



# Acetylcholinesterase activity and oxidative stress indices in cerebellum, cortex and hippocampus of rats exposed to lead and manganese

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## Abstract

Human exposure to metals is a major health concern in the world as a result of increased industrialization. The present study was designed to investigate the toxic effect of lead and manganese alone and in combination on different regions of rat brain, namely cerebellum, cortex and hippocampus. Adult male Wistar rats were exposed to 100 ppm lead as lead acetate, 100 ppm manganese as manganese chloride, and lead and manganese in combination in their drinking water for 4 weeks. Following either lead or manganese exposure, acetylcholinesterase activity and the antioxidant enzymes, superoxide dismutase and catalase activities were inhibited. Likewise, malonylaldehyde level, a marker of lipid peroxidation and protein carbonyl groups used to assess oxidatively modified proteins were markedly increased by exposure to either metals. The non-enzymatic antioxidants, reduced glutathione and total thiol groups were significantly ( $p < 0.05$ ) depleted by these metals. However, these aspects were more pronounced in animals exposed to the mixture of both lead and manganese. The metals displayed additive, as well as, potentiation effect in their interactions in the brain regions. It can thus, be concluded that the mixture of lead and manganese demonstrated a higher neurotoxic effects than does either metals alone.

**Keywords:** Lead; Manganese; Coexposure; Brain Regions; Acetylcholinesterase; Oxidative Stress.

## 1. Introduction

Increased industrialization has resulted in an escalation of human exposure to metals, a situation which has become an environmental and public health concern of global magnitude (Toscano and Guilarte, 2005). Human exposure to chemical mixtures is particularly widespread in socioeconomically disadvantaged populations (Yang, 2016) and among populations living near hazardous waste sites (Mellard et al. 2011). Some of these metals, owing to their environmental persistence and propensity to bioaccumulate in the brain have been implicated in the etiology of various neurodegenerative diseases (ND) (Weiss, 2011), altogether leading to an increase in incidence of neurological diseases. Mining and metal smelting activities, among others, have contributed to these metals accumulating in the atmosphere and soil (ATSDR, 2007; Dhatrik and Nandi, 2009). Chief among these metals are lead (Pb) and manganese (Mn).

Pb is one of the key environmental heavy metal pollutants and exposure to this metal occur from a variety of sources. Besides metal smelting operations, increased Pb levels in the environment are principally attributed to combustion of leaded gasoline, contaminated water supply by industrial waste, as well as, occupational settings (Jemiola-Rzeminska, 2007; Andrade et al. 2013). After gastrointestinal absorption, Pb is predominantly deposited in the brain, kidney and blood, which can induce neurological, nephrotoxic and hematological effects respectively (ATSDR, 2007; Hodgson, 2010). Manganese, on the other hand, is an essential element involved in a variety of important

metabolic processes, but excessive exposure to the metal may result in adverse health effects on the nervous system, lungs, arteries, heart and liver (Jiang and Zheng, 2005; ATSDR, 2007). The main route of exposure to Mn is the diet, although excessive Mn levels through drinking water has been reported (ATSDR, 2007). Exposure to Mn in occupational and environmental settings have been associated with disabling neurological effects in humans. The brain's ability to retain Mn than other organs makes it more susceptible to manganese toxicity (Shukla et al. 1996). Excessive exposure to Mn has been reported to cause accumulation in the brain, leading to irreversible brain disease, similar to Parkinson's disease. Progressive neurological damage by manganese leads to the permanent neurological disorder known as manganism with the resultant tremor, difficulty in walking and facial muscle spasm (Huang, 2007; Stepens et al., 2008).

Lead and manganese, both neurotoxic elements readily cross the blood-brain barrier and they often occur in mixtures (Yokel, 2009; Dhatrik and Nandi, 2009; Balbuena and Erich, 2011). The mechanisms for lead neurotoxicity have been reported to include increased spontaneous release of neurotransmitters, oxidative stress and disruption of calcium metabolism (Bressler et al. 1999). Biological mechanisms for manganese toxicity have also been suggested to involve calcium metabolism, oxidative damage to neuronal cells, and impaired dopamine neurotransmission (Normandin et al., 2004; Aschner, 2006). With the brain being their major target organ, and both having similar biochemical mechanisms of toxicity, as well as, there being a potential for joint exposure, manganese and lead are important chemicals to examine for interactive effects.



