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Comparative evaluation of N-acetylcysteine (NAC) and N-acetylcysteine amide (NACA) on glutamate and lead-induced toxicity in CD-1 mice

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Abstract

Recent studies indicate that there is interaction between the glutamatergic neurotransmitters system and lead neurotoxicity. Previously, we have demonstrated the potential effects of glutamate in lead-induced cell death in PC12 cells and the protective role of the novel thiol antioxidant, N-acetylcysteine amide (NACA). The current study 1) investigated the potential effects of glutamate on lead exposed CD-1 mice and 2) evaluated the protective effects of NACA against glutamate and lead toxicity in CD-1 mice, and 3) compared the results with N-acetylcysteine (a well-known thiol antioxidant). Oxidative stress parameters, including glutathione (GSH), oxidized glutathione (GSSG), GSH/GSSG, and malondialdehyde (MDA) levels, were evaluated. Blood and tissue lead levels, glutamate/glutamine (Glu/Gln) ratios, GS activity, and phospholipase-A₂ (PLA₂) were also analyzed. Results indicated that lead and glutamate decreased GSH levels in the red blood cells, brains, livers, and kidneys. Exposure to glutamate and lead elevated the MDA levels and PLA₂ activity. NACA and NAC provided protection against the detrimental effects of lead by decreasing the blood and tissue lead levels, restoring intracellular GSH levels, and decreasing the MDA levels. NACA and NAC also increased the GS activity thereby decreasing Glu/Gln levels. However, NACA appeared to have better chelating and antioxidant properties than NAC, due to its higher lipophilicity and its ability to cross the blood-brain barrier.

Keywords

NACA; NAC; antioxidant; glutamate; oxidative stress; lead; CD-1 mice

INTRODUCTION

Lead's toxicity is largely due to its capacity to mimic calcium and to become a substitute for it in many of the fundamental cellular processes that depend on calcium. Both are divalent cations; however, lead has the ability to disrupt the physiological effects of Ca²⁺ at very low concentrations (as compared to those of Ca²⁺) (Jadhav *et al.*, 2000). Lead is able to cross the BBB and may activate PKC by mimicking calcium. Furthermore, it alters the functioning of the enzyme, PKC, a virtually ubiquitous protein which is of crucial

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importance in numerous physiological functions. Increased PKC activities modulate glutamate receptors by releasing neurotransmitter (glutamate) (Chen and Huang, 1992). Sodium ions may enter a cell through glutamatergic receptors, in conjunction with calcium, and thereby depolarize the cell membrane. Depolarization, in turn, opens voltage sensitive calcium channels through which lead may enter the cell. Activation of glutamate receptors and the opening of a calcium channel may increase intracellular calcium levels. Lead may activate calcium-sensitive enzymes, including phospholipase (PL) and through activation of PL, inositol 1, 4,5-triphosphate (IP3) and diacylglycerol (DAG) are formed. IP3 releases calcium from intracellular stores and DAG activates PKC which, in turn, has a key role in the production of reactive oxygen species (ROS).

Activation of phospholipase has a role in the generation of platelet-activating factor arachidonic acid, which inhibits the reuptake of glutamate released from synaptic space. This vicious cycle generates ROS that leads to widespread neuronal damage. On the other hand, lead may affect astrocytic function, because of its longer half lives in astrocytes. The function of astrocytes is to convert potentially toxic glutamate to non-toxic glutamine catalyzed by an astrocyte-specific enzyme called glutamine synthetase (GS). Since lead has broad coordination chemistry and the ability to bind sulfhydryl groups, it may inhibit GS by blocking sulfhydryl groups or by displacing Mg^{2+} at the active site of the enzyme. This leads to further accumulation of glutamate. Glutamate toxicity has been implicated in many neurodegenerative conditions and its role in lead toxicity cannot be ignored. The interaction of glutamate with lead may result in neuronal damage. The toxicity of glutamate generally involves increased Ca^{2+} influx, increased generation of ROS, mitochondrial dysfunction, and oxidative stress which, in turn, may cause apoptosis (Chandra *et al.*, 2000). In a nutshell, glutamate excitotoxicity, lead neurotoxicity, and oxidative stress are considered to be related phenomena causing neuronal damage (Naarala *et al.*, 1995; Loikkanen *et al.*, 2003).

