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EFFECT OF N-ACETYL L -CYSTEINE (NAC) AGAINST OXIDATIVE STRESS - INDUCED NEUROTOXICITY DUE TO LEAD, CADMIUM AND COMBINATION IN WISTAR RATS

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ABSTRACT

Lead (Pb) and cadmium (Cd) are potent neurotoxic heavy metals, which induce oxidative stress and membrane disturbances in brain. N Acetyl L cysteine (NAC) is an effective antioxidant and free radical scavenger against oxidative stress. The present study was designed to investigate the neuroprotective efficacy of NAC in protecting the Pb, Cd alone and co exposure induced changes in the activity of acetylcholinesterase (AChE), levels of lipid peroxidation, protein carbonyls, non-enzymatic antioxidants, enzymatic antioxidant status, membrane bound ATPases and histopathology in the brain of rats. To evaluate the neurotoxicity the present study was taken up with a total of 48 rats, divided uniformly into eight groups. The rats of group I and II were kept as control and NAC control @ 300mg/ kg given by oral gavage. Group III, IV and V served as toxic control group *viz.,* lead, cadmium and combination of lead + cadmium @ 1000, 300 and 1000+300 ppm, respectively given in the form of mash feed. Whereas group VI, VII and VIII served as NAC therapeutic groups (lead, cadmium and combination of lead + cadmium @ 1000, 300 and 1000+300 ppm, respectively and NAC @300mg/ kg). The experiment was carried out for 3 months and tissues were collected for antioxidant profile estimation and histopathological study was conducted at the end of the experiment. There was a significant decrease in concentrations of GSH, GST and significant increase in concentration of TBARS, protein carbonyl in the individual and co exposed toxic group and protein carbonyls formation was more significant in co exposed group, as other parameters were appreciably increased, thus represented synergistic effect lies in the co-administration of both metals. Similarly significant decrease in other parameters like Na^+K^+-ATPase, Mg^{2+}-ATPase, membrane transporter, lipid profile, and neurotransmitters like Ach and Glutamate were noticed. There was a significant alteration in the neurotransmitters in the co exposed group as compared to individual toxic group. Biometals like zinc (Zn) and copper (Cu) did not show any significant correlation with increased accumulation of lead and cadmium in toxic control group. Histopathology of brain revealed vacuolation and degeneration of few areas of cerebral cortex in lead exposed group 3, and mild to moderate degenerative changes and vacuolation in group 4, and lymphoid aggression and degenerative changes in purkinji layer cells in group 5.It concluded that the co existence of these metals showed positive pharmacodynamic interaction and NAC treatment showed significant improvement by replenish GSH pool in the biological system.

KEY WORDS: Rats, Lead, Cadmium, N Acetyl L cysteine, GSH, GST, TBARS, and Protein carbonyl.

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INTRODUCTION

Lead and cadmium are the common environmental heavy metal pollutants and have widespread distribution in air, water and soil in both urban and periurban areas[1]. Both natural and anthropogenic sources including mining, smelting and other industrial processes are responsible for human and animal exposure. In recent years, the levels of these heavy metals caused potential adverse effects on animal and human health[2]. Due to such concerns, the scientific community has focused its attention on the toxicological correlation between these trace metals. These pollutants, many a times, are co-pollutants leading to concurrent exposure to living beings and resultant synergistic deleterious health effects.

The central nervous system of newborn animals is highly susceptible to these metals, It has been demonstrated that Pb and Cd are particularly dangerous to the developing central nervous system (CNS) due to lack of a functional blood-brain barrier (BBB). Cadmium affects intercellular junctions, with abnormal topological distribution of tight junction proteins such as claudin-2, claudin-3 and claudin-5[3]. Lead exposure selectively alters the cellular level of claudin-1, which, in turn, reduces the tightness and augments the permeability of tight blood cerebrospinal fluid (CSF) barrier. The immature barrier appears to be more vulnerable to lead toxicity than the mature, well developed brain barrier, the fact possibly contributing to lead-induced neurotoxicity among young children[4].

Exact mechanism of action of lead and cadmium induced neurotoxicity is still unknown but oxidative stress could be response for their production of toxicity[2] and several other mechanisms may be involved in lead-induced neurotoxicity; one of the most important is the ability of lead to mimic or, in some cases, to inhibit calcium-mediated regulation of cell functions[5]. Other mechanisms that may be involved in lead toxicity include disruption of the pro-oxidant/anti-oxidant balance resulting in oxidative stress[6 and 7] and interference with nitric oxide (NO) production[8].

