observations. Results of DMR Test have been represented by small letters. Common letter between any two bars indicates their similarity while two different letters indicate significant difference at 5% level

of Pb^{2+} exposure ($p < 0.05$, Fig. 2). This observation supports earlier research reports which showed that lead was a potent inhibitor of Na^+K^+ATPase activity in various organ tissues (Rogers et al. 2003; Spokas et al. 2002). There is evidence that treatment of Na^+K^+ATPase with lipid peroxidation derived aldehyde products, namely 4-HNE, induces alterations in the conformation of the

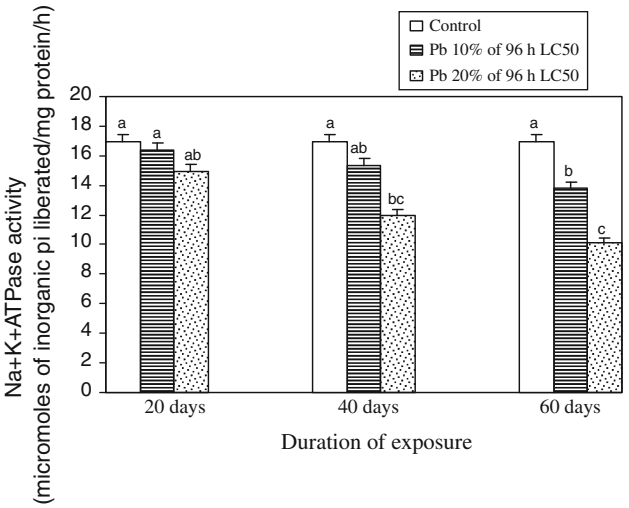

Fig. 2 Variation of Na^+K^+ATPase activity (μmoles of Pi liberated/mg protein/h) of fish synaptosomal fraction exposed to 0% (0.00 mg L^{-1}), 10% (37.8 mg L^{-1}) and 20% (75.6 mg L^{-1}) of 96 h LC_{50} of Pb^{2+} at different duration (20, 40, 60 days) of exposure. Data are mean ± SEM of eight observations. Results of DMR Test have been represented by small letters. Common letter between any two bars indicates their similarity while two different letters indicate significant difference at 5% level

enzyme molecule affecting its activity (Miyake et al. 2003; Zamai et al. 2002). Besides, the peroxidative damage inflicted due to lipid peroxidation causes structural and functional derangement of phospholipid bilayer of membranes wherein important enzymes embedded in the plasma membrane gets dysfunctional. Na^+K^+ATPase, a key transmembrane enzyme responsible for maintaining the ionic homeostasis of the cell, may get partially inactivated due to such peroxidative damage inflicted by Pb^{2+} exposure.

We have demonstrated that the activity of mitochondrial complex II (Succinate:ubiquinone oxidoreductase) – III (Ubiquinone:cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase) were significantly affected with increase in time and concentration of Pb^{2+} exposure (Fig. 3b, c) and it is suggested that the production of ROS in fish brain mitochondria may be enhanced due to inhibition of mitochondrial electron transport chain (Fig. 4b). The partial inactivation of mitochondrial electron transport chain may have direct impact on the level of ATP production in brain mitochondria. The present study has shown a decline in ATP production in mitochondria, the drop in energy production was profound with increase in Pb^{2+} concentration and time of exposure period (Fig. 4a). Na^+K^+ATPase, an energy dependent antiport protein gets inactivated due to diminution of ATP production due to a deficient mitochondrial electron transport chain activity.

In conclusion, it can be suggested that Pb^{2+} exposure induces toxicity in the piscine brain in the form of

