Calcium Addition Potentially Reverses Lead and Manganese Induced Enzymatic and Behavioral Alterations in Rats

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Abstract
Chronic exposure to lead (Pb) or manganese (Mn) is known to alter variety of neurological and behavioral functions. In this study, we have examined the neuro-behavioral perturbations in rats exposed to both Pb and Mn, and the protective effect of calcium supplement. Rats (3 months old) were exposed to Pb (0.2% through drinking water) and Mn (intraperitonially daily at a concentration of 2.5 mg/kg body wt) for a period of 3 weeks.

A separate batch of animals received calcium (0.02%) in drinking water together with Pb and exposed to Mn. Following exposures, the neurochemical alterations were assessed by determining the activity changes in synaptosomal acetyl cholinesterase (AChE) and mitochondrial ATPases in different brain regions (cortex, hippocampus and cerebellum). Behavioral studies included both cognitive (water maze tasks) and non-cognitive (open field, exploratory and locomotory tasks). Combined exposure to Pb and Mn resulted in behavioral dysfunctions (decrease in latency, swim distance, swim speed in water maze task and decrease in open field behavior and locomotor activity). Similar to the changes observed in motor and cognitive behavior, decrease in the activity of both AChE and ATPases were also observed in the brain regions of Pb+Mn exposed rats. However, the animals which received calcium together with Pb+Mn showed reversal effect in behavioral as well as the activity of the enzymes suggesting protective role of calcium supplementation against the Pb and Mn induced neurotoxicity. The results of this study support our earlier findings on the protective role of calcium and zinc against Pb and Mn induced neurotoxicity.

Keywords: Behavioral perturbations, Water maze, Lead, Manganese, Locomotor activity, cholinergic system, Calcium protection.

Introduction
The nervous system is the primary target for the low levels of Pb-exposure and the developing brain appears to be especially vulnerable to Pb/Mn-neurotoxicity (1-6). Pb-exposure at very low doses produces serious adverse effects on the central nervous system of children and infants, and these effects last for several years (7). Perinatal exposure to low levels of Pb has been shown to exert behavioral and neurochemical alterations in both suckling and adult rats (8). Pb is known to exert its neurotoxic effects by competing with calcium for calcium receptors coupled with second messenger functions (9) and in some cases, to inhibit the actions of Ca$^{2+}$ as a regulator of cell function (9).
Manganese (Mn) is an essential trace metal that is present in all tissues of mammals. This element can act both as a nutrient and a toxicant to the brain (10). The higher intake of Mn, either through the air or the diet, may result in severe pathologies, particularly in the central nervous system (CNS) leading to manganism, a Parkinson-like disorder (11). The toxic effects of Mn have been widely studied using animal models, notably concerning the accumulation of Mn in tissues and interactions with various other elements (e.g., iron [Fe], zinc [Zn] copper [Cu]) after subchronic or chronic administration (12).

The adverse effects of Mn on the nervous system probably result from the failure of protective enzymes capable to detoxify critical amounts of Mn or to alter its oxidation potential. A multifactor hypothesis is more likely, and could involve iron-induced oxidative stress and the direct interaction of Mn with dopaminergic terminal mitochondria, leading to selective mitochondrial dysfunction and subsequent excitotoxicity (13,14). In this study, we have examined the alterations in brain enzymes and behavioral perturbations in rats exposed to both lead and manganese since both metals coexist in environment originating from different exposure sources. We have also studied the protective effects of calcium against the alterations caused by Pb+Mn.

Animal exposure to Pb and Mn: Animals were exposed to 0.2% lead acetate (Sigma) by adding Pb-acetate to de-ionized drinking water and Mn (2.5 mg/kg) (Sigma) intraperitonially daily for a period of 21 days. Control rats received only deionized water without Pb, intraperitonially saline (0.9%) injections daily up to 21 days.

Calcium (Ca^{2+}) was supplemented as 0.02% in 0.2% Pb- water. The behavioral tasks in control, Pb+Mn-exposed and calcium supplemented 3 months old rats were observed in the open-field, water maze, exploratory behavior chamber and locomotory activity recorder. The brain regions (cerebral cortex, cerebellum and hippocampus) of control, Pb+Mn-exposed, and Ca^{2+} supplemented rats were collected and used for biochemical assays.

