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Zinc and calcium reduce lead induced perturbations in the aminergic system of developing brain

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Abstract

Since alterations in monoamines and monoamine oxidase (MAO) have been postulated to play a role in toxic effects of lead (Pb) on the central nervous system, we have examined the protective effects of calcium (Ca^{2+}) and zinc (Zn^{2+}) supplementation on Pb-induced perturbations in the levels of monoamines and the activity of MAO. Swiss albino mice were lactationally exposed to low (0.2%) and high (1%) levels of Pb-acetate via drinking water of the mother. Pb-exposure commenced on postnatal day (PND) 1, continued up to PND 21 and stopped at weaning. Ca^{2+} or Zn^{2+} (0.02% in 0.2% Pb–water or 0.1% in 1% Pb–water) was supplemented separately to the mother up to PND 21. The levels of monoamines (epinephrine, norepinephrine, dopamine and serotonin) and the activity of MAO in the brain regions such as hippocampus, cortex, cerebellum and medulla of young (1 month old) and adult (3 month old) mice were determined in the synaptosomal fractions. The synaptosomal monoamines though increased with low level (0.2%) Pb-exposure, significantly decreased with high level (1%) Pb-exposure in all the brain regions in both the age groups. In general, the young mice seem to be more vulnerable to Pb-neurotoxicity. Ca^{2+} or Zn^{2+} supplementation significantly reversed the Pb-induced perturbations both in the levels of monoamines and in the activity of MAO. However, the recovery in monoamine levels and MAO activity was more pronounced with Ca^{2+} supplementation as compared to Zn^{2+}. These results provide evidence that dietary Ca^{2+} and/or Zn^{2+} provide protection against Pb-induced neurotoxic effects.

Introduction

Lead (Pb), an environmental toxicant for centuries is a highly neurotoxic agent that causes functional and structural abnormalities in the brain (Struzynska *et al*. 2002). Developing nervous system has long been recognized as a primary target site for Pb-induced toxicity (Needleman *et al*. 1979; Wilsonet *et al*. 2000).

Since monoamines are thought to regulate motor activity in rodents (Dubas & Hrdina 1978; Govoni *et al*. 1980; Silbergeld 1985), alterations in the functioning of monoamines have been postulated to play a role in toxic effects of Pb on the central nervous system (Sauerhoff & Michaelson 1973; Silbergeld & Goldberg 1975). Pb-induced brain dysfunction has been proposed to involve over activation of biogenic amine systems mainly at high Pb levels (Sauerhoff & Michaelson 1973). Pb has been shown to alter a number of neurotransmitter systems including dopamine, norepinephrine, epinephrine, serotonin and γ-aminobutyric acid systems (ATSDR 1999). Pb-induced effects on the dopaminergic system include changes in the synthesis, turnover and reuptake of dopamine, changes in the levels of dopamine and its metabolites, as well as changes in the number of dopamine receptors (Cory-Slechta 1995). Inspite of several

publications addressing the action of Pb on neurotransmitter systems and the associated enzymes such as acetylcholinesterase (AChE), MAO and nitric oxide synthase (NOS) (Govoni *et al*. 1979; Reddy *et al*. 2002, 2003), it is often difficult to compare these studies because reports are highly variable and many observations lack confirmation by other laboratories.

Nutritional factors are thought to play an important role in Pb-poisoning. Pb toxicity can be reduced by supplementation of certain essential metals. One of such metal that can affect the absorption of Pb is calcium (Ca^{2+}). Pb is known to exert its neurotoxic effects by competing with Ca^{2+} for calcium receptors coupled with second messenger functions (Hammond *et al*. 1984; Sturges & Harrison 1985; Bressler & Goldstein 1991), and in some cases to inhibit the actions of Ca^{2+} as a regulator of cell function (Habermann *et al*. 1983; Bressler & Goldstein 1991). Another metal that can alter Pb-induced toxicity is Zinc (Zn^{2+}). As dietary Zn^{2+} increases, Pb absorption and its subsequent toxicity decreases, indicating that Zn^{2+} exerts its effect on Pb in the gastrointestinal tract.

Therefore, the present study was designed to determine the effect of lactational Pb-exposure on the brain aminergic system and to examine the protective effects of supplementation of Ca^{2+} and Zn^{2+} in Pb-treated mice.

Material and methods

Maintenance of the animals

Swiss albino mice were obtained from Indian Institute of Science, Bangalore. Mice were allowed to acclimatize for atleast 1 week before experiments started and were housed in clear plastic cages with hardwood bedding in a room maintained at 28 ± 2 °C and relative humidity $60 \pm 10\%$ with a 12 h light/day cycle. Standard mice chow (Sai Durga feeds and foods, Bangalore, India) and water were available *ad libitum*.

Chemicals

The chemicals used in this study were purchased from Sigma, USA and Qualigens, India.

Animal exposure

Swiss albino mice were lactationally exposed to 0.2% and 1% Pb by adding Pb-acetate to deionized drinking water of the mother. All pups, 24 h after birth (PND1) were pooled and new litters consisting of eight males were randomly selected and placed with each dam. Pb-exposure was continued up to PND21 and stopped at weaning. Control animals, received only deionized water without Pb.

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.