Therefore, antioxidants with strong chelating potential play a major role in the treatment of lead poisoning because oxidative stress, induced by the presence of lead in several organs, appears to be a component of the molecular mechanism of lead toxicity. Induction of harmful free radicals by lead, and subsequent depletion of a cell's antioxidant defenses, can result in generalized disruption of the prooxidant/antioxidant balance in Pb-burdened tissues and contribute to tissue injury via oxidative damage to critical biomolecules (Ercal *et al.*, 2001). Glutathione (GSH) is the principal intracellular thiol responsible for scavenging ROS and maintaining the oxidative balance in tissues. Cysteine and glutathione delivery compounds have been used to protect normal cells from redox imbalance (Ozols *et al.*, 1988). Therefore, thiol-containing compounds have gained special attention due to their profound role in maintaining tissue redox balance. In the past decade, a large number of natural thiols were used to combat lead-induced toxic effects. One of the most widely used thiol antioxidants has been N-acetylcysteine (NAC). The natural L-isomer of NAC (LNAC), a mucolytic agent and the drug of choice in acetaminophen (Tylenol) intoxication, indirectly replenished GSH through deacetylation to cysteine, preventing oxidative damage through scavenging of ROS. However, NAC has numerous side effects, including being pro-oxidant, suppressing respiratory burst, and causing a toxic ammonia accumulation in case of liver problems. Moreover, NAC's failure to provide significant antioxidant effects *in vivo*, may be due to its low solubility and tissue distribution (Cotgreave *et al.*, 1987; Cotgreave, 1997).

Researchers have recently developed a new strategy for improving the pharmacokinetic properties of these agents. This was achieved by neutralizing the carboxylic group of NAC, which made the drug more lipophilic and cell-permeating. This compound was shown to cross the blood-brain barrier, scavenge free radicals, chelate copper, and attenuate myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis in a multiple sclerosis mouse model (Offen *et al.*, 2004). Further evidence of the efficient

membrane permeation of NACA was demonstrated in a recent study (Grinberg *et al.*, 2005) which assessed NACA to determine its antioxidant and protective effects in a model using human red blood cells. Our findings, with regard to NACA, also convince us that it has the ability to protect against HIV protein-induced oxidative effects (Banerjee *et al.*) and radiation-induced oxidative damage (Wu *et al.*, 2008). In our research report, we show that NACA protects PC-12 cells against glutamate-induced cytotoxicity by preventing glutamate-induced loss of cellular GSH and inhibiting lipid peroxides (Penugonda *et al.*, 2005; Penugonda *et al.*, 2006). In addition, our recent findings provide evidence that NACA has better antioxidant and chelating properties when compared to those of NAC *in vitro* (Ates *et al.*, 2008). With this background, we evaluated the protective effects of NACA against the toxic effects of glutamate and lead and compared them to the effects of NAC in an *in vivo* model.

MATERIALS AND METHODS

The N-(1-pyrenyl)-maleimide (NPM) was purchased from Aldrich (Milwaukee, WI, USA). N-acetylcysteine amide (NACA) was purchased from David Pharmaceuticals, USA. High-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). The PLA2 kit was purchased from R&D Systems, Inc. MN. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Animal study design

All procedures involving animals in this study are consistent with the guidelines set by the National Institute of Health have been reviewed and approved by the Animal Care and Use Committee at Missouri University of Science and Technology. Adult male CD-1 mice, weighing 38-40g, were obtained from Charles River Laboratories. They were housed in a temperature-controlled (25 °C) room that was equipped to maintain a 12 h light-dark cycle. Standard rat chow (Purina rat chow) and water were given *ad libitum*, over the entire study. No change in food or water consumption was observed among the groups throughout the study. Also, there were no significant changes in the animals' weights. Twenty-four mice were randomized into six groups (n=4).

- I. Control: no lead acetate, no NAC, no NACA
- II. Lead only 1300: lead acetate (1300 ppm), no NAC, no NACA
- III. NAC only: no lead acetate, NAC (500 mg/kg body weight NAC orally mM), no NACA
- IV. NACA only: no lead acetate, no NAC, NACA (500 mg/kg body weight NACA orally)
- V. Lead 1300+NAC: lead acetate (1300 ppm), NAC (500 mg/kg body weight NACA orally), no NACA
- VI. Lead 1300+NACA: lead acetate (1300 ppm), no NAC, NACA (500 mg/kg body weight NACA orally)