Behavioural Studies

Open-Field Behavior: The open field test has been widely used to assess emotional reactivity/anxiety. It provides measures of locomotor activity. The horizontally directed activity (or locomotion) is measured by the number of line crossings, and vertically directed activity (or exploration) is measured by the frequency of rearings (15). The open-field behaviour of three months age rats was assessed in a wooden box measuring 90 x 90 x 30 cm high. The floor of the arena was divided into 36 equal squares by black lines. Immediately after a rat was placed in the centre of the open field, the movement of the animal was scored. The number of squares crossed with all paws (crossings), the standings on the hind legs (rearings), placing the nose against wall or floor (sniffing), wiping, licking, combing or scratching of any part of the body (grooming) were counted in all sessions. All the activities measured were combined together to assess the mean total behaviour in each session. Testing was carried out on five consecutive days in five minute sessions in control, Pb-exposed and supplemented animals.

Materials and Methods

Procurement and maintenance of experimental animals: Young albino rats (Wistar) of 3 months age were purchased from IISc, Bangalore and maintained in the animal house of Dept. of Zoology, S.V. University. The animals were housed in clear plastic cages with hardwood bedding in a room maintained at 28±2° C and relative humidity 60±10% with a 12 hour light/day cycle. The animals were fed with standard pellet diet supplied by Sri Venkateswara Traders, Bangalore and water ad libitum.
**Locomotory Activity:** Locomotor activity of the rat was studied with Opto-Varimex mini (Columbus Instruments, USA). The data was taken as total duration 30 minutes to each animal during the 5 minutes session interval.

**Exploratory Behaviour:** Exploratory behaviour was evaluated in the hole board. The apparatus was an open-field arena with four equally spaced holes (3 cm in diameter) in the floor. Each rat was placed individually in the centre of the arena for 5 min, during which we recorded head-dip count and head-dipping duration, in seconds. A head dip was scored if both eyes disappeared into the hole. Head-dipping duration data are expressed as total duration during the 5-min session. The results for head dip are expressed as number of counts, and for head-dipping duration in seconds.

**Morris water maze:** Spatial discrimination learning not only involves place learning, which is learning a position in space which in this case is the position of the hidden platform in the Morris water escape task, but also involves non-spatial components like procedural learning (such as learning to search for an escape platform) and visual or other sensorimotor processes together with possible motivational/emotional processes necessary for executing the task. After acquisition of the spatial water escape task, a probe trial can reveal whether the rats have actually learned the position of the platform. Furthermore, a spatial discrimination reversal task after the acquisition of the spatial task, measures mainly place learning because the rats are already familiar with the procedural component.

The water maze is a circular water tank measuring 1.85 m in diameter and 0.7 m deep constructed according to a basic design similar to that of Morris (16). Four points along the circumference of the water tank were designated arbitrarily North (N), South (S), East (E), and West (W), thus dividing the maze into four quadrants. The pool was filled to a depth of 30 cm with water made opaque with white, non toxic water-based paint. A circular submerged platform (diameter 12.5 cm) remained below the surface of water. All parameters involving time and distance are measured in seconds. Testing was carried out on five consecutive days. Control, Pb+Mn treated and Ca²⁺ supplemented rats were subjected to water maze learning tasks.

**Biochemical Studies**

**Preparation of mitochondrial fraction:** Mitochondrial fractions were prepared by following the method of Lai and Clark (17) by homogenizing in 5 volumes (w/v) of Sucrose-EDTA-Tris buffer (SET) buffer (0.25 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4). The homogenate was first centrifuged at 800 g for 10 min at 4°C, and then the supernatant was centrifuged at 10,000 g for 20 min. Then the pellet of mitochondrial fraction was suspended in SET buffer.

**Estimation of AChE:** The specific activity of AChE was determined as described by Ellman et al., (18). The reaction mixture contained 3.0 ml of 0.1M phosphate buffer (pH 8.0), 20μl of 0.075 M acetylthiocholine iodide and 100 μl of 0.01 M 5, 5-dithiobis 2-nitrobenzoic acid. The reaction was initiated with the addition of 100 μl of crude homogenate. The contents were incubated for 30min at room temperature and the color absorbance was measured at 412nm in spectrophotometer (Hitachi, Model U-2000). The enzyme activity was expressed as μ moles of ACh hydrolyzed /mg protein/h.

**Estimation of Adenosine Triphosphatase (ATPase) activity:** Na⁺K⁺ and Mg²⁺-ATPase activities in the tissues were estimated following the method of Tirri et al., (19). Briefly, 1% homogenates of the tissues were prepared in 0.25 M ice cold sucrose solution. Homogenates were
divided into two parts. One part was centrifuged at 1400g and the supernatant thus obtained was used as an enzyme source for Mg\(^{2+}\)ATPase, while the other part of the homogenate was used for the estimation of the total ATPase.