Methods

Isolation of synaptosomal fractions

Synaptosomes were isolated from brain homogenates using Ficoll-sucrose gradients (Cotman & Matthews 1971). 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 750 *g* for 10 min. The pellets were discarded. The supernatants were centrifuged at 17,000 *g* for 20 min. The pellets were suspended in 10 ml 0.32 M sucrose and were layered on a two step discontinuous Ficoll-sucrose gradient consisting 13% and 7.5% Ficoll and centrifuged at 65,000 *g* for 45 min. 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 at 17,000 *g* for 30 min. The supernatant was discarded and the pellet (synaptosomal fraction) was suspended in 0.32 M sucrose.

Estimation of catecholamines

The levels of monoamines were determined according to the method of Kari *et al*. (1978). Synaptosomal fractions were taken in acid butanol to give a final concentration of 50 mg/ml and centrifuged at 800 *g* (−4 °C) for 10 min. Residues were

discarded and to the supernatant 2.5 ml of distilled water and 2.5 ml of heptane were added. The contents were thoroughly mixed and centrifuged at 1000 *g* for 5 min. To the aqueous phase, 200 mg of acid alumina was added followed by 1.5 ml 2 M sodium acetate. The contents were mixed thoroughly for 5 min and the pH was adjusted to 8.0 with 1 N sodium hydroxide. Then the samples were centrifuged at 1000 *g* for 5 min. The supernatant was collected and used for the estimation of serotonin whereas the catecholamines were extracted from the alumina as described below.

The acid alumina was washed by vortexing the tubes twice with 2.0 ml of distilled water and then centrifuged at 1000 *g* for 5 min. The supernatant was discarded and the walls of the tubes were blotted with strips of filter paper. The alumina was then vortexed for 5 min with 2.0 ml of 0.2 N acetic acid to elute the catecholamines. The tubes were centrifuged at 1000 *g* for 5 min. The supernatants were transferred to 0.1 ml of 0.1 M EDTA and the pH was adjusted to 6.3. This was followed by the addition of 0.1 ml of 0.1 N iodine solution. The tubes were mixed thoroughly and allowed to stand for 2 min. Then 0.2 ml of alkaline sulphite solution was added. The contents were mixed and allowed to stand for 2 min at room temperature. Then the pH of the solution was adjusted to 5.4 with 5 N acetic acid. The samples with known amount of different amine standards were separately run to serve as internal standards. The fluorescence of epinephrine was read in a Perkin-Elmer LS55 Luminescence Spectrophotometer with excitation and emission wavelengths of 410 and 500 nm respectively with a bandwidth of 10/10 nm.

Norepinephrine was estimated by heating the same solution for 2 min in a boiling water bath. The tubes were cooled and the fluorescence of norepinephrine was read with excitation and emission wavelengths of 385 and 485 nm respectively with a slit width of 10/10 nm. After the estimation of norepinephrine, the same solution was again heated for 5 min in a boiling water bath. Then the tubes were cooled and the fluorescence of dopamine was read with excitation and emission wavelengths of 320 and 370 nm respectively with a slit width of 10/10 nm.

The amine content of each tissue sample was calculated by the method of Ansell & Beeson (1968) and expressed as μg amine/g wet weight of tissue.

Estimation of serotonin

After trapping the catecholamines from the tissue samples into the alumina, 1.5 ml of supernatant (as described earlier under the estimation of catecholamines) was taken, and to this 0.1 ml of cysteine, 1.5 ml of hydrochloric acid and 0.1 ml of *O*-pthaladehyde (OPA) solution were added. The tubes were kept at room temperature for 20 min. Then 0.1 ml sodium metaperiodate was added to each tube and the tubes were heated at 80 °C in a boiling water bath for 20 min. The samples were cooled and fluorescence of serotonin was read in a Perkin-Elmer LS55 Luminescence Spectrophotometer with excitation and emission wavelengths of 360 and 470 nm respectively with a bandwidth of 20/10 nm.

The amount of serotonin was calculated by the method of Ansell & Beeson (1968) and expressed as μg amine/g wet weight of tissue.

Spectrophotometric assay of monoamine oxidase (MAO)

The activity of MAO was estimated by the method of Green & Haughton (1961). The assay mixture containing of 1.0 ml of semicarbazide hydrochloride (0.05 M, pH 7.4), 1.6 ml of phosphate buffer (0.2 M, pH 7.4) and 0.4 ml of synaptosomal fraction was incubated for 20 min at 37 °C in a water bath with a shaking device. The reaction was started by adding 0.4 ml of tyramine hydrochloride (0.1 M, pH 7.4). After 30 min of incubation, the reaction was stopped by adding 1.0 ml of 0.5 N acetic acid and kept in boiling water bath for 30 min. The contents were centrifuged for 10 min at 1000 *g*. To 2.0 ml of supernatant, 2 ml of 2,4-dinitriphenylhydrazine (0.5 mg/ml in 2 N HCl) was added. After keeping at room temperature for 15 min, 5 ml of benzene was added. The tubes were vortexed and the aqueous layer was discarded. The benzene layer was washed with 4 ml of distilled water followed by the addition of 4 ml of 0.1 N NaOH solution and the contents of the tubes were mixed thoroughly. The benzene layer was discarded and the NaOH layer was allowed to stand at room temperature for 1 h. The absorbance of the samples was measured at 425 nm in a UV/VIS spectrophotometer (Hitachi, Model U-2000). The activity of MAO was calculated using the molar extinction coefficient of 9500 and

expressed as μ moles of p-hydroxy phenyl acetaldehyde formed/gram wet weight of tissue/hour.