Lead has been shown to cause neurotoxicity by interfering with chemical neurotransmission in the central nervous system[9]. Numerous studies in humans and animals indicate that lead exposure can promote brain dysfunction and may present as behavioral changes and defective cognitive function [9, 10 and 11]. In addition to damaging the central nervous system, lead can also poison the motor neurons within the peripheral nervous system. The cholinergic system plays a pivotal role in many of the central nervous system functions, including cognitive behavior. Impairment of this system appears to be associated with the behavioral disturbances and deficit in learning and memory usually observed in humans and animals[9 and 12]. Lead also interferes with excitatory neurotransmission by glutamate[13]. The glutamate N-methyl d-aspartate (NMDA) receptor thought to be associated with neuronal development and synaptic plasticity thus disrupts long-term potentiation, which compromises the permanent retention of newly learned information.

The mechanisms implicated in cadmium-induced neuronal apoptosis[14] are lipid peroxidation[15]. Inhibition of striatal activity of Na^+/K^+ ATPase[16] as well as interaction with calcium, as cadmium can substitute it in calcium-calmodulin complex, inhibits the calcium pumps and channels, and replace it in various calcium receptors[17]. It is important to remark that interaction with calcium in the neurotransmission process has been implicated in alterations in the content and release of neurotransmitters[18]. The levels of excitatory neurotransmitters (glutamate and aspartate) were found decreased, while the inhibitory neurotransmitters (glycine and GABA) were increased in the amygdala of Cd -exposed animals, suggesting that Cd affects the balance in excitation/ inhibition of the synaptic neurotransmission[19]. Contents of dopamine, serotonine and norepinephrine in adult male rats were found decreased in all brain regions after a 24 h exposure to Cd[20]. Cd also disturbs the metabolism of Cu and Zn. The observed Cd^{+2}

induced alterations in the trace element metabolism may also be a contributory factor in disturbing the ontogenic profile of the lipids metabolism [21].

Most of the studies were carried out on exposure to a single metal but meager literature is available on co exposure of these metals. Some studies showed that co exposure of these metals had additive effects on acetylcholine release at the neuromuscular junction of frogs[22] and inhibited the activity of some enzymes in the brain[23]. While some studies showed that Pb and Cd had additive effects [23 and 25] found that combined exposure to Pb and Cd produced smaller effects than exposure to Pb or Cd alone. Because the long-term accumulation of metals in tissues is the major cause of metal toxicity[26], it is of interest to study the toxic effects of Pb and Cd when they are co-administered during the entire perinatal period.

Hence, the present study was undertaken to determine the toxico dynamic interaction of lead and cadmium alone, there co exposure and accessing the potency of NAC to counter effect.

MATERIALS AND METHODS

i. Chemical Reagents

Lead acetate, cadmium acetate and all other chemicals used in our experiments were purchased from SRL Pvt. Ltd., Mumbai.

ii. Animals

Male albino rats of *Wistar* strain weighing about 200-250 g were procured from National Institute of Nutrition, Hyderabad Andhra Pradesh. The animals used in this study were approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (approval no. 5/I/10/2010).

iii. Experimental procedure

The study was carried out on 48 female *Wistar* rats randomly divided into 8 groups with six rats in each group. All the groups were maintained as per the following schedule for 3 months.

Group 1: Normal control.
Group-2: N-acetyl-L-cysteine (NAC) @ 300 mg / kg body weight.
Group-3: Lead toxic control (lead acetate @ 1000 ppm in feed).
Group-4: Cadmium toxic control (cadmium chloride @ 300 ppm in feed).
Group-5: Lead and cadmium toxic control @1000 and 300 ppm, respectively, in feed.
Group-6: Lead and NAC, respectively @ 1000 ppm in feed and @ 300 mg / kg body weight
Group-7: Cadmium and NAC, respectively @ 300 ppm in feed and @ 300 mg / kg body weight
Group-8: Lead, cadmium and NAC, respectively @1000, 300 ppm in feed and @ 300mg / kg body weight

Lead and cadmium were used in the form of lead acetate and cadmium chloride given in the form of mash feed, NAC administered by oral gavage by diluting in the distilled water.

iv. Collection and preparation of samples

Brain tissues were collected at the end of 3rd month. Rats were fasted overnight and sacrificed by cervical decapitation, removed the brain, weighed, dissected and washed in ice-cold saline. Some pieces were immediately homogenized (1:10, w/v) in a cold (4^0c) buffer containing Tris base (20 mmol/L), EDTA (1 mmol/L), and sucrose (0.5 mmol/L), KCl (150 mmol/L) with the pH adjusted to 7.4. Homogenates were centrifuged at 4000 g for 20 min at 4^0c, and collected clear supernatant for estimation of lipid peroxidation levels and proteins using serum bovine albumin as the standard as per the procedure described by[27].

v. Measurement of Glutathione (GSH)

Brain GSH levels were measured as per the procedure described by[28]. 100 µl of 25% trichloroacetic acid was added to 400 µl of homogenate, centrifuged and collected supernatant and used as sample. To 2.0 ml of 0.6 mM 5-5' dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate (pH 8), 0.1 ml of sample and 0.9 ml of 0.2 M phosphate buffer was added and the absorbance was read at 412 nm against a

reagent blank. The standards (0.05-5 mg/ml) were also treated in the same way.