Group I, III and IV (n=4) animals received deionized water for 5 weeks. Group II, V, and VI (n=4) animals were exposed to 1300 ppm lead acetate in their drinking water for the same 5 weeks. During the 6th week, Group I served as the control and these mice were given only standard rat chow and deionized water. Animals in Group II received standard rat chow and deionized water. Groups III and IV were used as NAC and NACA only groups. Group III received 500 mg NAC/kg body weight orally in drinking water whereas Group IV was given 500 mg NACA/kg body weight orally in drinking water. Animals in Group V received 500 mg NAC/kg body weight orally in drinking water after a 5-week lead treatment. Animals in

Group VI received 500 mg NACA/kg body weight orally in drinking water after a 5-week lead treatment. At the end of the 6th week, the animals fasted overnight and were anesthetized with urethane intraperitoneally. Blood samples were collected with lead-free needles via intracardiac puncture. The animals were then sacrificed and samples of the livers, kidneys, and brains were obtained. Tissue samples were kept in an antioxidant buffer containing mono and dibasic sodium phosphates, butylated hydroxytoluene, diethylenetriaminepentaacetic acid, aminotriazole, and sodium azide, at -70°C until their analyses. 50 μL of whole blood was used for blood lead analysis while the remainder of the blood was centrifuged at 3000 rpm for 10 min to obtain red blood cells (RBCs). The RBCs were analyzed in 24 hours for oxidative stress studies and kept at 4°C until their analyses. The measurements in the current study for NACA and NAC only groups did not show statistically significant differences between them and measurements of the control group. Therefore, the data obtained for NACA and NAC only groups is not included in the figures and tables.

Animal study design to evaluate the potential effects of glutamate on lead exposed CD-1 mice

Sixteen mice were randomized into four groups (n=4):

- I.** Control: no lead acetate, no GLU (glutamate)
- II.** Lead only 1300: lead acetate (1300 ppm orally)
- III.** GLU only: no lead acetate, GLU (2000 mg/kg body weight GLU orally)
- IV.** Lead 1300+GLU: lead acetate (1300 ppm), GLU (2000 mg/kg body weight GLU orally)

Group I and III (n=4) animals received deionized water for 5 weeks. Group II, and IV (n=4) animals were exposed to 1300 ppm lead acetate in their drinking water for the same 5 weeks. During the 6th week, Group I served as the control and these mice were given only standard rat chow and deionized water. Animals in Group II received standard rat chow and deionized water. Group III was used as the GLU only group, and received 2000 mg GLU/kg body weight orally in drinking water. Animals in Group IV were given 2000 mg GLU/kg body weight orally in drinking water after 5-weeks of lead treatment. At the end of the 6th week, the animals were fasted overnight and anesthetized with urethane intraperitoneally. Blood samples were collected with lead-free needles via intracardiac puncture. The animals were then sacrificed and samples of their brains were obtained. Brain tissue samples were kept at -70°C in an antioxidant buffer containing mono and dibasic sodium phosphates, butylated hydroxytoluene, diethylenetriaminepentaacetic acid, aminotriazole, and sodium azide, until they were analyzed.

HPLC System

The HPLC system (Thermo Electron Corporation) consisted of a FinniganTM SpectraSYSTEM SCM1000 Vacuum Membrane Degasser, FinniganTM SpectraSYSTEM P2000 Gradient Pump, FinniganTM SpectraSYSTEM AS3000 Autosampler, and FinniganTM SpectraSYSTEM FL3000 Fluorescence Detector ($\lambda_{\text{ex}} = 330\text{nm}$ and $\lambda_{\text{em}} = 376\text{nm}$). The HPLC column was a Reliasil ODS-1 C_{18} column (5 μm packing material) with 250×4.6 mm (Column Engineering, Ontario, CA, USA). The mobile phase was 70% acetonitrile and 30% water and was adjusted to a pH of about 2.5 through the addition of 1 mL of both acetic acid and o-phosphoric acid. The NPM derivatives were eluted from the column isocratically at a flow rate of 1mL/min.

Determination of GSH

Cellular levels of GSH were determined by using the method of Winters *et al.* (Winters *et al.*, 1995). Cell or tissue samples were homogenized in serine–borate buffer. Twenty (20) μL of the diluted homogenate were added to 230 μL of HPLC grade water and 750 μL of N-(1-Pyrenyl)maleimide (NPM, 1 mM in acetonitrile). The resulting solutions were incubated at room temperature for 5 minutes. The reaction was stopped by addition of 5 μL of 2N HCl. The samples were then filtered through a 0.2 μm filter and injected into the HPLC system.