Data analysis

Standard statistical procedures such as student *t*-test and ANOVA were used to analyze the data for the significance level.

Results

Monoamine oxidase

The specific activity of MAO was determined in the synaptosomal fraction of cerebral cortex, hippocampus, cerebellum and medulla of control, Pb-exposed and Ca^{2+} and Zn^{2+} supplemented mice of 1 and 3 months age. As shown in Figures 1a and b, the MAO activity increased from 1 to 3 months age. The activity levels of MAO were significantly higher in the medulla than in cerebral cortex, hippocampus and cerebellum in both age groups (Figures 1a and b). Exposure to Pbresulted in a significant decrease in the MAO activity in all the four brain regions of both young and adult mice. The decrease in MAO activity was more pronounced in young mice as compared to 3-months-old mice. High level (1%) Pb-exposure produced greater decrease in MAO activity (61.01% in cerebral cortex, 72.03% in hippocampus, 77.02% in cerebellum and 69.89% in medulla of young mice; 55.0% in cerebral cortex, 62.01% in hippocampus, 63.53% in cerebellum and 60.68% in medulla of adult mice) as compared to low level (0.2%) Pb-exposure. The supplementation with Ca^{2+} or Zn^{2+} greatly reduced the alterations in MAO induced by Pb-exposure. However, the recovery was more with Ca^{2+} supplementation as compared to Zn^{2+} supplementation (Figures 1a and b).

Monoamines

The levels of monoamines (norepinephrine, epinephrine, dopamine and serotonin) showed a marginal increase with age from 1- to 3-month-old control mice (Figures 2–5). All the monoamines examined showed a marginal increase in level in the brain regions exposed to low level (0.2%) Pb. The increase in dopamine was 37.40% in cerebral cortex, 44.91% in hippocampus, 41.88% in cerebellum and 40.53% in medulla; the increase in norepinephrine was 30.63% in cerebral cortex, 41.94% in hippocampus, 46.72% in cerebellum and 34.96% in medulla; the increase in epinephrine was 29.34% in cerebral cortex, 41.86% in hippocampus, 39.78% in cerebellum and 34.92% in medulla and the increase in serotonin was 32.35% in cerebral cortex, 51.89% in hippocampus, 40.95% in cerebellum and 41.98% in medulla (Figures 2–5). The monoamine levels showed a significant decrease in mice exposed to high level (1%) Pb. The decrease in dopamine was 56.17% in cerebral cortex, 62.43% in hippocampus, 48.10% in cerebellum and 42.98% in medulla; the decrease in norepinephrine was 35.38% in cerebral cortex, 42.39% in hippocampus, 39.95% in cerebellum and 32.90% in medulla; the decrease in epinephrine was 34.34% in cerebral cortex, 44.07% in hippocampus, 36.97% in cerebellum and 38.77% in medulla and the decrease in serotonin was 39.60% in cerebral cortex, 54.56% in hippocampus, 45.39% in cerebellum and 48.09% in medulla (Figures 2–5). Among the four brain regions, cerebral cortex documented higher levels of dopamine; medulla documented higher levels of norepinephrine, epinephrine and serotonin whereas cerebellum showed significantly lower levels of all monoamines. All the four brain regions showed significantly higher levels of dopamine followed by norepinephrine, serotonin and epinephrine. Interestingly, low level (0.2%) Pb-exposure increased the levels of monoamines, whereas high level (1%) Pb-exposure significantly decreased monoamine levels in all the brain regions in both age groups of mice (Figures 2–5) suggesting age and dose dependent changes in monoaminergic system. Addition of either Ca^{2+} or Zn^{2+} to Pb reduced the effects of Pb on aminergic system as seen from the marginal and non-significant alterations in monoamine levels especially with Ca^{2+} (Figures 2–5).

Discussion

The results of the present study showed that Pb-exposure perturbs the mouse brain synaptosomal aminergic system with a decrease in synaptosomal MAO activity and alterations in monoamine levels in a dose and age dependent