Simultaneous exposure to multiple metals occur frequently in the environment (De Rosa et al. 2004; Cory-Slechta et al. 2005), but the effects of combined exposure have not been fully studied. There is, therefore, a growing need to study metal mixtures exposure and the consequent impacts on health status. Data available support the hypothesis that coexposure to multiple metals may cause neurotoxic effects not seen with exposure to a single metal at the same dose. Coexposure to Pb and Mn have been described, with resultant decreased learning of conditioned avoidance responses more than either Pb or Mn alone, and gestational exposure to both Pb and Mn reduced brain weight to a greater extent than either metal alone (Chandra et al. 1983). In another study, coexposure to these metals affected learning in an additive manner while decreasing spontaneous motor activity and norepinephrine content of the brain in contrast to single exposures (Chandra et al. 1981). Administration of mining waste, consisting of manganese and lead, among other metals has been associated with lower levels of DOPAC and homovanillic acid in rats (Rodriguez et al. 1998).

The brain is an organ that is divided into different anatomical and physiological areas which may be affected differently by toxicological exposure. The effects of multiple metals on different brain regions have not been fully explored. Consequently, studies on Mn and Pb coexposure induced neurotoxicity in different regions of the brain are sparse. The present study was, therefore, designed to investigate lead and manganese coexposure induced changes in acetylcholinesterase activity and oxidative stress indices in the cerebellum, cortex and hippocampus regions of the rat brain. This was to gain further insight into the roles of metal mixtures in the etiology of neurodegenerative diseases.

## 2. Methods

### 2.1. Animals and treatment

Male adult Wistar rats weighing between 130-150 g were used for this study. Animals were housed in plastic cages and maintained in a 12-h light and dark cycle. The animals were fed standard food pellet and water ad libitum. The study was approved by the institutional animal ethical committee. After an acclimatization period of 14 days, the animals were randomly divided into 4 groups of 7 rats each. Treatments were performed for 30 days according to the following protocol: group 1 (control group) received distilled water; group 2 (Pb) was exposed to 100 ppm of lead in the form of lead acetate, in drinking water; group 3 (Mn) was exposed to 100 ppm manganese as manganese chloride in drinking water; group 4 (Pb+Mn) was exposed to the mixture of these 2 metals with the same doses and conditions of each single exposed group. The metal concentrations were chosen based on previous studies (Nakade et al., 2015; Al-Harbi, 2013). At the end of the exposure period, animals were sacrificed by cervical dislocation and the brain was removed from the skull, washed in ice-cold normal saline, weighed and dissected into different brain regions, i.e. the cortex, hippocampus and cerebellum. The dissected brain regions were weighed and stored at -20°C until used.

### 2.2. Biochemical assays

The brain regions (cortex, cerebellum and hippocampus) were homogenized in a 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% potassium chloride, to yield a 10% (w/v) homogenate. The preparation was then centrifuged at 10,000 g for 10 min at 4°C and the supernatant was collected and used for biochemical analyses.

Acetylcholinesterase (AChE) activity in the different brain regions was determined by Ellman's method (Ellman et al. 1961), using acetylcholine iodide as a substrate. In this method AChE in samples hydrolyzes acetylthiocholine iodide into thiocholine and butyric acid. The thiocholine reacts with 5, 5-dithiobis-2-

nitrobenzoic acid to form 5- thio-2-nitrobenzoic acid. The yellow colour developed is measured spectrophotometrically at 412 nm.

The quantitative measurement of lipid peroxidation was performed according to the method of Buege and Aust (1978). The malondialdehyde (MDA) formed after a series of peroxidation reaction as an end-product was measured by the reaction with thiobarbituric acid at 532 nm. The results were expressed as micromoles of MDA per milligram of protein, using the molar extinction coefficient of MDA-thiobarbituric chromophore ( $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ).

The levels of reduced glutathione (GSH) in brain homogenate were determined by the method of Moron et al. (1979) based on the reaction with Ellman's reagent (19.8 mg DTNB in 100 ml of 0.1% sodium citrate). The levels of total sulphhydryl groups (TSH) was measured after the reaction with 5,5-dithiobis-2-nitrobenzoic acid using the method of Ellman (1959).

Protein carbonyl content was estimated by Levine et al. method (1990). SOD activity was determined by the method described by Misra and Fridovich (1972). CAT activity was determined according to the method of Clairborne (1988), using  $\text{H}_2\text{O}_2$  as a substrate. Protein concentration was determined by the method of Lowry et al. (1951).

### 2.3. Statistical analysis

The statistical analysis was performed using the GraphPad Prism 6.0 package. The data were expressed as mean  $\pm$ SD. Level of significance among the groups was evaluated using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. p values of  $< 0.05$  were considered significant.