Determination of GSSG

GSSG was measured by incubation of 40 μL straight tissue homogenate, 44 μL of HPLC grade water, and 16 μL of 2-vinyl pyridine for 1 hour in order to block any preexisting GSH. After 1 hour, 95 μL of 2mg/ml solution of NADPH and 5 μL of a 2 unit/ml glutathione reductase were mixed with the solution. A 100- μL aliquot of this solution was then quickly removed and mixed with 150 μL of HPLC grade water and 750 μL of NPM (1 mM in acetonitrile). After a 5-minute incubation period, the reaction was stopped by adding 5 μL of 2N HCl. The samples were filtered through a 0.2 μm pore size filter and injected into the HPLC system (Winters *et al.*, 1995).

Determination of MDA

To prepare the solution, 350 μL of straight cell homogenate, 100 μL of 500 ppm BHT (butylated hydroxytoluene), and 550 μL of 10% TCA (trichloroacetic acid) were combined and boiled for 30 minutes. The tubes were cooled on ice and centrifuged for 10 minutes at 2500 rpm. Five hundred (500) μL of the supernatant were removed and 500 μL of TBA (thiobarbituric acid) were added. The tubes were boiled again for 30 minutes, and then cooled on ice. From this solution, 500 μL were removed, added to 1.0 ml of n-butanol, vortexed, and centrifuged for 5 minutes at 60 g to facilitate a phase separation. The top layer was then filtered through 0.45 μm filters and injected onto a 5 μm C₁₈ column (250 $\times$ 4.6 mm) on a reverse phase HPLC system. The mobile phase consisted of 69.4% 5mM sodium phosphate buffer (pH = 7.0), 30% acetonitrile, and 0.6% THF (tetrahydrofuran). The excitation wave length was 515 nm; the emission wave length was 550 nm (Draper *et al.*, 1993).

Determination of Glutamate and Glutamine

Glutamate and glutamine levels were determined by using the method of Tcherkas. (Tcherkas and Denisenko, 2001). One hundred and twenty (120) μL of the diluted tissue homogenate were added to 380 μL of methanol to precipitate the protein by centrifugation at 6000 g for 5 minutes. Forty (40) μL of supernatant, or amino acid standard mixture, were mixed with 80 μL of 0.8 M Iodoacetic acid solution (pH-10.5) and 120 μL of 0.1 M sodium borate buffer (pH-11.5). After a 30-second incubation time at room temperature, 20 μL of OPE-2ME (200 μL of 0.4 M o-phthalaldehyde mixed with 400 μL of 2-Mercaptoethanol plus 1400 μL of 0.1 M sodium borate buffer) reagent were added to the sample. After a 3-minute incubation time, 10 μL of reaction mixture were injected on the HPLC system. These o-phthalaldehyde-2-mercaptoethanol-fluorescent adducts were separated within 30 minutes on a Reliasil ODS-1 C₁₈ column with an isocratic elution using 17% methanol, 0.04 M sodium phosphate buffer (pH 7.0), and 0.002 M Na₂EDTA, and detected fluorimetrically (excitation 340 nm, emission 450 nm).

Glutamine synthetase measurement

Glutamine synthetase activity was determined spectrophotometrically by the method of Kingdon *et al.* (Kingdon *et al.*, 1968) and was expressed in units/mg protein, as described in

Sigma assay procedures. The assay was based on the regeneration of hydrolyzed ATP, coupled with the oxidation of NADH in the presence of excess phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase. This method measured the exponential disappearance of NADH at 340 nm in the presence of cellular homogenates.

Phospholipase A₂-Assay

This assay was performed in accordance with the procedure provided with the Phospholipase A₂- Assay kit (R&D Systems, Inc. MN). This assay used a specific substrate for PLA₂ that converted into a sulfhydryl molecule. The presence of the sulfhydryl product was detected colorimetrically using Ellman's reagent, DTNB, which formed a yellow product with the sulfhydryl. The amount of PLA₂ in the sample was compared to the amount of PLA₂ in the standard by evaluating the yellow color generated.

Blood and Tissue Lead Determinations

The blood samples were diluted in Triton X-100 (5%) and mixed well to disrupt the cells. The solution was then analyzed by graphite furnace AA, using a phosphate modifier. An ashing step was included in the furnace temperature program to eliminate the organic matrix, and deuterium background correction was used along with the secondary Pb absorption line at 283.3 nm to minimize the background that might be present. These were run along with whole blood QC samples to assure accuracy. Aqueous standards were used for a 5-point calibration curve.