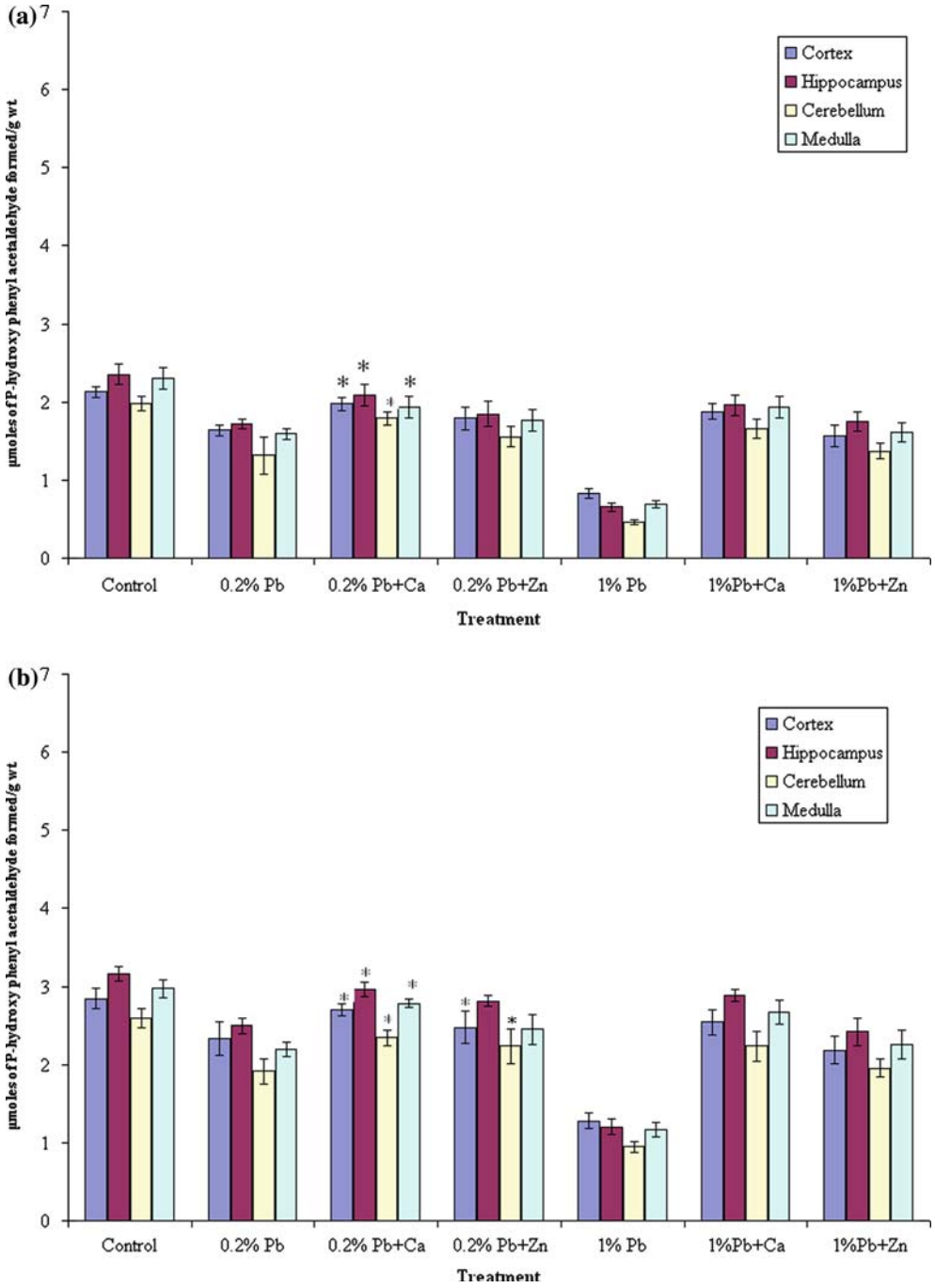

Figure 1. Effect of Pb-exposure and calcium/zinc supplementation to Pb on MAO activity in cerebral cortex, hippocampus, cerebellum and medulla. Male mice were exposed to either deionized water (control) or Pb-acetate (0.2% and 1%) or calcium/zinc together with Pb in deionized water from PND1 through PND21. MAO activity was determined in the synaptosomal fractions of brain regions in 1 month (a) and 3 months (b) old control, Pb-exposed, calcium/zinc supplemented mice. The enzyme activity was expressed as μ moles of p-hydroxy phenyl acetaldehyde formed per gram weight per hour. Values are mean $\pm$ SD of six separate experiments. All the values are significant at 1% level except the values marked with (*) as evaluated by two way ANOVA.

