Protective effects of Calcium and Zinc on Lead toxicity

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Abstract
Alteration in the cholinergic system is one of the major pathological hallmarks of lead (Pb) toxicity. Pb-exposure alters acetylcholinesterase (AChE) activity in brain regions which control learning and cognitive behavior. The present study is focused on the beneficial effects of calcium (Ca2+) and zinc (Zn2+) supplementation on Pb-induced alterations in AChE activity in the brains of developing and adult mice. Mice were lactationally exposed to Pb (0.2% and 1%) and supplemented with either calcium (Ca2+) or zinc (Zn2+) and the synaptosomal AChE enzyme activity was analyzed in cortex, hippocampus, cerebellum and medulla of brains excised on postnatal day (PND) 14, 21, 28 and 3 months. The histochemical staining for AChE was also performed in the sections of mice brain. Exposure to Pb resulted in a significant decrease in the activity and staining intensity of AChE. Interestingly the supplementation with Ca2+ or Zn2+ reversed the Pb-induced effects on AChE activity and expression. These findings strongly support that calcium and zinc supplementation significantly protects the brain from the Pb-induced alterations in cholinergic system.

Keywords: lead, calcium, zinc, acetylcholinesterase, synaptosomes

Introduction
Inorganic lead (Pb) represents a long lived and persistent environmental hazard to the nervous system. It has been proposed that cholinergic neurotransmitter system may be involved in the pathophysiology of Pb poisoning (34, 35, 52). One principal constellation of Pb toxicity includes alterations in motor coordination and cortical function and as such, draws attention to Pb mediated impairments in cholinergic neurotransmission (30). Conflicting effects of Pb on acetylcholinesterase (AChE) have been reported by several authors. Tomlinson et al., (56) have shown the inhibitory effects of Pb on AChE activity in vitro, however, Modak et al., (35) found that AChE activity was unchanged in the cortex and cerebellum of rats exposed to Pb before and after weaning. A generalized reduction in brain cholinergic function has been reported in Pb-treated mice (8) and rats (1). The sensitivity of the brain regions to Pb was further supported by the in vitro studies conducted using different concentrations of Pb, which showed dose dependent inhibitory actions on AChE activity in a manner similar to the presence of eserine (47).

Several authors linked the reduction of AChE in the hippocampus region with short-term memory disturbance, which could result in incomplete understanding and consequently, abnormal communication skills and stated that exposure to Pb decreases the levels of acetylcholine causing less than one-fifth of normal concentration and a strong negative IQ (51). Schwartz et al., (50) suggested that cognitive function can progres-
sively decline as a result of past occupational exposures to a neurotoxicant. Exposure to Pb even at low concentrations; impairs both sensory and motor functions (32).

Since Pb is an element, its neurotoxic actions might be due to interactions with other essential elements. It has been suggested that Pb might compete with other elements, especially divalent and monovalent cations such as calcium (Ca\(^{2+}\)), magnesium (Mg\(^{2+}\)), sodium (Na\(^+\)) and iron (Fe\(^{2+}\)). Ca\(^{2+}\) - Pb interactions are also related to important clinical effects of Pb both in the cell and at molecular level. Pb blocks entry of Ca\(^{2+}\) into nerve terminals. Pb negatively affects normal Ca\(^{2+}\) balance in cells, which is essential for normal cell function. Zinc (Zn\(^{2+}\)) counteracts the inhibitory action of Pb on hematopoietic enzymes. Interactions between Zn\(^{2+}\) and Pb have been investigated at absorptive and enzymatic sites (18). Zn\(^{2+}\) and Pb compete for similar binding sites on the metallothionein like transport protein in the gastrointestinal tract (25). The competition between Zn\(^{2+}\) and Pb might decrease the absorption of Pb, thus reducing Pb toxicity.