The main difference in the blood and tissue analysis was basically the sample preparation. Tissue samples were weighed and dissolved in concentrated nitric acid (0.15g tissue/ml acid), and then allowed to digest for at least 24 hours. A dilution was prepared from these solutions and standards were prepared in a similar matrix.

Determination of Hemoglobin

Hemoglobin levels were measured by the method of Drabkin (Drabkin *et al.*, 1944). 50 μ L of diluted RBCs were added to 2.5 ml of Drabkin's reagent, containing 0.2 g/L K₃Fe(CN)₆, 0.05 g/L KCN, 1.0 g/L NaHCO₃. The absorbance measurements were taken at 540 nm.

Determination of protein

The Bradford method was used to determine the protein content of the tissue samples (Bradford, 1976). Concentrated Coomassie Blue (Bio-Rad) was diluted 1:5 (v/v) with distilled water; then 2.5mL of the diluted dye was added to 50 μ L of the sample. The mixture was incubated at room temperature for 5 minutes and absorbance was measured at 595 nm by a spectrophotometer. A standard curve was constructed by using bovine serum albumin (BSA) ranging between 0-1mg/ml.

Statistical analyses

The data was given as the mean $\pm$ SD. Statistically significant analysis was determined using analysis of variance (ANOVA), followed by Tukey's multiple comparison test ($p < 0.05$ was considered significant).

RESULTS

Antioxidant and chelator effects of N-acetylcysteine and N-acetylcysteine amide on lead-exposed CD-1mice

Blood and Tissue Lead Levels—Blood, liver, kidney, and brain lead levels were determined using Graphite Furnace Atomic Absorption (Table 1). Lead levels in the blood

of lead-treated animals were $48 \pm 3 \mu\text{g/dL}$, which represented a significant elevation when compared to the control group. Blood lead levels were reduced to $37 \pm 3 \mu\text{g/dL}$ by NACA treatment and to $43 \pm 3 \mu\text{g/dL}$ by NAC treatment.

The results of lead determinations in brain, kidney, and liver tissues were consistent with lead levels found in the blood. Brain, kidney, and liver lead levels were significantly elevated in the lead-treated group as compared to those of the control group. NACA (500mg/kg) and NAC (500mg/kg) treatments significantly reduced the tissue lead levels (Table 1). However, NACA showed a better chelating ability when compared to NAC.

GSH and GSSG Results—GSH and GSSG levels were measured in RBCs, as well as in the livers, brains, and kidneys in all groups. Figures 1, 2, and 3 shows the GSH, GSSG and GSH/GSSG values obtained from the RBCs. Oral administration of lead acetate to mice decreased glutathione/glutathione disulfide ratios. Administration of NACA (500mg/kg), or NAC (500mg/kg), to lead-treated mice for 7 days, prior to sacrifice, significantly enhanced the GSH levels in the RBCs ($p < 0.05$). However, in the NAC-treated group the GSH levels were not as high as those in the control group, whereas treatment with NACA had increased the GSH levels slightly above those of the control mice's RBCs. A significant increase in GSSG levels was observed in the RBCs of mice treated with lead for 5 weeks (Group II). In both the NACA and NAC-treated groups, GSSG levels were returned to the same levels as those in the control mice's RBCs.

The GSH and GSSG levels and the GSH/GSSG ratio of the brain and liver tissues can be seen in Tables 2 and 3, respectively. In both tissues, lead exposure significantly decreased the GSH levels, along with the GSH/GSSG ratios, and elevated the GSSG levels. In the livers, administration of NACA or NAC, following lead exposure, resulted in considerable improvement in GSH levels. However, the increase in GSH levels with NAC treatment, after lead exposure, was not statistically significant.

Interesting results were observed from examination of kidney tissue samples. Lead exposure enhanced the GSH levels in the kidneys, but not significantly, as opposed to levels in the RBCs and other tissues. In the kidneys, administration of NACA, following lead exposure, resulted in a significant increase in GSH levels, whereas NAC treatment showed only a slight increase in GSH levels when compared to that of the lead-exposed group (Table 4).

MDA Results—Lead exposure enhanced lipid peroxidation by elevating the MDA levels. As shown in Figure 4, MDA levels in the RBCs of lead-exposed animals' were higher than those of the controls. Treatment with NACA (500 mg/kg) or NAC (500 mg/kg) significantly decreased the MDA levels in RBCs.