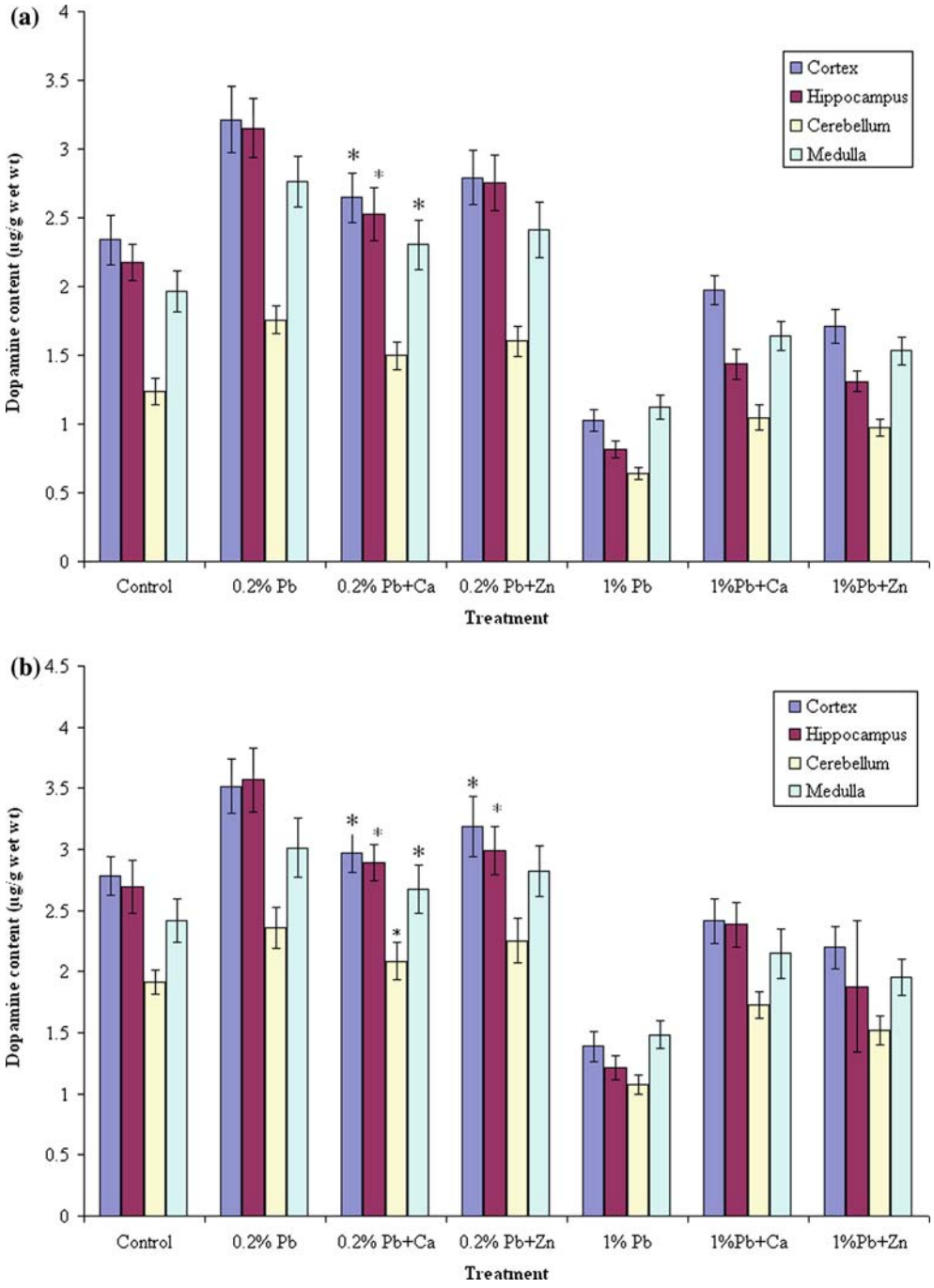

Figure 2. Effect of Pb-exposure and calcium/zinc supplementation to Pb on dopamine levels in cerebral cortex, hippocampus, cerebellum and medulla. Male mice were exposed to either deionized water (control) or Pb-acetate (0.2% and 1%) or calcium/zinc together with Pb in deionized water from PND1 through PND21. Dopamine levels were determined in the synaptosomal fractions of brain regions in 1 month (a) and 3 months (b) old control, Pb-exposed and calcium/zinc supplemented mice and expressed as microgram per gram wet weight. Values are mean ± SD of six separate experiments. All the values are significant at 1% level except the values marked with (*) as evaluated by two way ANOVA.