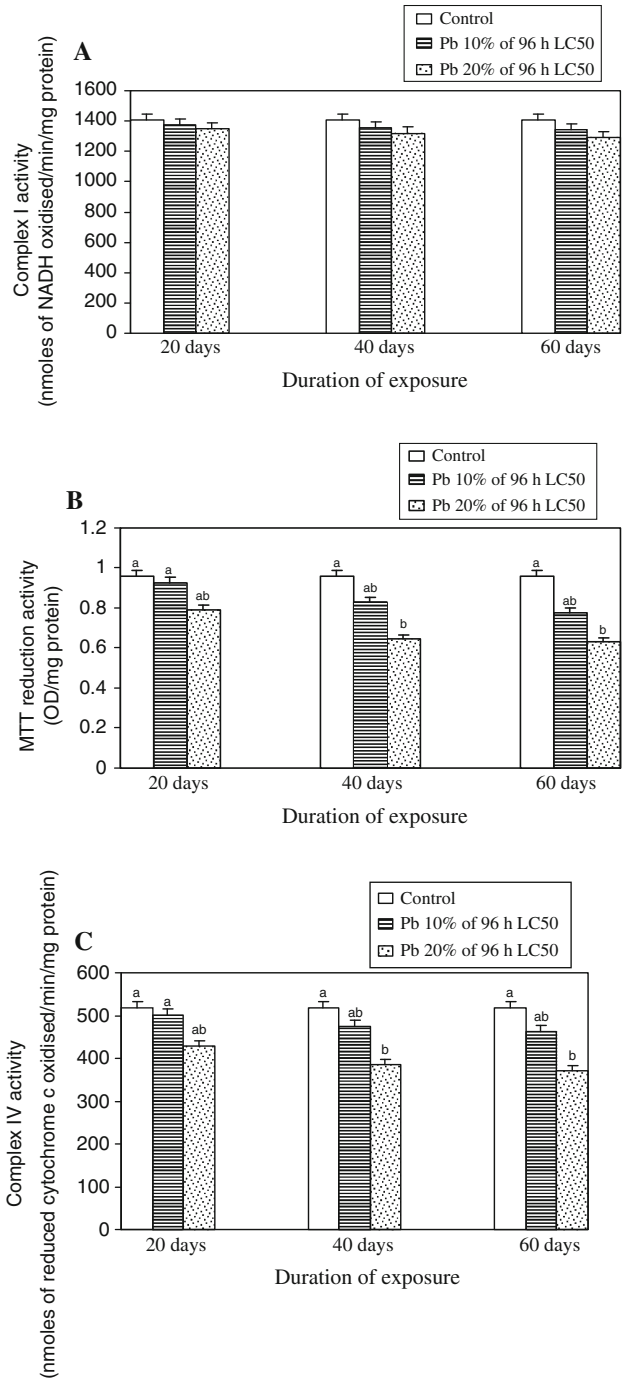

Fig. 3 Variation of complex I assay (**a**), MTT reduction assay (**b**) and complex IV assay (**c**) of fish brain mitochondrial fraction exposed to 0% (0.00 mg L^{-1}), 10% (37.8 mg L^{-1}) and 20% (75.6 mg L^{-1}) of 96 h LC_{50} of Pb^{2+} at different duration (20, 40, 60 days) of exposure. Data are mean ± SEM of eight observations. Results of DMR Test have been represented by small letters. Common letter between any two bars indicates their similarity while two different letters indicate significant difference at 5% level

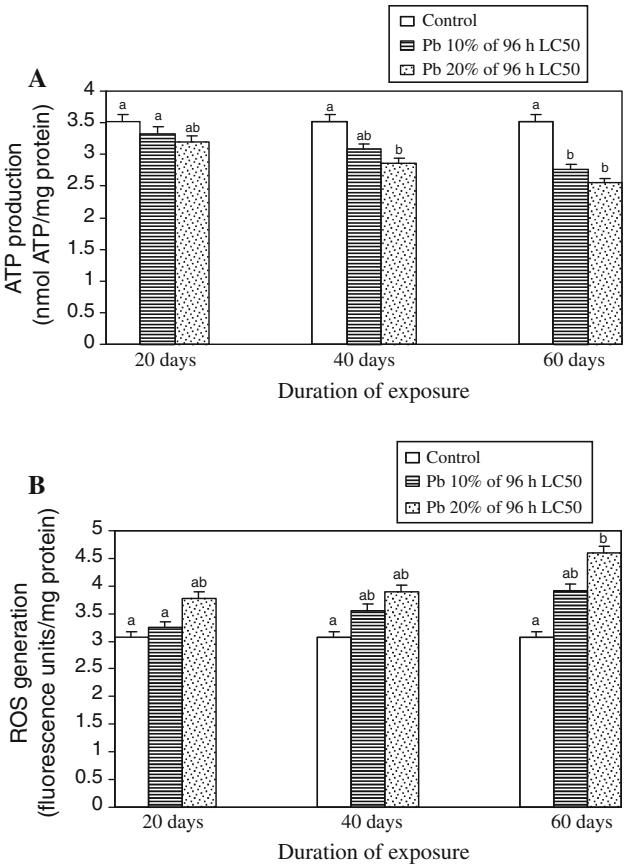

Fig. 4 Variation of Adenosine Triphosphate (ATP) production (**a**) and Reactive Oxygen Species (ROS) production (**b**) of fish brain mitochondrial fraction exposed to 0% (0.00 mg L^{-1}), 10% (37.8 mg L^{-1}) and 20% (75.6 mg L^{-1}) of 96 h LC_{50} of Pb^{2+} at different duration (20,40,60 days) of exposure. Data are mean ± SEM of eight observations. Results of DMR Test have been represented by small letters. Common letter between any two bars indicates their similarity while two different letters indicate significant difference at 5% level

oxidative stress induced inactivation of Na^+K^+ATPase with the involvement of deficient mitochondrial electron transport chain. The partial inactivation of Na^+K^+ATPase as observed in this study may be the end result of a number of biochemical mechanisms acting individually or cooperatively. Oxidative stress induced lipid peroxidation along with its aldehyde end products seem to have an inhibitory effect on Na^+K^+ATPase activity. This impounding effect on Na^+K^+ATPase is further aggravated by the inhibition of neural ATP production in mitochondrial electron transport chain of piscine neurone due to Pb^{2+} exposure. So it can be concluded that Pb^{2+} exerts toxicity in piscine brain by inhibiting partially the Na^+K^+ATPase activity possibly due to the inhibition of mitochondrial electron transport chain and production of Na^+K^+ATPase inhibitory peroxidative end products produced as a result of Pb^{2+} induced oxidative stress.

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