## 3. Results

AChE activity was significantly inhibited in the cortex, cerebellum and hippocampus by all treatments (Fig. 1). The effect of lead was most profound in the cortex, where the activity of the enzyme was inhibited by 58% by the metal. This was followed by the hippocampus and cerebellum by 55% and 21% respectively. Manganese reduced the activity by 26%, 29% and 28% in cerebellum, cortex and hippocampus respectively ( $p < 0.05$ ). Co-administration of the two metals significantly lowered the enzyme activity by 14%, 32% and 36%, respectively ( $p < 0.05$ ).

Although CAT activity in the hippocampus of rats treated with single metals or combination was depressed compared to control, the activity did not differ significantly from each other ( $p > 0.05$ ). Their effects were however, more pronounced in the cortex where lead, manganese and their combination inhibited the enzyme by 67%, 59% and 70% respectively ( $p < 0.05$ ). In the cerebellum, manganese yielded the highest level of inhibition, with a lowering of the activity by 45% while lead and their combination reduced the activity by 12% and 23% respectively (Fig. 2).

Lead and manganese exposures significantly ( $p < 0.05$ ) lowered SOD activity in the cortex and hippocampus of the rats (Fig. 3). In the cerebellum, however, manganese did not display any effect on the enzyme activity. Co-administration of lead and manganese, on the other hand, markedly inhibited superoxide dismutase activity in all the three regions of the brain, more than either metals, giving rise to 54%, 93% and 84% inhibition in the cerebellum, cortex and hippocampus, respectively.

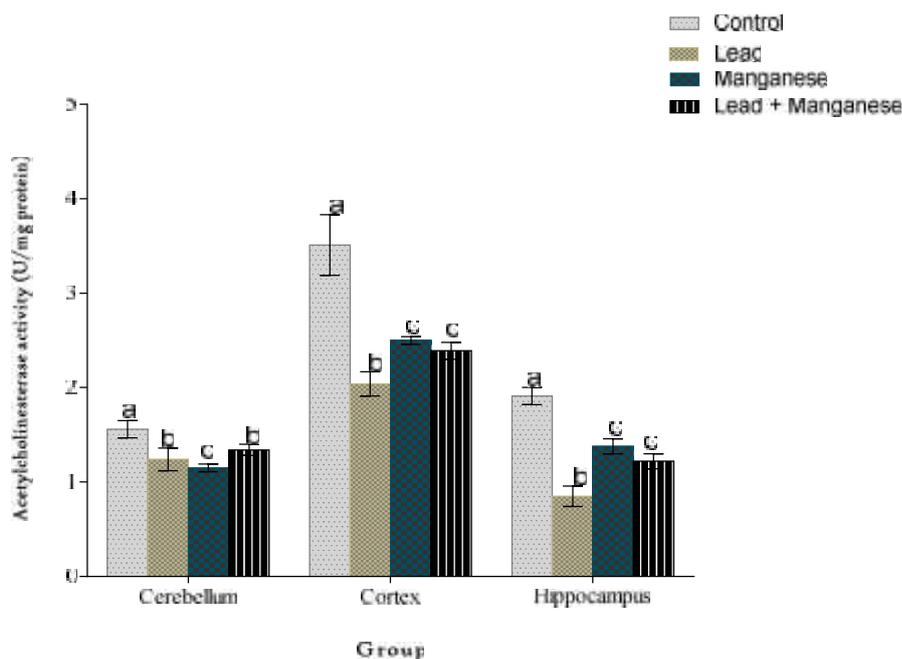
As shown in Figure 4, the level of MDA as a marker of lipid peroxidation was observed to increase in all the three regions of brain as a result of exposure to either lead or manganese. Lead increased MDA level by 15%, 51% and 94% while manganese increased lipid peroxidation by 13%, 46% and 26% in the cerebellum, cortex and hippocampus, respectively. In the three regions, combined exposure caused increases in MDA contents that were significantly higher than that of either lead or manganese alone, with the effect most pronounced in the cerebellum. Moreover, the metal mixture displayed an additive effect on MDA concentration in the cerebellum.

Figure 5 depicts the effects of lead, manganese and their combination on GSH concentration in the brain. Lead exposure significantly decreased GSH level in all three brain regions. Cerebellum underwent 27% depletion of GSH while the cortex and hippocampus had 24% and 17% reduction of the molecule. Manganese elicited a 13% decrease in the cerebellum while showing no effect on GSH contents in both the cortex and hippocampus. Conversely, lead and manganese mixture resulted in significant depletion of GSH concentrations in the regions ( $p < 0.05$ ).