The MDA levels in the brains (Table 2), livers (Table 3), and kidneys (Table 4) of the lead-exposed group were significantly elevated, when compared to the control group's MDA levels. Mice that received NACA or NAC during the final week of the experiment, after lead exposure, had significantly reduced MDA levels in their tissue. NACA was shown to be efficient in suppressing elevated MDA levels due to lead treatment.

Glutamate and Glutamine Results—Glutamate and glutamine levels were measured in brain tissue samples; Table 2 shows the Glu/Gln values obtained from these samples. More than a two-fold increase was observed in the Glu/Gln values in lead-exposed animals, as compared to those of controls. Groups that received NACA had lower ratios than those of lead-exposed animals that were not treated.

Glutamine Synthetase Results—With reference to the above data, glutamine synthetase activity was significantly decreased in the lead-exposed group. NACA and NAC reversed the decrease in GS activity, following lead exposure (Table 2).

Potential effects of glutamate on lead-exposed CD-1 mice

GSH Results—GSH levels were measured in the RBCs, as well as in the brains of all groups. Oral administration of lead acetate to mice decreased GSH levels, while administration of glutamate (2g/kg) potentiated the effect by further decreasing the GSH levels. Similar results were even observed in brain tissue samples (Table 5).

MDA Results—MDA levels in the RBCs and brains of the lead-exposed group were significantly elevated when compared to the control group's MDA levels. Inclusion of glutamate enhanced the lipid peroxidation by further elevating the MDA levels (Table 5).

Phospholipase A₂ activity—The activity of PLA₂ was measured only in brains (Table 5). More than a three-fold increase in the activity of PLA₂ was observed in lead-exposed animals, as compared to that of the controls. Co-exposure of lead and glutamate resulted in almost a five-fold stimulation of PLA₂ activity.

DISCUSSION

Recent studies indicate that lead-induced toxicity has a glutamatergic component (Lasley and Gilbert, 2000). The toxic effects of lead are attributed to its deposition in astrocytes and its continuous release into the synaptic spaces (Holtzman *et al.*, 1987). This may result in the inhibition of astrocytic function. Astrocytes play an important role in the conversion of potentially toxic glutamate to non-toxic glutamine by the enzyme – glutamine synthetase. This leads to excessive accumulation of extracellular glutamate with lead exposure. Furthermore, with lead exposure, expressions of neuronal and glutamate transporters in adult brains were shown to result in impaired clearance of glutamate (Struzynska *et al.*, 2005). Most noteworthy with this scenario is that, in the treatment of lead poisoning, a therapeutic agent should not only chelate lead from systemic circulation but also from different sites. It is well known that brain tissue is particularly vulnerable to lead. The major concern with current conventional chelators is their poor BBB permeability and their harmful side effects.

In this study, we evaluated the involvement of a glutamatergic component in lead-induced toxicity and also investigated a compound, NACA, as a potential chelator against the entire toxic phenomenon of lead in comparison with NAC in an *in vivo* model.

NACA was found to be effective in lowering blood lead levels, when compared to NAC (Table 1). This may be attributed to the greater permeability of NACA, than that of NAC. It is well known that lead has a high affinity for thiol groups and depletes GSH levels. GSH plays an important role in antioxidant defense and redox regulation (Drake *et al.*, 2002). In previous studies, it was shown that, after 5 weeks of lead exposure, GSH levels in mice were decreased (Ercal *et al.*, 1996). Furthermore, we also showed that GSH levels in PC-12 cells exposed to lead were significantly decreased, as compared to the control values (Aykin-Burns *et al.*, 2005). Studies indicate that a decreased cellular GSH level is cytotoxic when followed by the depletion of ATP (Redegeld *et al.*, 1992). Depletion of GSH may result in mitochondrial dysfunction (Heales *et al.*, 1995) because electron transport complexes are susceptible to the attack of ROS *in vitro* (Benzi and Moretti, 1995). It has been reported that GSH has an important role in protecting against the mitochondrial damage induced by sustained exposure to ROS (Bolanos *et al.*, 1996). Furthermore, depletion of GSH, induced by glutamate exposure, was shown to be involved in the impairment of energy metabolism

(Pereira and Oliveira, 2000). This may be due to decreased GSH levels, followed by ROS accumulation, and mitochondrial dysfunction. When taken together, lead and glutamate toxicity disturbs Ca^{2+} homeostasis, mitochondrial dysfunction, generation of ROS and ultimately leading to oxidative stress. Our results indicated that decreased GSH levels are accompanied by increases in GSSG levels in RBCs, livers, and brain tissues, following exposure to lead. The ratio of GSH/GSSG was proposed as a sensitive indicator of oxidative stress, and our data supports a decrease in this ratio in lead-exposed animals (Figure 5). GSH, a tripeptide natural antioxidant, has an ability to scavenge ROS. The decrease in the GSH/GSSG ratio indicated enhanced ROS generation in lead-exposed animals.