vi. Measurement of Glutathione S-transferase (GST)

Brain GST levels were measured as per the procedure described by[29]. Homogenate was centrifuged at 1,05,000 g for 60 min and supernatant was used as enzyme source. 1 ml of phosphate buffer, 0.1 ml of 1-Chloro-2, 4-dinitrobenzene (CDNB) and 0.1 ml of supernatant was taken into a cuvette and adjusted the volume to 2.9 ml with distilled water. The mixture was incubated for 5 min at 37^0 C. The reaction was started by addition of 0.1 ml of glutathione and absorbance was read for 5 min at 340 nm. Reaction mixture without homogenate was used as blank.

vii. Measurement of Thiobarbituric acid reacting substances (TBARS)

Brain TBARS levels were measured as per the procedure described by[30]. 500 µl of supernatant from the homogenate, 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid were taken in a tightly Stoppard tube. The tube was heated to boiling temperature for 45 min. cool the tube and the contents were centrifuged. The supernatant was read at 532 nm against blank. The concentration of test samples was obtained using molar extinction coefficient of MDA.

viii. Measurement of protein carbonyls

Brain protein carbonyl levels were measured as per the procedure described by[31]. 1 ml of homogenate supernatant containing 1 mg protein was added to 4 ml of 10 mM dinitrophenyl hydrazine (DNPH) in 2.5 M HCl. Samples were vortexed and incubated at room temperature for 1 hour in dark. Then protein was precipitated by adding 5 ml of 20% trichloroacetic acid and centrifuged at 3000 rpm for 10 min, supernatant was discarded to collect protein precipitate, which was washed thrice with 4 ml of ethanol: ethyl acetate (1:1) solution. Final protein precipitate was re-dissolved in 2 ml of 6 M guanidine HCl in 20 mM of potassium phosphate and kept at 37^0C for 10 min, and centrifuged to remove the insoluble substances and the absorbance was read at 372 nm against 2.5 M HCl blank. Known concentration of bovine serum albumin dissolved in 6 M guanidine HCl in 20 mM of potassium phosphate was used as standard.

ix. Measurement of Na^+/K^+ and Mg^{2+} ATPase

Na^+/K^+ and Mg^{2+} ATPase measured as per procedure described by[32]. Enzyme preparation (microsomal preparation) and ATP solution were thawed and kept on ice. Reaction mixture (0.5 ml) was taken into 2 test tubes. 0.2 ml of 10 mM ouabain was added to one tube, while equal amount of distilled water was added to the other. 100 µl of microsomal preparation (containing 3 mg protein/ml) was added to both the tubes and pre-incubated for 5 min. To make total volume of incubation mixture to 1 ml, 100 µl of water was added to both the tubes. Reaction was initiated by adding 100 µl of 30 mM ATP solution at room temperature. The reaction was terminated by adding 1 ml of 10% TCA after 30 min. Tubes were immediately transferred to ice. After 10 min, the tubes were centrifuged for 5 min to remove the precipitate. The supernatant was used for estimation of phosphate by the method[33]. The difference in the activity between the absence and presence of ouabain was taken as Na^+-K^+ ATPase activity. Activity in the presence of ouabain was taken as Mg^{2+} ATPase activity.

x. Estimation of lipids in organs

a. Extraction of lipids from organs was done as per procedure described by[34]
b. Estimation of total lipids in organs; The mixture obtained after washing thrice with chloroform – methanol – potassium chloride mixture (1:10:10 v/v) was evaporated to dryness and the amount of total lipids was obtained by gravimetric method (difference in the weights of empty centrifuge tube and

centrifuge tube with residue). The values were expressed as mg/ g tissue

c. Estimation of cholesterol in lipid extract; the total cholesterol was estimated by enzymatic method using standard kits supplied by Qualigens Pvt.Ltd., Mumbai.
d. Estimation of Phospholipids as per the procedure described by[35]. Lipid samples were transferred into clean glass tubes and the solvent was completely evaporated. To the residue, 0.65 ml perchloric acid was added and the tubes were placed in a heated block for about 30 min or until the yellow colour disappears. After cooling, 3.3 ml water, 0.5 ml of molybdate solution and then 0.5 ml of ascorbic acid solution were added to the test tubes. The tubes were agitated on a vortex after each addition. The tubes were placed in a boiling water bath for 5 min. The absorbance of cool samples (including the standards) was read at 800 nm. Standards (1 to 5 ug P/tube) were diluted in 3.3 ml of water and 0.65 ml perchloric acid. Digestion is not necessary before adding reagents. Classically, 5 µg of phosphate (P) gives an absorbance of 0.9. The amount of phosphorus was calculated directly on a molar basis from the amount of P and on a weight basis after multiplying the amount of P by 25. The values were expressed as mg/g tissue.