In the present study, we investigated the extent of dose related Pb effects on AChE activity in different regions of mouse brain and the extent to which Ca\(^{2+}\) / Zn\(^{2+}\) supplementation has protection over Pb-induced cholinergic perturbations.

Materials and Methods

Materials: All chemicals used in the study were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animals: Pregnant Swiss albino mice were obtained from Indian Institute of Science, Bangalore. The animals were housed in clear plastic cages with hardwood bedding in the animal facility and maintained at 28 ± 2 °C and relative humidity 60 ± 10% with a 12 h light/day cycle. Standard mice chow (Sai Durga feeds and foods, Bangalore, India) and water were made available ad libitum. The protocol and animal use were approved by Institution Animal Ethical Committee, S.V. University.

Animal exposure: Mice were lactationally exposed to 0.2% and 1% Pb by adding Pb-acetate to deionized drinking water of the mother. Pb-exposure dose was obtained from the published work and our earlier studies (13, 42 - 45, 48, 60 ). Pb-exposure commenced on PND 1, continued up to PND 21 and stopped at weaning. Litters consisting of eight males were randomly selected and placed with each dam. Control mice received only deionized water.

Calcium and zinc supplementation: Calcium (Ca\(^{2+}\)) or Zinc (Zn\(^{2+}\)) was supplemented as 0.02% in 0.2% Pb-water and 0.1% in 1% Pb-water and is separately given to the mothers up to PND 21 and stopped at weaning. The supplemented doses and protocols were obtained from previously published works (5, 42 - 45).

Isolation of tissues: Control, Pb-treated and Ca\(^{2+}\)/Zn\(^{2+}\) supplemented mice were sacrificed by cervical decapitation at PND 14, PND 21, PND 28 and 3months. After decapitation, the brain was removed onto ice-cold glass plate and different brain regions (cortex, hippocampus, cerebellum and medulla) were quickly dissected, snap frozen on dry-ice and stored at -80 °C until use. The postnatal time periods were selected from published works and based on developmental differentiation and cellular maturation (5, 42 – 45, 54).

Isolation of synaptosomal fractions: Synaptosomal fractions were isolated from brain homogenates of cortex, hippocampus, cerebellum and medulla using Ficoll - sucrose gradients (12). The cerebral cortex, cerebellum, hippocampus and medulla were isolated in cold conditions. The
tissues were weighed and homogenized in 10 ml of ice-cold homogenizing buffer and the volume was brought up to 25 mL with homogenizing buffer. The homogenates were centrifuged at 750g for 10 minutes. The pellets were discarded. The supernatants were centrifuged at 17,000g for 20 minutes. The pellets were suspended in 10 ml of 0.32 M sucrose and were layered on a two step discontinuous Ficoll-sucrose gradient consisting 13% and 7.5% Ficoll solution and centrifuged at 65,000g for 45 minutes. The milky layer was formed at the interface of 13% and 7.5% Ficoll. The milky layer fraction was diluted with 9 volumes of 0.32 M sucrose and centrifuged again 17,000g for 30 minutes. The supernatant was discarded and the pellet (synaptosomal fraction) was suspended in 0.32 M sucrose and used for enzyme assay.

**Estimation of acetylcholinesterase activity (AChE) (EC 3.1.1.7):** AChE activity was estimated following the method of Ellman et al., (15). 2% (w/v) tissue homogenates were prepared in 0.25 M sucrose solution. The reaction mixture contained 3.0 ml of phosphate buffer (pH 8.0), 20 µl of 0.075 M acetylcholine iodide (substrate) and 100 µl of 0.01 M 5, 5-Dithiobis-2-Nitrobenzoic acid (DTNB). The reaction was initiated with the addition of 100 µl of crude homogenate/synaptosomal fraction. The contents were incubated for 30 minutes at room temperature and the color absorbance was measured at 412 nm in a UV/VIS spectrophotometer (Hitachi, Model U-2000) against a blank. The enzyme activity was expressed as µ moles of ACh hydrolyzed/mg protein/hr.