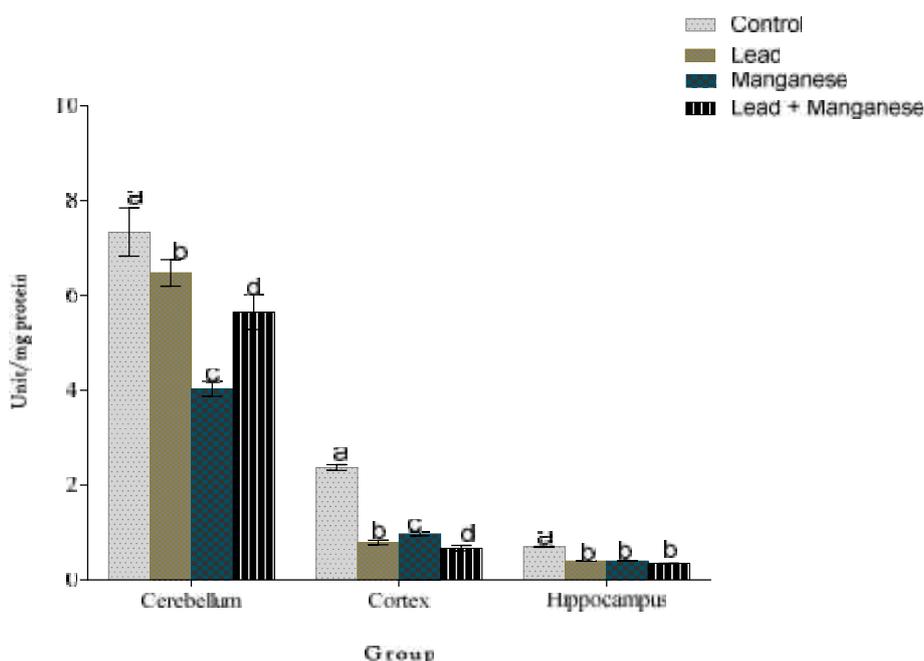
Protein carbonyl groups were significantly increased in the brain regions by exposure to lead and manganese singly and in combination ( $p < 0.05$ ). The increase in protein carbonyl groups was of similar trend in the three brain regions (Fig. 6). Lead induced marked oxidative modification of proteins yielding 175%,

50% and 145% in the cerebellum, cortex and hippocampus, respectively. Manganese on the other hand increased the oxidative modified proteins by 158%, 40% and 70% respectively. However, the combined treatment yielded higher percentage of protein carbonyl groups, generating 210%, 83%, and 145% above the control in the cerebellum, cortex and hippocampus respectively.

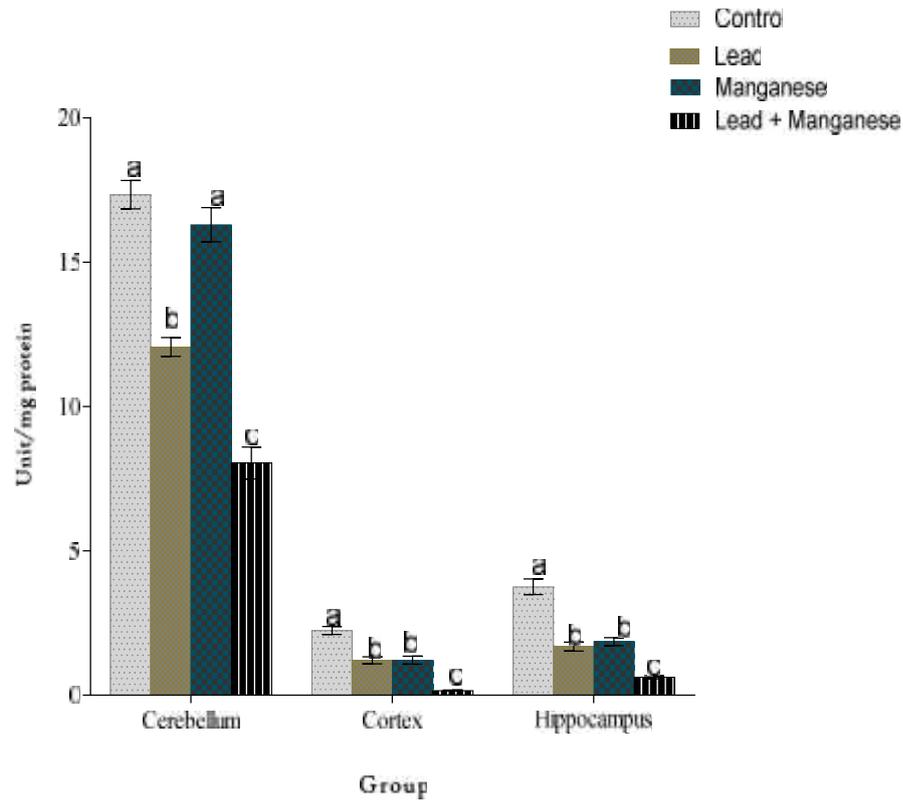
Total thiol groups were significantly ( $p < 0.05$ ) depleted by exposure to the metals singly and in combination (Fig. 7). Lead exposure caused 27%, 31% and 27% reduction in total thiol contents in the cerebellum, cortex and hippocampus, respectively, while manganese reduced same by 15%, 21% and 14% respectively. Total thiol groups were decreased the mixture of both metals by 43%, 23% and 34% respectively.