xi. Estimation of neurotransmitter

Brain was collected and quickly frozen in liquid nitrogen, weighed accurately and placed in pyrex glass tubes. These tubes were placed in boiling water for 5 min to terminate the action of AChE enzyme and also to release the bound ACh. The tissues were then homogenized in 1 ml phosphate buffer with pH 7.0. To the homogenate, 1 ml of alkaline hydroxylamine hydrochloride followed by 1 ml of 50% hydrochloric acid solution (1:1 HCl: H_2O) were added. The contents were mixed thoroughly and centrifuged. To the supernatant, 0.5 ml of 0.37 M ferric chloride solution was added and the brown colour developed was read at 540 nm against a reagent blank in a spectrophotometer as per described by[36]. The ACh content was expressed as µ moles of ACh/g wet weight of tissue. The glutamate content of the brain was estimated employing kits supplied by Bio Vision Research Products, 980 Linda Vista Avenue, mountain Avenue, CA 94043, USA. The colour developed was measured at 450 nm in a microplate reader. The concentration was expressed as nmol/µl or mM.

xii. Metal Analysis

The concentration of Pb and Cd estimated as per the procedure described by[37] by digesting the tissue samples in a reagent grade of nitric acid-perchloric acid (2:1) mixture until the samples became colorless. Then, the acid mixture was evaporated and the remaining precipitate was dissolved in a few drops of concentrated HCl. The samples were diluted in 10 mL of distilled water, and then the Pb and Cd concentrations were measured by Atomic Absorption Spectrophotometer (Model; F S 120), at department of Environmental pollutants and micro nutrients, Agricultural Research Institute, Rajendranagar, Hyderabad.

xiii. Micro nutrients analysis

Ashing procedure: Weigh sample into 50 ml quartz crucible and dry in an oven (120 ± 20ºC) overnight until the sample is thoroughly dry. Place the sample into cool muffle furnace and raise the temperature of the oven to 450 ± 20ºC (50ºC/h). Next day, remove the samples from the oven and cool to room temperature. Then treated with acid digestion and the final solutions of the samples are diluted in 0.2% nitric acid. Each batch should include reagent blank and control sample containing all the reagents in the same volumes. The concentration of Zn and Cu were estimated by Atomic Absorption Spectrophotometer as per the procedure described by[38] (model Varian 240) at department of Animal Nutrition, College of

Veterinary Science Rajendranagar, Hyderabad

xiv. Histopathology

Approximately 0.2g of brain tissue was preserved in 10% Neutral buffer formalin for histopathological study, the fixed tissues were processed and stained with Hematoxylin and Eosin (H & E) stain as described by[39] at department of Veterinary Pathology, College of Veterinary Science, Rajendranagar, Hyderabad.

xv. Analysis of variance

Results were expressed as mean ± S.E. One-way analysis of variance (ANOVA) by SPSS (Statistical Package for Social Sciences) (Ver. 10.00) followed by Duncan test was used to analyze the results with $p < 0.05$ considered significance.

RESULTS

1. Oxidative stress parameters

The concentration of GSH (µ moles/mg protein) and the activity of GST (µ moles/min/mg protein) in brain homogenate revealed a significant ($p<0.05$) decrease in the toxic control groups 3, 4 and 5 as compared to groups 1 and 2, and the lowest concentration of GST was observed in combination group 5, though it did not differ significantly as compared to individual toxic control groups 3 and 4 as depicted in the table 1. The concentration of GSH and GST in NAC treated groups 6, 7 and 8 showed a significant ($p<0.05$) increase as compared to that of toxic control groups and was comparable to groups 1 and 2

The concentration of TBARS (n moles of MDA released/mg protein) and protein carbonyls (n moles/mg protein) in brain homogenate revealed a significant ($p<0.05$) increase in toxic control groups 3, 4 and 5 as compared to groups 1 and 2 and protein carbonyls showed significant change in the co exposed group, while appreciable increased TBARS which was not significantly differed at 0.05% level. The TBARS concentration and protein carbonyls in NAC treated groups 6, 7 and 8 showed a significant ($p<0.05$) decrease as compared to that of toxic control groups and the values were comparable to groups 1 and 2.

Table1

Concentrations of oxidative biomarkers, hepatic enzymes lipid profile and glycogen in different groups of rats.