**AChE Histochemistry:** AChE histochemistry was performed as described by Hedreen et al., (22), modified method of Karnovsky and Roots (26). Mice of one month old were anesthetized with sodium pentoborbitol and perfused with 50 mM phosphate buffered saline (PBS, pH 7.4, 4°C) followed by 4% paraformaldehyde (4°C) through cardiac catheter. Brains were removed and postfixed in paraformaldehyde for 2 hours. This was followed by cryoprotection in 10, 20, and 30% sucrose gradients. Eight micron thick sections were cut in a freezing monotome (Cryostat, Bright Instrument Company Ltd, England). The sections were rinsed in 0.1 M phosphate buffer (pH 6.0) and incubated in a solution containing 32.5 ml of 0.1 M phosphate buffer (pH 6.0); 2.0 ml of 0.1 M sodium citrate; 5 ml of 0.03 M cupric sulphate; 1.0 ml of 0.0005 M potassium ferricyanide; 25 mg of acetylthiocholine iodide and 9.5 ml of distilled water. Sections were also incubated with the AChE inhibitor, eserine (10^-4 M), in the above media to study the inhibitory effect and non-specific staining. The sections were incubated for 20 minutes at room temperature and then dehydrated in ethanol series, cleared in xylene and mounted in permount.

**Estimation of protein content:** Protein content of the tissues was estimated by the method of Lowry et al., (29).

**Statistical analysis of the data:** The data obtained from six separate samples were expressed as mean ± SD. Significance between various age groups and treatments was analyzed by two-way analysis of variance (ANOVA). Significance of each age group among different treatments was analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls Multiple comparison post hoc test using Standard Statistical Software Package to compare the effects among various groups. The 0.05 level of probability was used as the criterion for significance.

**Results**

The specific activity of AChE was determined in the synaptosomal fractions of cerebral cortex, hippocampus, cerebellum and medulla.
regions of control, Pb-exposed (low and high doses) and Ca\(^{2+}\)/Zn\(^{2+}\)supplemented mice at different postnatal periods (PND 14, 21, 28) and at 3 month age.

The results of the present study showed that the specific activity of AChE was significantly higher in the synaptosomal fraction of hippocampus followed by cerebral cortex, cerebellum and medulla. The activity levels of AChE were significantly increased from PND 14 to 3 months in an age dependent manner. In the synaptosomal fractions of various regions of control brain, the specific activity was found to be 11.74, 13.96, 15.82 and 18.38 µ moles of ACh hydrolyzed/mg protein/hr in cortex (Fig 1a); 13.2, 15.12, 17.16 and 19.47 in hippocampus (Fig 1b); 10.46, 12.28, 14.82 and 17.52 in cerebellum region (Fig 1c) and 9.72, 11.61, 13.62 and 15.2 in medulla region (Fig 1d) at PND 14, PND 21, PND 28 and 3 month respectively.

The 0.2% Pb-exposure resulted in a decrease of 59.59% in hippocampus, followed by cerebellum (56.27%), medulla (53.93%) and cerebral cortex (48.50%) in the synaptosomal AChE. The inhibitory effect of high dose (1%) Pb was more in cerebral cortex (70.20%) followed by hippocampus (68.70%), cerebellum (66.40%) and medulla (64.70%) (Fig 1).