A majority of ROS is most likely derived from mitochondria (Chen *et al.*, 2003). This organelle is regarded as the vital target of lead toxicity (Belyaeva *et al.*, 2008), in line with the vital role of mitochondria in cell death induction. Increased generation of ROS may trigger lipid peroxidation. MDA is an end product of the lipid peroxidation process which can be defined as oxidative deterioration of polyunsaturated lipids. Our results indicated high RBC, brain, liver, and kidney MDA concentrations in lead-exposed animals.

One of the acknowledged targets of lead is synaptic transmission (Struzynska and Rafalowska, 1994). The toxic effects of lead are attributed to its deposition in astrocytes and its continuous release into the synaptic spaces. The activation of glutamatergic receptors in lead-exposed synapses may result from an increased glutamate release due to an increased intracellular accumulation of glutamate in astrocytes (Mattson *et al.*, 1999; Toscano and Guilarte, 2005). Our results indicate an increased Glu/Gln ratio, accompanied by decreased GS activity in lead-exposed animals (Table 2). This may be possible because GS, the astrocyte-specific enzyme, converts the toxic glutamate to non-toxic glutamine. Therefore, this observation suggests that, under lead exposure, brain tissue is vulnerable to the excitotoxic cell damage arising from the impaired clearance of the released glutamate. Furthermore, activation of glutamatergic receptors may alter the activity patterns of a calcium-sensitive enzyme such as phospholipase. Increased PLA_2 activity was observed in lead-exposed animals, when compared to that in control animals (Table 5). Administration of glutamate potentiated the detrimental effects of lead by altering the GSH and MDA levels in the RBCs and brains (Table 5).

In conclusion, the results of GSH, MDA, and PLA_2 activity indicate the involvement of free radicals in potentiation of lead-induced toxicity by glutamate. In order to counteract the glutamate and lead neurotoxicity, the data presented in this study suggest that NACA may be more effective than NAC. Considering current chelation therapy and the toxic effects of chelators, multiple-acting NACA may be effectively included in the treatment of lead poisoning. Further research is warranted to evaluate the pharmacokinetic properties of this novel thiol antioxidant in animals.

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Fig. 1.

Effect of NAC and NACA on GSH levels in RBCs of CD-1 mice after exposure to lead. CD-1 mice were exposed to 1300 ppm lead acetate in their drinking water for 5 weeks followed by 500 mg NAC or NACA/kg body weight orally for a week. The symbols and bars represent the mean and SD values, respectively (n=4 for each point). Significance indicated by: * $p < 0.001$ compared to corresponding values of the control group; # $p < 0.05-0.005$ compared to corresponding values of the lead-only group.

Fig. 2. Effect of NAC and NACA on GSSG levels in RBCs of CD-1 mice after exposure to lead. CD-1 mice were exposed to 1300 ppm lead acetate in their drinking water for 5 weeks followed by 500 mg NAC or NACA/kg body weight orally for a week. The symbols and bars represent the mean and SD values, respectively (n=4 for each point). Significance indicated by: *p<0.05 compared to corresponding values of the control group; #p<0.05 compared to corresponding values of the lead-only group.

Fig. 3. Effect of NAC and NACA on GSH/GSSG levels in RBCs of CD-1 mice after exposure to lead. CD-1 mice were exposed to 1300 ppm lead acetate in their drinking water for 5 weeks followed by 500 mg NAC or NACA/kg body weight orally for a week. The symbols and bars represent the mean and SD values, respectively (n=4 for each point). Significance indicated by: * $p < 0.05$ compared to corresponding values of the control group; # $p < 0.05-0.005$ compared to corresponding values of the lead-only group.