S.No	parameters	Control group		Toxic control group			Therapeutic group		
		Group1 control	Group2 NAC control	Group3 Lead	Group4 Cadmium	Group 5 Lead +Cd	Group 6 Lead + NAC	Group 7 Cadmium+ NAC	Group 8 Lead + NAC
1	GSH (µ moles/mg protein)	4.91±0.39^{bc}	5.07±0.34^{c}	2.36±0.24^{a}	2.60±0.14^{a}	2.49±0.30^{a}	4.02±0.35^{b}	4.35±0.18^{bc}	4.28±0.46^{bc}
2	GST (µ moles/min/mg protein)	2.86±0.32^{c}	2.83±0.16^{c}	0.89±0.03^{a}	0.91±0.06^{a}	0.85±0.04^{a}	2.32±0.12^{bc}	2.27±0. 12^{bc}	1.95±0.09^{b}
3	TBARS (n moles of MDA released/mg protein)	2.71±0.10^{a}	2.64±0.08^{a}	5.65±0.29^{b}	5.71±0.23^{b}	6.21±0.53^{b}	2.68±0.04^{a}	2.66±0.03^{a}	2.74±0.05^{a}
4	Protein carbonyls (n moles/mg protein)	0.56±0.02^{a}	0.64±0.03^{a}	2.19±0.10^{bc}	2.01±0.11^{b}	2.28±0.12^{c}	0.68±0.03^{a}	0.65±0.05^{a}	0.70±0.05^{a}
5	Na^{+}/K^{+} ATPase (µ moles of Pi liberated/mg protein/30 min)	8.08±0.26^{b}	8.03±0.22^{b}	3.60±0.12^{a}	3.56±0.07^{a}	3.08±0.10^{a}	7.85±0.14^{b}	8.12±0.16^{b}	8.13±0.11^{b}
6	Mg^{2+} ATPase (µ moles of Pi liberated/mg protein/30 min)	4.19±0.19^{b}	3.87±0.11^{b}	1.08±0.04^{a}	0.99±0.06^{a}	0.95±0.03^{a}	3.96±0.11^{b}	3.92±0.09^{b}	4.24±0.19^{b}
7	Concentration of Ach (µM/g)	17.60±0.47^{d}	18.03±0.90^{d}	3.54±0.28^{a}	6.21±0.89^{b}	5.57±0.57^{b}	13.48±0.52^{c}	16.39±0.47^{d}	14.27±0.24^{c}
8	Concentration of Glutamate (n moles/g)	21.07±0.53^{f}	20.73±0.34^{ef}	13.96±0.07^{a}	16.93±0.36^{b}	11.92±0.21^{c}	19.22±0.40^{d}	20.13±0.21^{de}	19.94±0.14^{de}
9	Total lipids (mg/g)	44.14±1.02^{a}	43.65±3.25^{a}	42.93±0.77^{a}	43.40±1.43^{a}	44.59±1.36^{a}	43.97±1.70^{a}	43.39±1.62^{a}	44.24±1.18^{a}
10	Cholesterol (mg/g)	2.02±0.09^{a}	2.08±0.13^{a}	4.66±0.03^{b}	4.60±0.05^{b}	4.67±0.06^{b}	2.11±0.19^{a}	2.12±0.16^{a}	2.29±0.11^{a}
11	Phospholipids (mg/g)	2.66±0.06^{a}	2.62±0.12^{a}	5.58±0.09^{b}	5.61±0.08^{b}	5.57±0.13^{b}	2.85±0.08^{a}	2.65±0.07^{a}	2.83±0.10^{a}
12	Zn levels(ppm)	8.95±0.94^{a}	8.91±0.86^{a}	9.04±0.92^{a}	8.70±0.79^{a}	9.20±0.98^{a}	8.87±0.83^{a}	9.11±0.92^{a}	9.04±0.87^{a}
13	Cu levels(ppm)	2.10±0.11^{a}	1.93±0.13^{a}	1.88±0.20^{a}	1.96±0.13^{a}	1.90±0.16^{a}	1.76±0.28^{a}	2.02±0.11^{a}	1.97±0.12^{a}
14	Pb levels (ppm)	BDL	BDL	6.91±0.19^{a}	BDL	7.09±0.24^{a}	1.63±0.07^{b}	BDL	1.58±0.07^{b}
15	Cd levels(ppm)	BDL	BDL	BDL	0.68±0.04^{a}	0.64±0.03^{a}	BDL	0.12±0.03^{b}	0.14±0.02^{b}

Values are mean ± standard error (n=6)

Means with different small alphabets as superscripts differ significantly ($p<0.05$) for vertical comparison

2. ***The activity of membrane bond enzyme Na^+/K^+ and Mg^{2+} ATPase***

The activity of Na^+/K^+ ATPase (µ moles of Pi liberated/mg microsomal protein/30 min) and activity Mg^{2+} ATPase (µ moles of Pi liberated/mg microsomal protein/30 min) in brain revealed a significant ($p<0.05$) decrease in the toxic control groups 3, 4 and 5 and lowest activity was noticed in the toxic combination group but did not significantly ($p<0.05$) differed with individual toxic control group as compared to groups 1 and 2. NAC treated groups 6, 7 and 8 showed a significant ($p<0.05$) improvement as compared to the toxic control groups and it was comparable to groups 1 and 2.