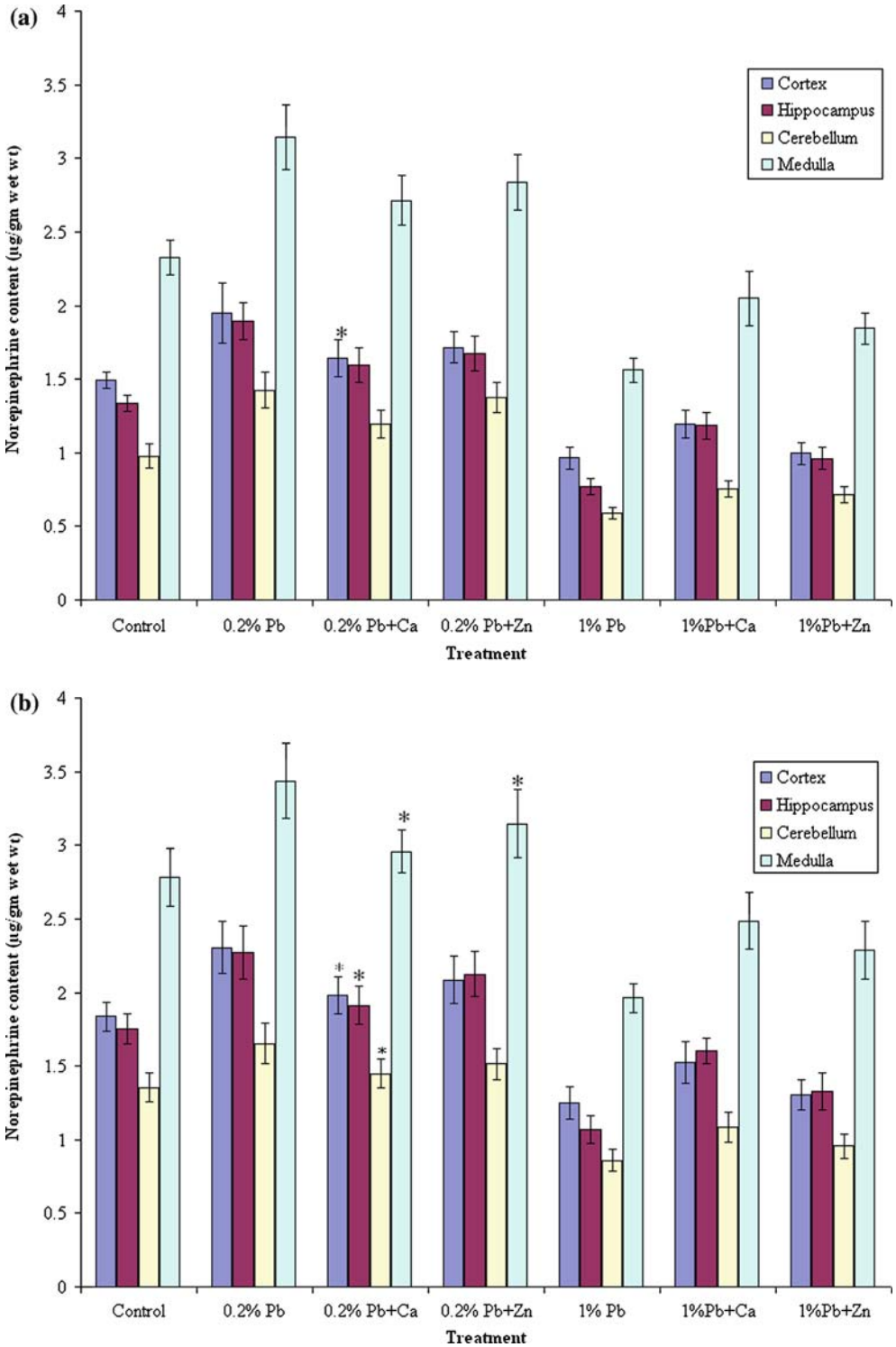

Figure 3. Effect of Pb-exposure and calcium/zinc supplementation to Pb on norepinephrine levels in cerebral cortex, hippocampus, cerebellum and medulla. Male mice were exposed to either deionized water (control) or Pb-acetate (0.2% and 1%) or calcium/zinc together with Pb in deionized water from PND1 through PND21. Norepinephrine levels were determined in the synaptosomal fractions of brain regions in 1 month (a) and 3 months (b) old control, Pb-exposed and calcium/zinc supplemented mice and expressed as microgram per gram wet weight. Values are mean ± SD of six separate experiments. All the values are significant at 1% level except the values marked with (*) as evaluated by two way ANOVA.

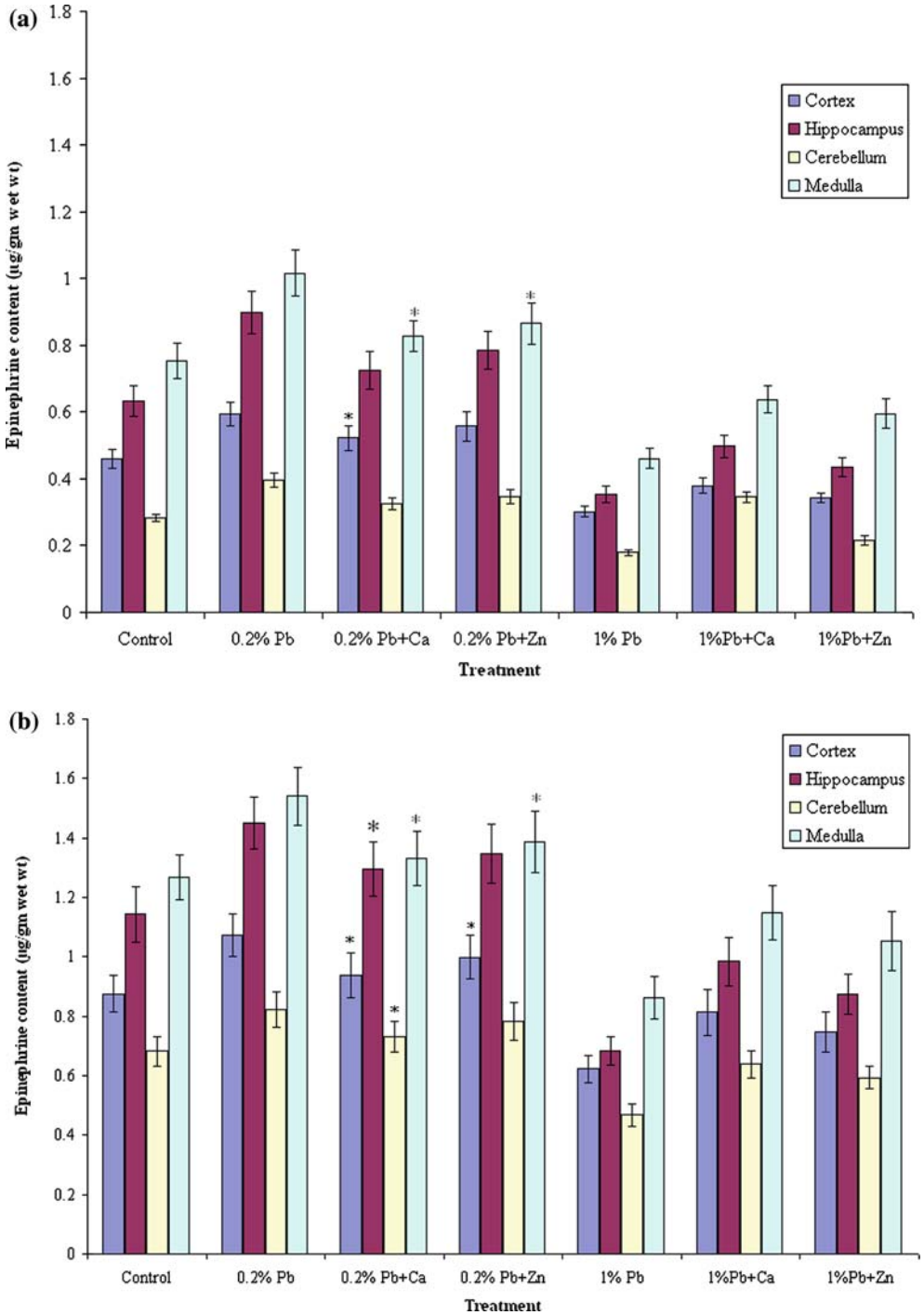

Figure 4. Effect of Pb-exposure and calcium/zinc supplementation to Pb on epinephrine levels in cerebral cortex, hippocampus, cerebellum and medulla. Male mice were exposed to either deionized water (control) or Pb-acetate (0.2% and 1%) or calcium/zinc together with Pb in deionized water from PND1 through PND21. Epinephrine levels were determined in the synaptosomal fractions of brain regions in 1 month (a) and 3 months (b) old control, Pb-exposed and calcium/zinc supplemented mice and expressed as microgram per gram wet weight. Values are mean ± SD of six separate experiments. All the values are significant at 1% level except the values marked with (*) as evaluated by two way ANOVA.