Fig. 4. Effect of NAC and NACA on MDA levels in RBCs of CD-1 mice after exposure to lead. CD-1 mice were exposed to 1300 ppm lead acetate in their drinking water for 5 weeks followed by 500 mg NAC or NACA/kg body weight orally for a week. The symbols and bars represent the mean and SD values, respectively (n=4 for each point). Significance indicated by: *p<0.05-0.01 compared to corresponding values of the control group; #p<0.005 compared to corresponding values of the lead-only group

Table 1

Effect of NAC and NACA on lead levels in blood and tissues of CD-1 mice

GROUP	Blood Lead ug/dl	Brain ppb	Kidney ppb	Liver ppb
Control	5.6 ± 0.5	226 ± 23	132 ± 14	177 ± 21
Lead only	48 ± 3 *	1509 ± 341 *	909 ± 100 *	1280 ± 292 *
Lead + NAC	43 ± 3 *	448 ± 168 *#	791 ± 49 *#	652 ± 165 *#
Lead + NACA	37 ± 3 *#	417 ± 130 *#	695 ± 102 *#	464 ± 86 *#

* Significance indicated by: p<0.005-0.01 compared to corresponding values of the control group;

p<0.005-0.01 compared to corresponding values of the lead-only group.

Table 2

Effect of lead exposure on brain tissue of CD-1 mice

GROUP	GSH (nmol/mg pro.)	GSH/GSSG	MDA (nmol/100mg pro.)	Glu/Gln	GS (U/mg pro.)
Control	14.55±1.3	33.6±7.4	15.6±4.1	0.88±0.12	237±18
Lead only	11.13±1.3 [*]	8.7±2.6 [*]	32±6.3 [*]	1.66±0.08 [*]	181±27 [*]
Lead + NAC	13.62±0.7	194±21 ^{*#}	23.5±1.1 ^{*#}	0.76±0.18 [#]	235±9 [#]
Lead + NACA	15.5±2.0 [#]	227±33 ^{*#}	20.6±2.2 [#]	0.63±0.11 [#]	248±17 [#]

^{*} Significance indicated by: p<0.05-0.01 compared to corresponding value of control group;[#] p<0.05-0.001 compared to corresponding value of lead-only group

Table 3

Effect of lead exposure on liver tissue of CD-1 mice

GROUP	GSH (nmol/mg pro.)	GSH/GSSG	MDA (nmol/100mg pro.)
Control	27 ± 3.5	6 ± 0.4	10.64 ± 2.1
Lead only	21.15 ± 3.5 *	3.3 ± 0.8 *	19.32 ± 1.8 *
Lead + NAC	23.56 ± 6.6	6.2 ± 0.7 [#]	12.13 ± 1.3 [#]
Lead + NACA	31 ± 5.4 [#]	6 ± 0.2 [#]	12 ± 1.3 [#]

[#]Significance indicated by: p<0.05 compared to corres

* p<0.05-0.01 compared to corresponding values of the control group;

[#] p<0.05 compared to corresponding values of the lead-only group.

Table 4

Effect of lead exposure on kidney tissue of CD-1 mice

GROUP	GSH (nmol/mg pro.)	MDA (nmol/100mg pro.)
Control	6.73± 1.5	18.52 ± 5
Lead only	8.4 ± 2.3	26.6 ± 3.2 *
Lead + NAC	10 ± 1	18 ± 4.8 [#]
Lead + NACA	16.39 ± 2.1 ^{*#}	16.3 ± 3.4 [#]

* Significance indicated by: p<0.05 compared to corresponding values of the control group

[#] p<0.05 compared to corresponding values of the lead-only group.

Table 5

Effects of lead and glutamate on RBCs and brain tissues

GROUP	RBCs		Brain tissues		
	GSH (nmol/mg pro.)	MDA (nmol/100mg pro.)	GSH (nmol/mg pro.)	MDA (nmol/100mg pro.)	PLA ₂ U/mL
Control	255 ± 22	29 ± 2	16 ± 2	17 ± 0.6	6.5 ± 1
Lead only	171 ± 9 [*]	35 ± 1 [*]	11.2 ± 1 [*]	42 ± 2 [*]	18 ± 2 ^{**}
GLU only	227 ± 32	32 ± 5	14.3 ± 1	34 ± 4 ^{**#}	9.6 ± 0.4 ^{**†}
Lead + GLU	154 ± 8 ^{**#}	38 ± 1 [*]	9.3 ± 2 [*]	49 ± 3 ^{**#}	26 ± 1.2 ^{**†}

* Significance indicated by: p<0.05-0.005 compared to corresponding values of the control group

p<0.05-0.005 compared to corresponding values of the lead-only group

**

p<0.05-0.001 compared to corresponding values of the control group

†

p<0.05-0.001 compared to corresponding values of the lead-only group.