**Mg\(^{2+}\)ATPase:** The reaction mixture for Mg\(^{2+}\)ATPase assay contained 0.5 ml of tris buffer (0.13 M; pH 7.4), 0.4 ml of substrate ATP, 0.5 ml of Magnesium chloride (0.05 M MgCl\(_2\)) and 0.2 ml of mitochondrial fraction (enzyme source). The contents were incubated at 37°C for 15 minutes and the reaction was stopped by the addition of 10% TCA. Zero time controls were maintained by adding TCA prior to the addition of homogenate/mitochondrial fraction. The contents were centrifuged at 1000g for 15 minutes and the inorganic phosphate was estimated in the supernatant fraction following the method of Fiske and Subbarow (20).

**Na\(^+\)K\(^+\)ATPase:** 1% (W/V) homogenate already set apart was used for the total ATPase assay. The reaction mixture in a final volume of 2.6 ml contained, 0.5 ml of Tris buffer (0.13 M; pH 7.4), 0.4 ml of substrate ATP, 0.5 ml MgCl\(_2\) (0.05 M), 0.5 ml potassium chloride (KCl, 0.05 M), 0.5 ml of sodium chloride (NaCl, 0.05 M) and 0.2 ml of crude homogenate/ mitochondrial fraction (enzyme source). The contents were incubated at 37°C for 15 minutes and the reaction was arrested by the addition of 1.5 ml of 10% TCA prior to the addition of homogenate. The contents were centrifuged and the inorganic phosphate was estimated in the supernatant fraction following the method of Fiske and Subbarow. The contents were centrifuged at 1000g for 15 minutes and the inorganic phosphate was estimated in the supernatant fraction following the method of Fiske and Subbarow (20).

**Estimation of protein content:** Protein content of the tissues was estimated by the method of Lowry et al. (21).

**Analysis of Data:** The data was subjected to statistical analysis (ANOVA and t-test as applicable) using standard statistical procedures.

**Results**

**Open field behavior:** The results presented in Fig.1 show decreased locomotor behavior in all responses (crossings, rearings, sniffings, groomings) of the open-field in Pb+Mn exposed rats compared to controls. The control rats showed higher locomotor activity (111.6 crossings, 14.2 rearings, 19.2 sniffings, 19.2 groomings) compared to Pb+Mn treated rats (31.7 crossings, 3.1 rearings, 3.7 sniffings, 4.3 groomings). However, the addition of calcium to Pb+Mn, showed a marginal reversal in the alterations observed in the locomotor behavior (46.88 crossings, 4.33 rearings, 6.77 sniffings, 7.33 groomings) of Pb+Mn treated rats.

**Locomotor activity:** The results of locomotor activity (Fig.2) showed a decrease in the total number of movements in the Pb+Mn treated rats than the control rats. The administration of calcium along with the Pb+Mn, showed increase in the locomotory activity than Pb+Mn exposed rats.

**Fig.1.** Open-field behavior of control, Pb+Mn exposed and Ca\(^{2+}\) supplemented rats. Rats were exposed to 0.2% lead acetate through drinking water and Mn (2.5 mg/kg) intraperitonially daily for a period of 21 days. Calcium (0.02%) was added in 0.2% Pb water. Each bar represents mean ± SD (n = 6). The values marked with asterisk (*) are significantly different from controls at p<0.05 – p<0.001.
than the control rats (head dip duration: 41.625 sec/5min and the number of head dippings: 5.875 times/5min). However, the administration of calcium showed a marginal increase in both head dip duration (5.66 sec/5 min) and number of head dippings (3.166 times/5 min) than the Pb+Mn alone treated rats.

**Water maze learning:** Behavioral assessments on water maze confirmed the impairment in performance of the water maze acquisition, reversal and working memory tasks in Pb+Mn exposed rats (Figs 4 to 6). All the rats easily learned to find the submerged platform except for the first day of training when all subjects performed at chance level. The Pb+Mn exposed rats took significantly longer time than control animals to find the hidden platform.

**Acquisition phase:** From the Fig. 4, it evident that Pb+Mn exposure significantly impaired the water maze acquisition performance. In the first day of training session, no specific alterations were

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**Exploratory behavior:** From the figure 3, it is evident that Pb+Mn treated rats showed a decrease in head dip duration (3.52 sec/5 min) and number of head dippings (1.91 times/5 min) than the control rats (head dip duration: 41.625 sec/5min and the number of head dippings: 5.875 times/5min). However, the administration of calcium showed a marginal increase in both head dip duration (5.66 sec/5 min) and number of head dippings (3.166 times/5 min) than the Pb+Mn alone treated rats.