The supplementation with Ca\(^{2+}\)/Zn\(^{2+}\) reversed the inhibitory effect caused by Pb-exposure on AChE activity in synaptosomal fractions. A maximum recovery of 57% in cortex, 110% in hippocampus, 72% in cerebellum and 73% in medulla from 0.2% Pb-induced inhibition on AChE activity was observed when 0.02% Ca\(^{2+}\) was supplemented with 0.2% Pb where as a recovery of 40% in cortex, 100% in hippocampus, 68% in cerebellum and 51% in medulla was observed with 0.02% Zn\(^{2+}\) supplementation to 0.2% Pb. A recovery of 124%, 98%, 87%, 106% in cortex, hippocampus, cerebellum and medulla respectively was observed with 0.1% Ca\(^{2+}\) supplementation to 1% Pb and a recovery of 111%, 70%, 72% and 54% was observed in cortex, hippocampus, cerebellum and medulla respectively from 1% Pb-induced inhibition on AChE activity when 0.1% Zn\(^{2+}\) was supplemented (Fig 1).
Fig. 1. Effect of Pb-exposure and calcium/zinc supplementation to Pb on acetylcholinesterase (AChE) activity in cerebral cortex (a), hippocampus (b), cerebellum (c) and medulla (d) regions of mouse brain. Male mice were exposed to either deionized water (control) or Pb-acetate (0.2% or 1%) or calcium and zinc together with Pb (0.02% in 0.2% Pb and 0.1% in 1% Pb in deionized water) from PND 1 through PND 21. AChE activity was determined in brain regions in PND 14, PND 21, PND 28 and 3 months old control, Pb-exposed, calcium/zinc supplemented mice. The enzyme activity was expressed as µ moles of ACh hydrolyzed/mg protein/hr. Values are mean ± SD of six separate experiments. Significance of each age group among different treatments was analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls Multiple comparison post hoc test. The 0.05 level of probability was used as the criterion for significance. *p < 0.05, **p < 0.01, ***p < 0.001 compared to controls, #p < 0.05, ##p < 0.01, ###p < 0.001 compared to 0.2% Pb treatment and $p < 0.05, $$p < 0.01, $$$p < 0.001 compared to 1% Pb treatment.

Both Ca²⁺/Zn²⁺ supplementation was found to be protective in reducing the Pb-burden. The supplementation of Ca²⁺ or Zn²⁺ to low dose Pb-exposure appears to be more effective than to high dose Pb. As compared to Zn²⁺, the Ca²⁺ supplementation was found to be more protective (Fig 1).

Histochemical staining of AChE revealed regional alterations in the brain of Pb-exposed mouse in a dose dependent manner. However, the alterations in AChE staining with 0.2% Pb-exposure were not significantly visible as compared to high level Pb-exposure. The AChE histochemical staining appeared intensely in cortical cell layers (Fig 2 (I)), dentate gyrus and CA3 of hippocampus (Fig 2 (II)), and molecular and granular cell layers of cerebellum (Fig 2 (III)) of control mouse (A). Pb-exposure (B) decreased the staining markedly in CA3 / CA2 areas and dentate gyrus of hippocampus (Fig 2 (II)). Pb also produced decrease in staining in molecular and granular cell layers of cerebellum and cortical layers (Fig 2). The supplementation with Ca²⁺/Zn²⁺ (C and D) increased AChE decreasing the Pb-burden on AChE staining (Fig 2).
Discussion

The results of the present study clearly demonstrate a significant inhibition in AChE activity by Pb in a dose and age dependent manner and recovery from the Pb-induced alterations in AChE activity and expression with the supplementation of Ca\(^{2+}\) or Zn\(^{2+}\) in mouse brain. AChE is an enzyme that terminates the signal transmis-