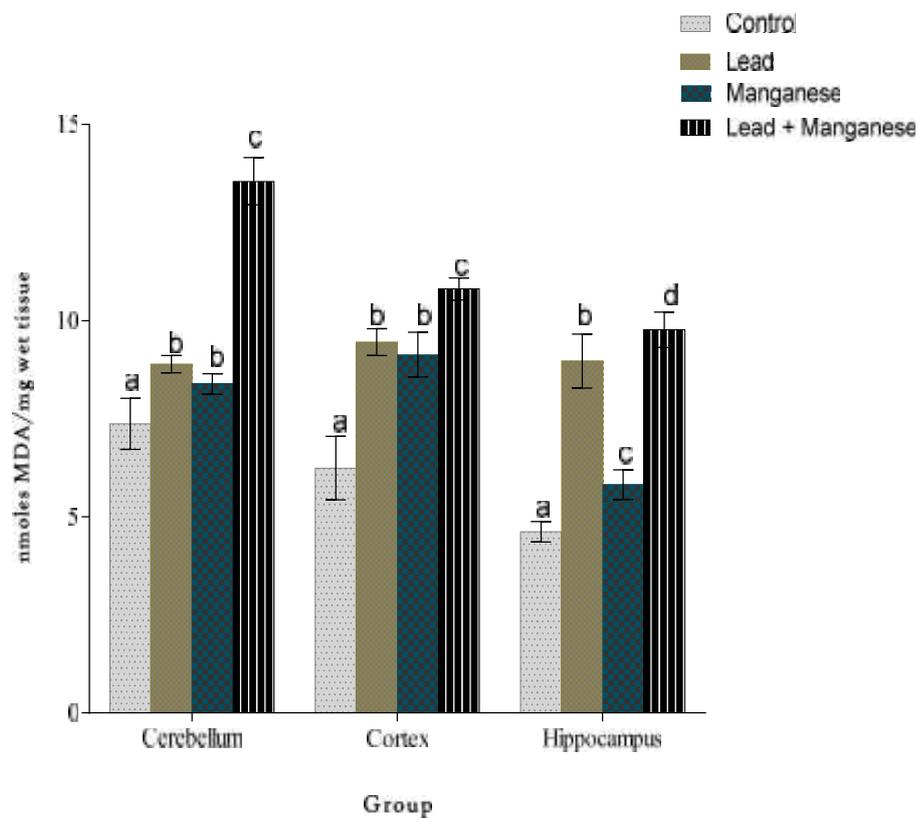
**Fig. 1:** Acetylcholinesterase Activity in Brain Regions of Rats Exposed to Lead, Manganese and Their Combination. Values are Mean  $\pm$  SD. Bars of the Same Compartment Carrying Different Letters of the Alphabet are Significantly Different from Each other ( $P < 0.05$ ).