**Water maze learning:** Behavioral assessments on water maze confirmed the impairment in performance of the water maze acquisition, reversal and working memory tasks in Pb+Mn exposed rats (Figs 4 to 6). All the rats easily learned to find the submerged platform except for the first day of training when all subjects performed at chance level. The Pb+Mn exposed rats took significantly longer time than control animals to find the hidden platform.

**Acquisition phase:** From the Fig. 4, it evident that Pb+Mn exposure significantly impaired the water maze acquisition performance. In the first day of training session, no specific alterations were
found between control and treated rats. Later, control rats quickly proceeded to the north quadrant where the platform is located and identified the hidden platform, whereas Pb+Mn treated rats took longer time to reach the hidden platform. Pb+Mn-exposed rats exhibited the tendency of peripheral swimming, thereby took longer time to reach the platform. The mean escape latency of controls was 12.17±3 on 1st day, 11.62±2.9 on 2nd day, 9.42±2.4 on 3rd day and 8.67±2.2 on 4th day. The Pb+Mn treated young rats exhibited increase in the escape latencies (24.56±1.2 on 1st day, 24.2±1.8 on 2nd day, 21.02±2.2 on 3rd day and 21.3±1.9 on 4th day). The escape latency however greatly reduced in Pb+Mn-exposed rats supplemented with Ca2+ (16.54±2.3 on 1st day, 17.86±1.7 on 2nd day, 16.32±2.3 on 3rd day and 16.2±3.1 on 4th day).

Reversal phase: During the spatial discrimination of reversal task, the submerged platform was shifted to south quadrant. All the groups initially spent more time in north quadrant and then reached to the south quadrant. In the first session, there was not much difference in all treated and control groups. From the Fig. 5, it is evident that the control rats showed mean latencies of 10.29±1.2 on 1st day, 10.01±1.5 on 2nd day, 8.27±0.9 on 3rd day and 8.72±0.9 on 4th day. The Pb+Mn treated young rats exhibited increase in the escape latencies (19.7±1.3 on 1st day, 18.02±1.9 on 2nd day, 18.01±2.1 on 3rd day and 16.09±2.2 on 4th day). The escape latency however greatly reduced in Pb+Mn-exposed rats supplemented with Ca2+ (14.408±1.5 on 1st day, 14.02±1.7 on 2nd day, 12.02±1.9 on 3rd day and 11.62±2.2 on 4th day).

Working memory: As the platform is changed to different quadrants in working memory, the escape latencies were greater than acquisition and reversal phases. However, all the control as well as Pb+Mn-exposed rats took lesser time to
locate the hidden platform kept in north quadrant from all the three directions suggesting the retention of earlier acquisition (Fig. 6). As in the case of acquisition and reversal phases, Pb+Mn-exposure exhibited greater impairments in working memory also.

**Acetylcholinesterase:** Among the different brain regions studied, the specific activity of synaptosomal AChE in control rats was recorded highest in hippocampus, followed by cerebral cortex and cerebellum. Incase of Pb+Mn treated animals, a decrease in the activity of AChE was observed in all the three brain regions i.e., hippocampus followed by cerebral cortex and cerebellum. The animals which received calcium together with Pb+Mn indicated significant recovery in AChE activity in all the three brain regions (Fig. 7).

**Adenosine triphosphatases:** The specific activity of Na\(^+\)K\(^+\) ATPase was recorded highest in cerebral cortex (0.282), followed by hippocampus (0.217) and cerebellum (0.146) in the control rats. Incase of Pb+Mn treated rats, a decrease in the Na\(^+\)K\(^+\) ATPase activity was recorded in all the brain regions, i.e., cerebral cortex (0.211) followed by hippocampus (0.172) and cerebellum (0.101). However, calcium supplementation to Pb+Mn, reversed the inhibitory effect of Pb+Mn in all the brain regions; i.e., cerebral cortex (0.256) followed by hippocampus (0.198) and cerebellum (0.124) (Fig. 8).