3. ***Lipid profile***

The mean cholesterol content (mg/g tissue) and mean concentration of phospholipids (mg/g tissue) in brain revealed a significant ($p<0.05$) increase in the toxic control groups 3, 4 and 5 as compared to groups 1 and 2, whereas mean total lipid concentration (mg/g tissue) revealed a non-significant difference in toxic control groups 3, 4 and 5 as compared to the remaining groups. NAC treated groups 6, 7 and 8 showed a significant ($p<0.05$) decreased in mean cholesterol content and mean concentration of phospholipids as compared to the toxic control groups.

4. ***Neurotransmitters***

The concentration of ACh (µM/g) in whole brain revealed a significant ($p<0.05$) decrease in the toxic control groups 3, 4 and 5 as compared to groups 1 and 2 and the lowest concentration was noticed in lead toxic control group 3, which was significantly ($p<0.05$) different as compared to other toxic control groups 4 and 5. Whereas the concentration of glutamate (nM/g) revealed a significant ($p<0.05$) decrease in the toxic control groups 3, 4 and 5 and the lowest concentration was found in combination toxic control group 5, which was differed significantly ($p<0.05$) when compared to other toxic control groups 3 and 4. The concentration of ACh and glutamate in NAC treated groups 6, 7 and 8 showed a significant ($p<0.05$) improvement and the values were comparable to groups 1 and 2.

5. ***Concentration of heavy metals and micronutrients***

The concentration of zinc and copper (ppm) in the brain was estimated as they are important in co factor regulation of certain anti oxidant enzyme, but brain homogenate revealed no significant differ in their concentration in the toxic control group.

The concentration of lead (ppm) in the brain revealed significant ($p<0.05$) increase in the toxic control groups 3 and 5 as compared to NAC treated groups 6 and 8, while it was not detectable in groups 1, 2, 3 and 6 whereas concentration of Cd (ppm) revealed a significant ($p<0.05$) increase in the toxic control groups 4 and 5 as compared to NAC treated groups 7 and 8 while it was not detectable in groups 1, 2, 4 and 7.

6. ***Histopathological studies***

The histopathology of brain revealed vacuolation and degeneration of few areas of cerebral cortex in lead exposed group 3 (Fig.1), and group 4 (Fig. 2) showed mild to moderate degenerative changes and vacuolation, while group 5 exhibited lymphoid aggression and degenerative changes around purkinje cells (Fig.3). The NAC treated group 6 showed mild degenerative changes (Fig. 4) and 8 showed mild vaculation in the cells (Fig. 5) when compared with their respective toxic control groups at the end of 3rd month. However, no evidence of pathological changes was observed in NAC group (Fig. 6).

DISCUSSIONS

Oxidative stress is a molecular mechanism that may explain Pb and Cd induced neurotoxicity[2].

The major forms of cellular damage in brain are lipid peroxidation, protein oxidation and thiol depletion. The present study demonstrated the elevated levels of lipid peroxidation by the thiobarbituric acid-reacting substances assay and protein carbonyls with reduced level of GSH and GST in the brain of lead and cadmium alone and co exposed and appreciable changes particularly in co exposed group treated rats might be due to the over production of free radicals and lipid peroxidative products, which leads to oxidative modifications of proteins. Oxidative damage to proteins leads to a loss of functional and structural efficiency, with increased levels of protein carbonyls, one of the most used biomarker of oxidative damage to proteins[40 and 41].

In the present study, the brain melondialdehyde (MDA) and protein carbonyls content increased significantly in the groups exposed to lead and cadmium alone as well as co exposure when compared to control group. Among exposure groups, co exposure group showed significantly increased levels of protein carbonyls where as MDA levels did not increase significantly at 5% level, thus representing the synergistic effect in the co-administration of both metals. These results are in agreement with[2] who revealed that perinatal exposure to low doses of Pb and Cd can produce alterations in lipid peroxidation and ultrastructure of rat brain and co-exposure to Pb and Cd produced more lipid peroxidation than exposure to Pb or Cd alone.

GSH is the most abundant non-protein thiol that maintains the cellular redox status and providing first line of antioxidant protection against oxidative stress in brain[42]. Thiols are potent chelators capable of mobilizing even intracellularly bound Pb and Cd and also provide an antioxidant defense function by removing them from the site of deleterious oxidant reactions[43]. GST have long been suspected to be important in protecting cells from oxidative stress by detoxifying some of the secondary reactive oxygen species (ROS) produced when they react with cellular constituents by catalysing conjugation of glutathione to the toxic reactive compounds[44]. Our study showed diminished levels of GSH and GST in lead and cadmium intoxication which could be due to increased utilization to overwhelm the production of free radicals by Pb and Cd and subsequent lipid peroxidation in brain. GSH is an important antioxidant defense, which forms complexes with Pb and Cd through the free sulphydryl group and, thereby, alters their distribution and excretion[45]. In agreement with this, the depletion of non-enzymic antioxidant levels in plasma may be due to increased utilization to reduce the Cd induced oxidative stress. The direct free radical scavenging ability of N Acetyl cysteine decreased the Pb and Cd induced lipid peroxidation, which could reduce the utilization of non-enzymic antioxidants and consequently leading to improvement of GSH, GST. These results are in accordance with the findings of[46 and 47], who reported the decreased GSH and increased GSSG and TBARS in brain tissue following lead exposure; brain GSH/GSSG ratio was dropped significantly. Similar results by[48 and 49] revealed that chronic Cd administration in rats resulted in a decline in GSH content, and a decrease in the activity of SOD and GST in rat brain and testis, indicating that Cd increases lipid peroxidation and oxidative stress. Treatment with NAC was able to counter these changes in this study.