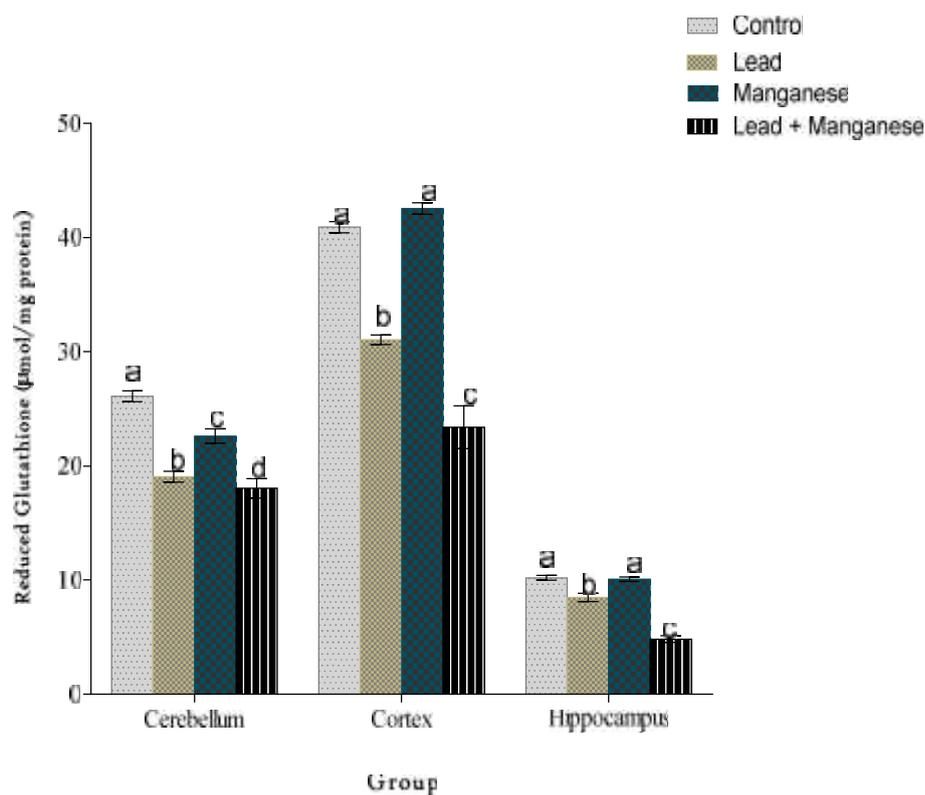
**Fig. 2:** Catalase Activity in Brain Regions of Rats Exposed to Lead, Manganese and Their Combination. Values are Mean  $\pm$  SD. Bars of the Same Compartment Carrying Different Letters of the Alphabet are Significantly Different from Each other ( $P < 0.05$ ).



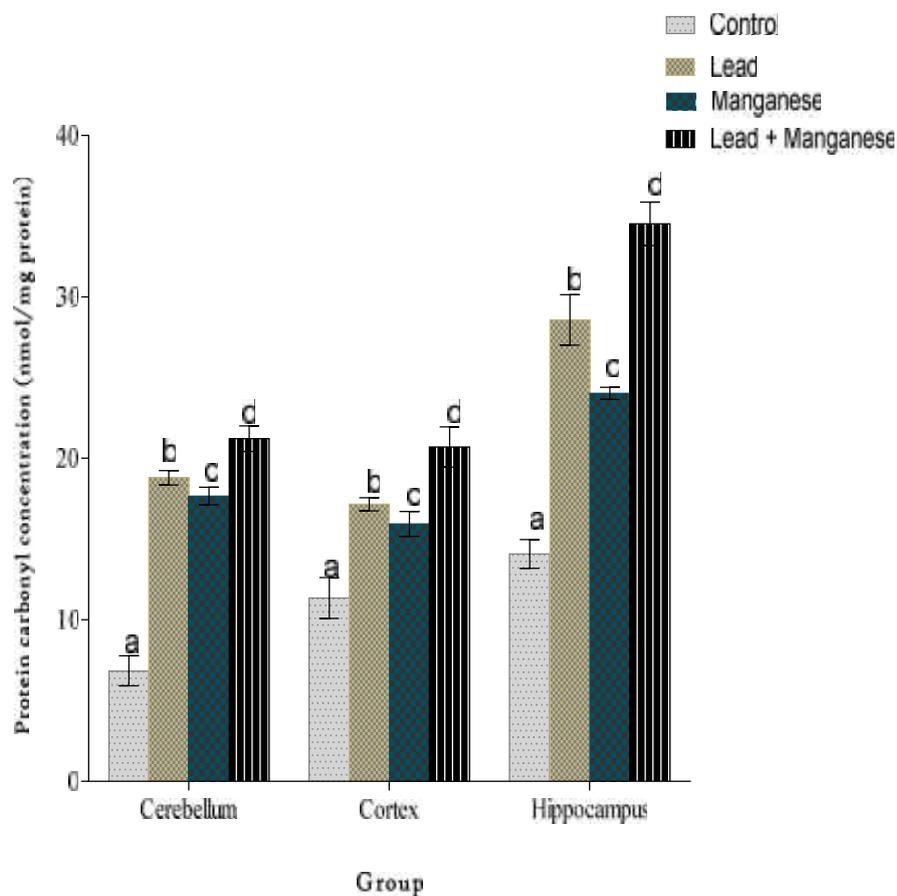
**Fig. 3:** Superoxide Dismutase Activity in Brain Regions of Rats Exposed to Lead, Manganese and Their Combination. Values are Mean  $\pm$  SD. Bars of the Same Compartment Carrying Different Letters of the Alphabet are Significantly Different from Each other ( $P < 0.05$ ).