In the control rats, the activity of Mg\(^{2+}\) ATPase was recorded highest in hippocampus (0.894) followed by cerebral cortex (0.783) and cerebellum (0.624). Pb+Mn treated rats exhibites significant decrease in Mg\(^{2+}\) ATPase activity in all the three brain regions. i.e., hippocampus (0.717) followed by cerebral cortex (0.712) and then cerebellum (0.526). Calcium supplementation to Pb+Mn, however, reversed this effect producing a marginal increase in the Mg\(^{2+}\) ATPase activity, i.e., hippocampus (0.768) followed by cerebral cortex (0.754) and cerebellum (0.601) (Fig. 9).

**Fig.7.** Effect of Pb+Mn exposure on AChE activity in cerebral cortex, hippocampus and cerebellum. Rats were exposed to 0.2% lead acetate through drinking water and Mn (2.5 mg/kg) intraperitonially daily for a period of 21 days. Calcium (0.02%) was added in 0.2% Pb water. Each bar represents mean ± SD (n = 6). The changes (values marked with asterisk *) are significant at p<0.001.

**Fig.8.** Effect of Pb+Mn exposure on Na\(^+\)K\(^+\) ATPase activity in cerebral cortex, hippocampus and cerebellum. Rats were exposed to 0.2% lead acetate through drinking water and Mn (2.5 mg/kg) intraperitonially daily for a period of 21 days. Calcium (0.02%) was added in 0.2% Pb water. Each bar represents mean ± SD (n = 6). The changes (values marked with asterisk *) are significant at p<0.001.

Calcium protection against lead and manganese toxicity
Discussion

Our recent work (22, 23) has shown inhibitory effect of Pb on AChE activity in the developing nervous system and reversal effect with calcium supplementation. Keeping in view of these findings, the present study was aimed at examining the combined effect of Pb and Mn-exposure on the activity of AChE and ATPases as well as locomotor and cognitive behaviour, and the protective effect of calcium supplementation.

Control animals showed higher frequency of all the open field responses (crossings, rearings, sniffings and grooming) where as significant decrease was observed in open field responses of Pb+Mn-exposed rats compared to controls. The open field test has been widely used to assess emotional reactivity/anxiety. It provides measures of locomotor activity (15). The memory of habituation in the open field is processed by the hippocampus, which is believed to be a target for metal neurotoxicity.

In the present study, the cognitive deficits in Pb+Mn-exposed rats were examined by water maze swim tasks. As the platform is changed to different quadrants in working memory, the escape latencies in working memory were greater than acquisition and reversal phases. However, all the control as well as Pb+Mn-exposed rats took lesser time to locate the hidden platform kept in north quadrant from all the three directions suggesting the retention of earlier acquisition.

The impaired performance in water maze swim tasks of Pb+Mn exposed rats can be attributed to motor impairments because there were significant differences between swim latencies in control and Pb+Mn exposed groups. These results demonstrate that chronic exposure to Pb+Mn, causes learning impairment by way of deficits in both working memory and reference memory. Thus, the results of our behavioral studies indicate that the ability needed to solve a complex task is more affected by the combined exposure of Pb and Mn. However Ca^{2+} supplementation significantly reversed the Pb+Mn induced behavioral impairments.

The nervous system is the primary target for the Pb-exposure and the developing brain appears to be especially vulnerable to Pb neurotoxicity (3-5). Pb has high affinity for the free sulfhydril groups in enzymes and proteins and its binding can alter their correct function in numerous processes (24, 25). This may be the reason for the observed inhibition of AChE activity. The alteration in the motor activity is one of the most studied effect of the intoxication, but learning impairments, in particular, caused by Pb has been described at very low doses and included a decrease in the intelligence quotient as well as memory (26). Therefore, a significant decrease in open field behavior and cognitive behavior in Morris water maze were observed with combined exposure to Pb+Mn.
A generalised reduction in brain cholinergic function has been reported in Pb-treated rats. Exposure to Pb resulted in a decrease in the AChE activity in cerebellum and hippocampus at various post natal time points (22). Neonatal exposure to Pb altered the muscarinic receptor density (27) which account for the deficits in central cholinergic functions (28). The results of our present study are in agreement with these findings.

The alterations in AChE activity during early postnatal development could be related with the fact that Pb crosses the blood brain barrier quite readily (29). The inhibitory effect of Pb on AChE activity also reflected in alterations in the motor activity (30, 5). The results of the present study showed maximum alterations in AChE content in hippocampus of Pb-treated rats. The cholinergic synapses are more in hippocampus as compared to cerebral cortex and cerebellum (31). AChE inhibition in this area leads to cognitive and non cognitive alterations as this is the principle area for memory and cognition. The fact that the hippocampus develops late (32) and sequester, Pb, primarily in the mossy fibre pathway (33) would put this structure at particular risk for damage following early Pb-exposure.