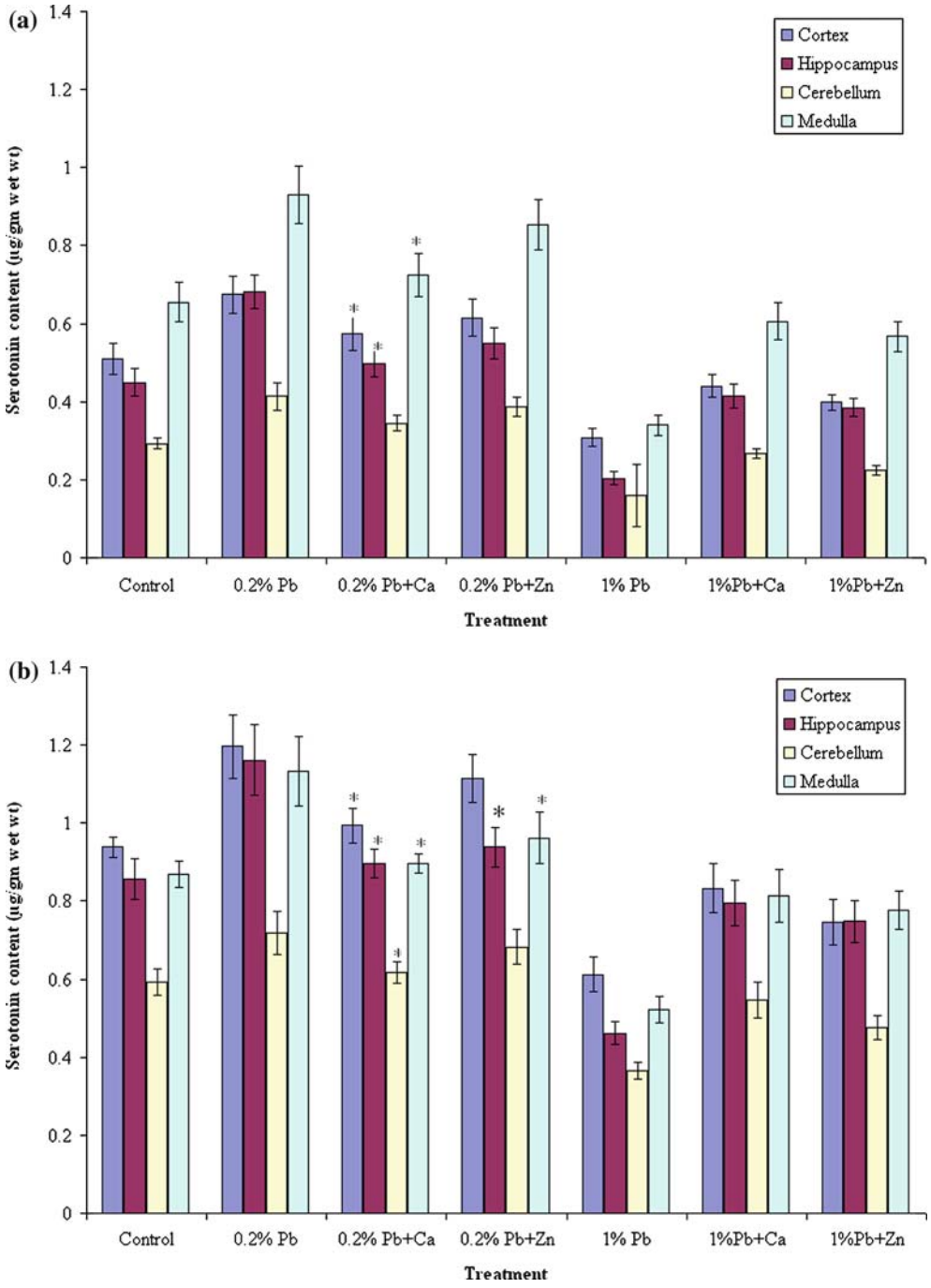

Figure 5. Effect of Pb-exposure and calcium/zinc supplementation to Pb on serotonin levels in cerebral cortex, hippocampus, cerebellum and medulla. Male mice were exposed to either deionized water (control) or Pb-acetate (0.2% and 1%) or calcium/zinc together with Pb in deionized water from PND1 through PND21. Serotonin levels were determined in the synaptosomal fractions of brain regions in 1 month (a) and 3 months (b) old control, Pb-exposed and calcium/zinc supplemented mice and expressed as microgram per gram wet weight. Values are mean ± SD of six separate experiments. All the values are significant at 1% level except the values marked with (*) as evaluated by two way ANOVA.

manner. Young mice were found to be more vulnerable to Pb-toxicity over adults of 3 months age.
High dose (1%)
Pb-exposure produced significant alterations as compared to low dose (0.2%).