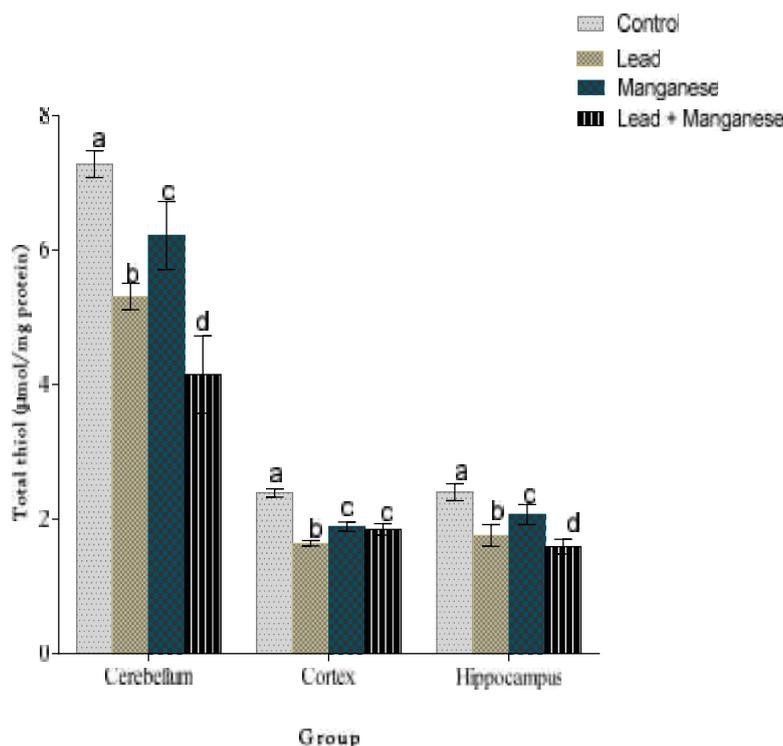
**Fig. 4:** Malondialdehyde Contents in Brain Regions of Rats Exposed to Lead, Manganese and their Combination. Values are Mean  $\pm$  SD. Bars of the Same Compartment Carrying Different Letters of the Alphabet are Significantly Different from Each other ( $P < 0.05$ ).



**Fig. 5:** Reduced Glutathione Contents in Brain Regions of Rats Exposed to Lead, Manganese and Their Combination. Values are Mean ± SD. Bars of the Same Compartment Carrying Different Letters of the Alphabet are Significantly Different from Each other (P < 0.05).



**Fig. 6:** Protein Carbonyl Contents in Brain Regions of Rats Exposed to Lead, Manganese and their Combination. Values are Mean ± SD. Bars of the Same Compartment Carrying Different Letters of the Alphabet are Significantly Different from Each other (P < 0.05).



**Fig. 7:** Total Thiol Concentration in Brain Regions of Rats Exposed to Lead, Manganese and their Combination. Values are Mean  $\pm$  SD. Bars of the Same Compartment Carrying Different Letters of the Alphabet are Significantly Different from Each other ( $P < 0.05$ ).

#### 4. Discussion

Exposure to Pb and excess Mn have been reported to enhance oxidative stress and instigate neurotoxicity (ASTDR 2007, Bokara et al. 2008). In this study, acetylcholinesterase activity and oxidative stress parameters were evaluated in different brain regions associated with the pathogenesis of neurodegenerative diseases (Griffiths et al. 1999, Andreassen et al. 1999). The present investigation demonstrated that combined exposure to lead and manganese effected such interactions that significantly elevated markers of neurotoxicity in the brain regions more than the individual metals alone.

A major hallmark in most neurodegenerative diseases include a marked reduction of nicotine acetylcholine receptors in brain regions such as the cerebral cortex and hippocampus (Oddo & LaFerla 2006). Among the earliest and severe biochemical changes to occur in such diseased states is the depletion of cholinergic markers such as acetylcholinesterase with the loss of the enzyme correlating with the degree of cognitive impairment (Perry & Perry 1980). The decreased activity of AChE may be associated with cholinergic hyperactivity, convulsion and status epilepticus (Olney et al. 1986). AChE inhibition may also precipitate symptoms such as confusion, headache and memory lapses (Ecobichon, 1996). Regarding AChE activity in this study, both lead and manganese caused a reduction in the cerebellum, cortex and hippocampus but their mixture did not display any interactive effect apart from the effect exhibited by the individual metals. AChE expression is substantially altered and its activity decreased in most brain regions in neurodegenerative diseases. AChE is an important regulator of neuronal processes, the decrease in AChE activity observed in our study further buttresses the suggestion that environmentally active metals contribute to the etiology of neurodegenerative diseases. The depletion of AChE could be ascribed to the increased lipid peroxidation provoked by the metals as a result of their generation of reactive oxygen species. Metal-induced increased lipid peroxidation rate has been reported to affect neurons, leading to the reduction of AChE (Flora et al. 2003).