Fig. 2. Alterations in histochemical staining for AChE in Pb-exposed and calcium/zinc supplemented cerebral cortex (I), hippocampus (II) and cerebellum (III) of control (A), 1% Pb-exposed (B), 1% Pb+ 0.1% Ca\(^{2+}\) (C) and 1% Pb+ 0.1% Zn\(^{2+}\) (D) of one month old mouse brain. Male mice were exposed to either deionized water (control) or Pb-acetate (1%) or calcium and zinc together with Pb (0.1% in 1% Pb in deionized water) from PND 1 through PND 21. AChE staining was performed in control, Pb-exposed, calcium/zinc supplemented mice brain sections of eight micron thickness cut in a cryostat. Areas marked with (→) show the changes in the intensity of AChE staining. CCL: cortical cell layers; DG: dentate gyrus; M: molecular layer; G: granular layer. Magnification – (cortex: 200X), (Hippocampus: 300X), (cerebellum: 600X).
activity can be observed in the central nervous system (2, 24, 37). In mouse brain, total AChE activity increases about 15 fold between embryonic day (ED) 9 to ED 19. This developmental change is characterized by a continuous increase in the amphiphilic tetrameric (G4). AChE form a transient increment in the amphiphilic monomer (G1) form which reaches a maximum by ED 17, and a gradual increase in both hydrophilic G1 and G4 forms through to ED 19 (23). The active site of AChE comprises two subsites critical for its functioning. One of these has traditionally been termed the anionic subsite because it was hypothesized to consist of one or more negatively charged groups that electrostatically interacted with the positively charged quaternary nitrogen (N) of the substrate. The other region of the active site is the so called esteratic subsite, which possesses a serine residue that is responsible for driving the hydrolytic reaction (17, 33, 55).

The results of the present study showed inhibitory action of Pb on AChE activity in all the four brain regions; cortex, hippocampus, cerebellum and medulla. It is noteworthy that the alterations in AChE activity were continued even after the Pb-exposure was with drawn. Heavy metal ions exert a potent inhibitory effect on different enzymes such as AChE by binding avidly to sulfhydryl (–SH) groups with similar affinities (7, 10). Pb has high affinity for the free sulfhydryl groups in enzymes and proteins and its binding can alter their correct function. The effect of Pb is observed to be more in the fetal brain due to the immature BBB (blood brain barrier) and the absence of protein complexes that sequester metals in mature tissues (4). The decrease in AChE activity in young mice could be related to the fact that Pb crosses BBB quite readily (27). This may be the reason for higher inhibition of AChE in the brain tissue of young mice as compared to three month old mice. Highest inhibition on PND 28 may be due to longer Pb-exposure time. The inhibition of AChE could also be due to the damage of the brain cells. Pb-toxicity may have induced the above conditions in mouse brain tissues and hence a decrease in AChE activity was observed. Pb disturbs the normal development of the brain, causing reductions in cellular development in the cerebellum (46), cerebral cortex (41) and hippocampus (11).

A review of previous reports suggests the possibility of functional association between the effects of early Pb-exposure, hippocampal damage and cholinergic deficiency (1). Pb-exposure produces a direct effect on AChE activity in the developing cerebellum leading to alterations in motor coordination (20). The susceptibilities of different brain areas to Pb-exposure could be related to local differences in their formation and maturation. The cholinergic synapses were more in hippocampus, followed by cortex, cerebellum and medulla. This may be the reason for higher AChE activity observed in hippocampal region as compared to cortex, cerebellum and medulla. It is known that the differential sensitivity to Pb neurotoxicity in the different brain regions is not due to a preferential Pb accumulation, but is possibly due to alterations in the biochemical or cellular processes that are uniquely associated with, or greatly enhanced in a particular region (36, 59).

The inhibition of AChE following Pb-exposure appeared to comprise a correlation between increases in the lipid peroxidation observed in our study (42). An inverse correlation between AChE and lipid peroxidation in brain has been reported by Sandhir et al., (49). Pb may also exert its neurotoxic effects via peroxidative damage to the membranes.

AChE activity inhibition was observed following chronic exposure of Pb to the young mice through their mother’s milk. The sensitivity of
immature brain to the neurotoxic effect of Pb and the increased resistance in the adult brain may be due to its defense mechanisms developing with age (62). The gradual recovery of AChE activity in three month mice can be attributed either to spontaneous reactivation of the enzyme or the denovo synthesis of the enzyme molecule or both (14). It is also dependent on the degree of exposure and its initial depression (39). The high dose of Pb-exposure resulted in higher inhibition of AChE activity. This may be due to more binding of Pb to -SH groups in addition to more accumulation of Pb in various regions of brain tissue.