In the present study, the decrease observed in MAO activity was significant in the synaptosomal fraction of cerebral cortex, hippocampus, cerebellum and medulla of 1% Pb-treated mice where as a marginal decrease in MAO activity was observed in 0.2% Pb-treated mice. Decrease in MAO activity can be attributed to the simultaneous increase observed in brain monoamines as we reported earlier for 0.2% Pb-exposed rats (Devi *et al*. 2005). However, the decrease in both MAO and monoamines in the brain regions exposed to high level Pb may be due to cellular damage (Alfano *et al*. 1983) and the high affinity of Pb for sulfhydryl groups in enzymes (Bagchi *et al*. 1997).

High level of Pb-exposure showed decreased levels of norepinephrine, epinephrine, dopamine and serotonin. These observations are in agreement with the decreased levels of monoamines reported for Pb-treated animals (Levina *et al*. 1973; Dubas & Hrdina 1978; Baksi & Hughes 1982; Sidhu & Nehru 2003; Devi *et al*. 2005). High dose Pb-administration to mice might have changed the morphology of the synaptosomes, as manifested in the decreased number of synaptic vesicles (Jablonska *et al*. 1994) contributing to alterations in synaptosomal monoamine levels. Dose related decrease in the activity of tyrosine hydroxylase together with alterations in the levels of catecholamines was also observed in Pb-exposed mice (McIntosh *et al*. 1988; Meredith *et al*. 1988). Pb-interferes with neuronal 5HT release by mechanisms involving Ca^{2+} (Pascal *et al*. 1989). 5HT levels decreased when Pb was administered at high doses (Antonio & Leret 2000).

Wince *et al*. (1976) reported that low level Pb enhanced the conversion of tyrosine to dopamine suggesting enhanced catecholamine synthesis. This could be a reason for the observed increase in monoamine levels with low level Pb-exposure in mouse brain. Pb activated release of norepinephrine at considerably lower concentrations was also reported by Tomsig & Suszkiw (1993).

Once inside the brain, Pb-induced damage occurs primarily in the cerebral cortex, cerebellum, hippocampus and medulla which may result in many morphological alterations in brain that can remain even after Pb levels have fallen (Chen *et al*. 1998). The hippocampus is an important target of neurotoxic agents and it accumulates Pb to a greater degree than other parts of the brain (Stoltenburg-Didinger 1994). Behavioral alterations following Pb-exposure have been related to hippocampal dysfunction (Altmann *et al*. 1991). Developmental exposure to Pb seems to alter the development of aminergic neurons mainly in the medulla, cerebral cortex and hippocampus. Hence, in the present study the monoamine alterations were more pronounced in these regions.

The differential susceptibilities of different Pb-exposed brain regions observed in the present study could be related to local differences in their formation and maturation, as well as the development of neurotransmitter systems. Dubas *et al*. (1978) also reported that depending on the dose, Pb has different effects on monoamine levels in different brain regions.

Pb affects the activity of the monoaminergic system during development of the central nervous system (Bressler & Goldstein 1991; Mejia *et al*. 1997; Antonio *et al*. 1999; Antonio & Leret 2000). Immature animals accumulate more Pb in the brain than adults because of their under developed blood–brain barrier (Willes *et al*. 1977). The fetal monoamine system represents a potential target for Pb. The first 3 weeks of postnatal life form the period of greatest neurotransmitter vulnerability to Pb (Widmer *et al*. 1991). This may be the reason for the observed neurotoxic effects of Pb on monoamines and MAO activity in 1 month old mice.

Usually the secretion of neurotransmitters and neurohormones is triggered by a rise in intracellular calcium (Cohen & Kloot 1985). Pb may block the influx of Ca^{2+} through membrane channels into the nerve terminal following the action potential (Boykin *et al*. 1991). The decrease in Ca^{2+} influx caused by Pb could be associated with an altered transmitter release (Antonio & Leret 2000, Antonio *et al*. 2002). Pb can enter through the same ion channels as Ca^{2+} and regulate the activity of these channels to uptake more Pb into the cell (Schuld 2005). Once inside, Pb may act in a mimetic role and will activate the Ca^{2+}-mediated synaptic vesicle release mechanisms. The overall effect is an increase in spontaneous neurotransmitter release

(Pages & Deloncle 1997). Animal studies have also shown higher retention of Pb in animals fed low-calcium diets, raising the possibility that low-calcium diets could affect the blood Pb levels of humans (Six & Goyer 1970; Mahaffey *et al*. 1973; Barton *et al*. 1978). Therefore, the supplementation with Ca^{2+} reversed the Pb-induced alterations in aminergic system of developing mouse brain.

Zn^{2+} influences both tissue accumulation of Pb and susceptibility to Pb toxicity. Supplementation of Zn^{2+} decreases Pb-gastrointestinal absorption, decreases Pb-tissue accumulation and thus decreases Pb-toxicity (Peraza *et al*. 1998). Lasley & Gilbert (1999) reported that Zn^{2+} levels significantly decreased as a function of increasing Pb concentrations.

As Ca^{2+} is more essential in the pathway of monoamine production, the supplementation with Ca^{2+} exhibited greater recovery in Pb-induced aminergic alterations as compared to Zn^{2+} supplementation. Ca^{2+} or Zn^{2+} supplementation thus plays a protective role against Pb-induced perturbations in aminergic system.

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