Normal brain functions are protected from oxidative stress by the antioxidant defense system consisting of SOD, CAT and GSH

among others. The basis of the antioxidant enzymes protective ability is the rapid dismutation of superoxide anion to  $H_2O_2$  by SOD. In so doing, the superoxide anion is prevented from participating in Haber-Weiss reaction to generate hydroxyl radicals.  $H_2O_2$  is subsequently converted to water and oxygen by CAT to protect the cell from its oxidizing action. In the present study, SOD and CAT activities were significantly reduced in the brain regions of lead, manganese and Pb+Mn groups compared to control. Furthermore, their activities in the combined group was significantly lower than in all other groups with the exception of CAT activity in the cerebellum. The reduction in SOD and CAT activities as observed in this study implies that the oxidant capacity of the metals were increased following enhanced metal metabolism in the brain regions.

The brain with a high rate of oxidative metabolism but relative to other organs, contains low levels of enzymatic and non-enzymatic antioxidants. In addition, there are present a high concentrations of unsaturated lipids making it susceptible to oxidative stress (Bondy 1997). Studies have shown that excessive production of free radicals in brain and the imbalance between oxidative species and antioxidant defenses are linked to the development of neurodegenerative diseases (Halliwell 2006). Exposure to lead and manganese, as well as, their mixture resulted in significant increase in the level of MDA compared to the control in the cerebellum, cortex and hippocampus, with the combined treatment significantly higher than the other groups ( $p < 0.05$ ). Interestingly, our results indicate in the cerebellum that the coadministration of lead and manganese displayed an additive interaction in their induction of lipid peroxidation as measured by MDA. The cerebellum contains a higher content of polyunsaturated fatty acids (PUFA) and oleic acid compared to the cerebral cortex and hippocampus (Budowski et al. 1987). This higher amount of PUFA in the cerebellum makes it more vulnerable to lipid peroxidation which may explain the higher MDA concentration observed in that brain region in this study. Oxidative stress in the brain resulting from the generation of reactive oxygen species (ROS) has been suggested to be responsible for metal-induced neurotoxicity (Adonaylo & Oteiza, 1999, ATSDR 2007). The brain is particularly vulnerable to oxidative damage, and free radical injury is known to mediate pathways of neuronal damage

and death (Pratico & Delanty 2000). ROS attacks cell components including membrane lipids, producing lipid peroxide and with the brain been rich in fatty acids, there is therefore, the propensity for the disruption of cell membrane structural integrity with damage to lipids, proteins and DNA in the tissue.

Evidence suggests that metals could cause the impairment of antioxidant defense system in the brain (Shukla et al. 1996, Antonio et al. 2003). In the brain, GSH is the most abundant non-protein thiol that maintains the cellular redox status and provides first line defense against oxidative stress (Dringer et al. 2000). Thiols are potent chelators capable of mobilizing even intracellularly bound metals and also provide an antioxidant defense function by removing metals from the site of deleterious oxidant reactions (Lyn 2003). The reduced concentrations of GSH and TSH in this study, with the exception of the effect of manganese in both cortex and hippocampus, could thus, be due to an increase in their utilization resulting from the increased generation of free radicals by lead and manganese and the attendant lipid peroxidation in the brain. In the cortex and hippocampus, manganese did not alter the level of GSH in these two brain regions. However, in the presence of lead, manganese appeared to have caused a potentiation of the former, initiating the generation of more free radicals, leading to a greater depletion of both GSH and TSH.

Protein carbonyl groups were assessed as markers of oxidative protein damage in this study. Our data showed an increase in the protein carbonyl contents in the cerebellum, cortex and hippocampus of the brain of the metal intoxicated rats. In the single metal treated animals, lead generated more carbonyl groups than did manganese. However, the combined treatment with both metals elicited the most pronounced of protein carbonyl induction. This is of significance as sulfhydryl chemistry has been recognized to play an important role in normal biology and in the cellular defense against pro-oxidant agents, free radicals, and electrophiles. The disruption of thiol-disulfide homeostasis is key in the pathogenesis of many diseases. More importantly, in the brain the modification of critical cysteine residues is crucial in signal transduction (Mieyal et al. 2008).

In conclusion, it was found that, although either lead or manganese alone possesses neurotoxic characters, when co-administered, their neurotoxic nature increases. The increase emanated from both additive and potentiation effects induced by their interaction together. The neurotoxicity is prompted by alteration of acetylcholinesterase activity and free radical generation in the brain regions. The study thus, gives evidence of higher neurotoxic effects of combined lead and manganese exposure, as compared to lead and manganese exposure alone.

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