The dentate gyrus and CA1 sub regions of the hippocampus have distinct developmental profiles, CA1 developing primarily prenatally in rodents, the dentate gyrus maturing much later in the postnatal period (6). Pb impaired LTP in area CA1 and dentate gyrus coincided with impaired spatial learning in the water maze (45). Van der Zee et al., (57, 58) established a learning related increase in immunoreactivity to the gamma isoform of protein kinase C (PKC) in hippocampal soma and dendrites. They found learning-related increase in immunoreactivity for muscarinic acetylcholine receptors in hippocampal pyramidal cells of area CA1-CA2 and a learning related decrease in the immunoreactivity of the non pyramidal neurons of the same region. The decrease of AChE activity with Pb-exposure can be correlated with the expression of AChE with histochemical staining in this study. The intense staining appeared in control brain and significantly loss of staining was noticed in Pb-exposed brain especially in CA3 and DG region of hippocampus, cortical cell layers, and molecular granular cell layers of cerebellum.

Supplementation with Ca2+/ Zn2+ reversed the Pb-induced inhibition in AChE activity and also the loss of AChE staining in different brain regions. However, the Ca2+ supplemented brain regions did not show significant loss of AChE staining confirming the protective effect of Ca2+. The reversal of inhibition in the activity of AChE by supplementation with Ca2+/ Zn2+ may be due to competition of these metals for similar binding sites and reducing the availability of binding sites for Pb. Supplementation with Ca2+/ Zn2+ reduces the gastrointestinal absorption of Pb and decreases Pb retention.

Ca2+ is a divalent cation just like Pb. Because the same transport mechanism is operative for absorption of Pb and Ca2+ from the gastrointestinal tract there is resulting competitive interaction between Pb and Ca2+ (38). Studies have shown that Pb has an inhibitory effect on the peripheral nervous system through stimulus coupled or Ca2+ dependent release of acetylcholine. Absorption of Pb by gastrointestinal tract is inversely related to the amount of Ca2+ present (21, 31). Furthermore Ca2+ supplements had a protective effect by significantly reducing blood Pb levels in pregnant women whose diets were deficient in Ca2+ (16). Ziegler et al., (61) observed an inverse relationship between dietary Ca2+ and Pb retention and absorption in young infants. Ca2+ and Pb compete for similar binding sites on intestinal mucosal proteins, which are important in the absorptive process (19). These shared binding sites on absorptive proteins would explain why sufficient Ca2+ decreases Pb absorption. The achievement of adequate rather than excessive dietary Ca2+ seems to be more useful in combating Pb intoxication (40).

Zn2+ counteracts the inhibitory action of Pb in animals (28). Zn2+ deficiency reduces the activity of numerous enzymes in the brain that may lead to a stop in cell multiplication at a crucial period in morphogenesis (3, 53). Potential mechanisms of interaction between Pb and Zn2+ include an inhibition of Pb gastrointestinal absorption by Zn2+, which has been demonstrated.
in rats (9). Indirect evidence includes the observation that supplemental Zn\(^{2+}\) decreased the concentrations of Pb in blood and in a variety of tissues, in Pb-exposed mice. Thus supplementation with Ca\(^{2+}\) or Zn\(^{2+}\) reduced the inhibitory effect of Pb on the activity of AChE enzyme and reversed the enzyme activity nearer to control level.

The changes produced in the levels of AChE during Pb treatment indicate the degree of neuronal impairment which in turn produces functional and structural damage to the tissue depending on the dose. The supplementation with Ca\(^{2+}/\)Zn\(^{2+}\) reduced the Pb-induced alterations in AChE activity, with Ca\(^{2+}\) supplementation being more protective as compared to Zn\(^{2+}\).

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**References**


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