Further, histopathplogy of brain in the present study revealed vacuolation and degeneration of few areas of cerebral cortex in lead exposed group 3, and mild to moderate degenerative changes and vacuolation in group 4, and lymphoid aggression and degenerative changes in purkinji layer cells in group 5.

Both these metals enhances the production of free radicals in the brain of adult rats and interfere with the antioxidant defense system which in turn leads to alteration of the structural integrity of membrane lipids and secondarily affect the membrane bound enzymes such as Na^+K^+-ATPase[50]. The saturation of lipid bilayer of several areas of

brain regions as a result of Lipid Per Oxidation (LPO) exposed animals causes disturbances in membrane fluidity and intracellular Ca^{2+} concentrations when exposed to these metals[51]. In the present study rats treated with Pb, Cd alone and their co exposed rats showed a significant inhibition in the activities of membrane bound ATPases which may be due to the increased membrane lipid peroxidation. The activity of these enzymes was improved in NAC-treated groups owing to the antioxidant potential. Similar reports of[52 and 53] which showed the decreased levels of membrane bound ATPase in the brain of cadmium intoxicated rats. Na^+K^+-ATPase is a key enzyme implicated in neural excitability, metabolic energy production. The role of Mg^{2+} ATPase is to maintain the high intracellular Mg^{2+} level in brain, changes of which can control the rate of protein synthesis and cell growth[54]. As transitional heavy metals compete with intra cellular Ca^{2+}, the alterations in Ca^{2+} level also leads to severe pathological lesions in brain[55]. The activities of the ATPase enzymes are affected by the exposure of Cd[56] indicating the alterations in membrane and neurotransmitter functions. The activities of Na^+/K^+ ATPase and Mg^{2+}-ATPase in brain and uptake of catecholamines have been found to be decreased by chronic *in vivo* Cd administration in rats and by *in vitro* Cd pre-incubation in rat brain synaptosomes[57]. An impairment of the cholinergic function was described in Pb-treated animals, including alterations in acetylcholine turnover rates[58]. The decreased activity of ATPases could also be due to the SH binding nature of Cd or through its oxidative stress in brain[56].

In the present study, estimation of the concentration of neurotransmitters ACh and glutamate revealed a significant reduction in the concentration of both in the toxic control groups; the lowest concentration of ACh was found in lead toxic group 3, whereas the lowest concentration of glutamate was found in lead control (group 3) and co exposed group (5). This data represented the positive pharmacodynamic interaction of co existence of these metals. An impairment of the cholinergic function was described in Pb-treated animals, including alterations in ACh turnover rate, AChE activity[59] and choline acetyltransferase activity[60]. Cd inhibited the K^+-evoked release of ACh, dopamine, serotonin, GABA and glutamate from rat brain slices[61]. Moreover, a decreased function of dopaminergic and serotonergic system of certain brain areas of rats after Cd exposure (21 days)[62]. On the other hand, decreased release of excitatory neurotransmitters – glutamate and aspartate, and an increased release of inhibitory neurotransmitters – glycine and GABA by 10–30 mM Cd using *in vivo* microdialysis in rat amygdala[63]. AChE was inhibited by high Cd concentrations *in vitro* and they found that Cd^{2+} is one of the metal inactivators of the enzyme (where Ca^{2+} is one of the activators) and is capable of inducing a conformational change in the protein, which leads to the formation of an ‘‘unreactive’’ enzyme[64].

Lipid molecules make-up between 30 and 80% of biological membrane by mass. Peroxidation of fatty acyl groups occurs mostly in membrane phospholipids. This in turn can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in several cellular dysfunctions, activation of lipid peroxidation can be correlated with the changes in lipid composition[65]. In our study, significant increased total cholesterol and phospholipids, but no significant difference in total lipid content was revealed. These results are in accordance with the findings of[66 and 67]; in brain exposed to cadmium and lead. As consequences of lipid peroxidation all the enzymes required for synthesis of lipid and cell membrane bound lipids get damaged. Phospholipidosis is due to enhanced cholesterogenesis or enhanced phospholipid biosynthesis due to enhanced free fatty acid availability[68]. Other studies have reported that decrease in total lipid content may be due to the decreased deposition of certain major lipid components[69]. A decrease in total lipid content was also reported by[70] who also

indicated that, Cd intoxication at an early stage of brain development retarded the level of cholesterol at the very initial stage of myelination, i.e day 15 of age (9%), which further decreased with the period of exposure, resulting in 15% decrease by the age of 45 days. A remarkable decrease in the cholesterol levels during postnatal development (20-60 days) was also reported in the whole brain of growing rat pups.