Since the cholinergic system is responsible for the behavioral manifestations, any alteration in the cholinergic system would be reflected in the behavior. In the present study, the observed alterations in cholinergic system have greatly influenced the open field behavior of rats exposed to Mn and Pb. The memory and spatial discrimination tasks in Morris water maze also decreased suggesting the hippocampal damage due to Pb+Mn exposure.

Supplementation with Ca2+ reversed the Pb and Mn induced inhibition in AChE activity. The reversal of inhibition in the activity of AChE by supplementation with Ca2+ may be due to competition of these metals for similar binding sites and reducing the availability of binding sites for Pb or Mn.

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+ (34). Studies have shown that Pb has an inhibitory effect on the peripheral nervous system through stimulus coupled or Ca2+ dependent release of acetylcholine (35) and this inhibitory effect of Pb at the neuromuscular junction and the ganglion was similar to the effect of reducing the concentration of Ca2+ in bathing media of neural preparations; so it is not surprising that this inhibitory effect of Pb can be overcome by the addition of Ca2+. Absorption of Pb by gastrointestinal tract is inversely related to the amount of Ca2+ present (36, 37). Furthermore Ca2+ supplements had a protective effect by significantly reducing blood Pb levels in pregnant women whose diets were deficient in Ca2+ (38, 39). Ziegler et al., (40) observed an inverse relationship between dietary Ca2+ and Pb retention and absorption in young infants.

Pb+Mn-exposure exerted inhibitory action on the activity of enzymes Mg2+, and Na+K+ATPases in the developing brain. Heavy metals such as Pb can bind to a number of sites on proteins including imidazole, histodyl, carbonyl and especially sulfhydryl side chains (41). Heavy metals have great affinity for ATPase system and they interact with enzyme molecule resulting in the inhibition (42). Pb has been reported to inhibit Na+K+ATPase of mammalian tissues (43, 44) and also interferes with mitochondrial function and blocks the O2 uptake. Bhaumik and Raychudhari (45) reported that the inhibition of Na+K+ATPase may be due to the flow of the Na+ K+ ions from the tissues to the blood. The decrease in the Mg2+ATPase activity might be due to low operation of oxidative pathway, resulting in
decreased formation of free energy and altered cellular energy metabolism (46).

The combination of Pb$^{2+}$ and Mn$^{2+}$ produced a pronounced decrease in the activity of Na$^+$ K$^+$-ATPase, but the magnitude of the change was the sum of the individual metal effects. (47). It is known that brain derived Na$^+$K$^+$ATPase is among the enzymes particularly affected by Pb (48-50). The decrease of Na$^+$K$^+$ATPase activity can change the gradients of Na$^+$ and K$^+$ across the cell membrane and can be the cause of the disturbances in neurotransmitters levels (50).

The observed high activity of ATPase in the cortex, cerebellum and hippocampus regions of the brain suggests the involvement of these regions in different behavioral functions. It is known that the level of ATPase parallels the metabolic demands of different regions of rat brain and the differential sensitivity to Pb+Mn neurotoxicity in these brain regions is not due to a preferential metal accumulation, but is possibly due to alteration of biochemical or cellular processes that are uniquely associated with, or greatly enhanced in a particular region (51, 8). Regional variations in AChE and ATPases activity levels observed in different brain regions could be due to structural and functional differences in brain regions. Addition of Ca$^{2+}$ reduced the effects of Pb+Mn on the activities of AChE and ATPases in brain regions. The supplemented Ca$^{2+}$ may compete for similar binding sites as that of Pb and Mn. Supplementation with Ca$^{2+}$ decreases Pb gastrointestinal absorption and decreases tissue accumulation (52). Thus Ca$^{2+}$ replaces Pb and Mn in the body reducing the Pb+Mn-burden in the body.

Thus, from the present study, it is evident that exposure to a combination of Pb and Mn inhibited the activities of both Mg$^{2+}$ATPase and Na$^+$K$^+$ATPase enzymes as well as AChE leading to decrease in motor and cognitive functions. Calcium supplementation significantly reversed these alterations in enzyme activities as well as behavioral functions suggesting therapeutic role of calcium for Pb+Mn induced neurotoxicity.

Acknowledgements
The authors acknowledge the financial support from CSIR Grant No. 37(1349)/08/EMR-II and DST Grant No.SR/SO/AS-72/2008.

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