Present study also aimed to evaluate the correlation between micro minerals (Zn and Cu) with bio metals (lead and cadmium), In our results, the data concluded that there was no correlation exists in between Zn and Cu concentration and accumulation of biometals. The rate of accumulation of Pb and Cd in alone and their co exposed groups were significantly higher where as NAC treated groups showed significantly decreased accumulation as NAC chelates heavy metals. Several studies[71 and 72] showed that inhibition of micronutrient utilization by several metabolizing enzymes in the presence of Pb and Cd contributes to the accumulation of these micronutrients in tissues. As Pb and Cd replace them from antioxidant enzymes by false substitution, the antioxidant enzymes fail to function properly, which results in free radical-induced tissue damage.

HISTOPATHOLOGY FIGURES

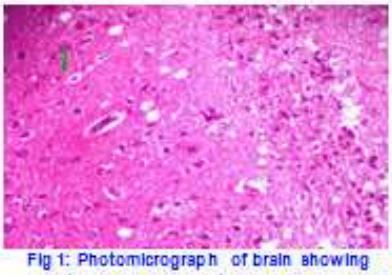

Fig 1: Photomicrograph of brain showing vacuolation and degeneration of few areas of cerebral cortex. H&E X200 (Group 3)

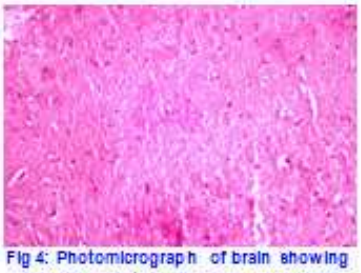

Fig 4: Photomicrograph of brain showing mild degenerative changes. H&E X200 (Group 6)

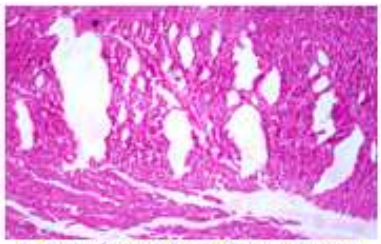

Fig 2: Photomicrograph of brain showing mild to moderate degenerative changes and vacuolation. H&E X200 (Group 4)

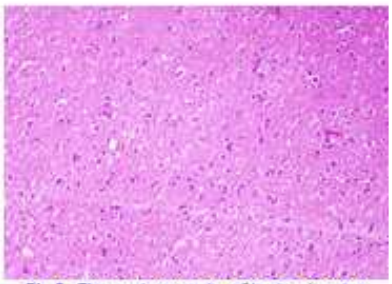

Fig 5: Photomicrograph of brain showing mild vaculation in the cells. H&E X200 (Group 8)

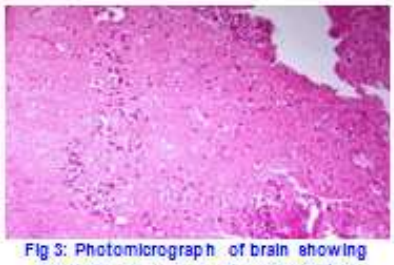

Fig 3: Photomicrograph of brain showing lymphoid aggression and degenerative changes around purkinje layer cells. H&E X200 (Group 5)

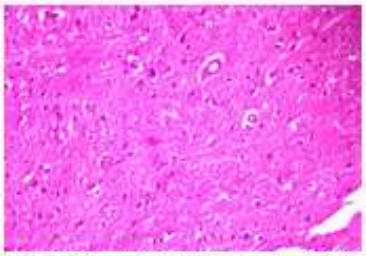

Fig 6: Photomicrograph of brain showing no lesions of pathological significance. H&E X200 (Group 2)

CONCLUSION

In conclusion, the present investigation enunciated that Pb, Cd and their combination induced toxicity to the biological system due to the excess generation of free radicals and impairment of antioxidant defenses. Toxic effects were more pronounced in the groups that received a combination of lead and cadmium suggesting positive toxicodynamic interaction. Supplementation of NAC as a chelating provided more pronounced effects particularly in the recovery of oxidative stress parameters suggesting that with the removal of Pb and Cd from the brain tissue, this antioxidant provides effective reversal in the altered parameters indicative of oxidative stress. It is, therefore, possible that restoration of altered anti oxidant parameters after concurrent NAC administration must be responsible for the decreased oxidative stress and consequently an oxidation of GSH. Reduced GSH is also utilized in the removal of lipid peroxide through the GPx reaction. Therefore, decreased level of TBARS after concurrent NAC treatment may save GSH from its higher utilization in this reaction and may eventually is responsible for the restoration of GSH status. Such a protective effect may be of great importance in view of the reports that GSH offers a first line of defense against lead toxicity[73].

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