Reversal of Lead-Induced Neuronal Apoptosis by Chelation Treatment in Rats: Role of Reactive Oxygen Species and Intracellular Ca$^{2+}$

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ABSTRACT

Lead, a ubiquitous and potent neurotoxicant causes several neurophysiological and behavioral alterations. Toxic properties of lead have been attributed to its capability to mimic calcium and alter calcium homeostasis. In this study, we have addressed the following issues: 1) whether chelation therapy could circumvent the altered Ca$^{2+}$ homeostasis and prevent neuronal death in chronic lead-intoxicated rats, 2) whether chelation therapy could revert altered biochemical and behavioral changes, 3) whether combinational therapy using two different chelating agents was more advantageous over monotherapy in lead-treated rats, and 4) what could be the mechanism of neuronal apoptosis. Results indicated that lead caused a significant increase in reactive oxygen species, neuronal nitric-oxide synthetase, and intracellular free calcium levels along with altered behavioral abnormalities in locomotor activity, exploratory behavior, learning, and memory that were supported by changes in neurotransmitter levels. A fall in membrane potential, release of cytochrome c, and altered bcl$_d$/bax ratio indicated mitochondrial-dependent apoptosis. Most of these alterations reverted toward normal level following combination therapy over monotherapy with calcium disodium EDTA (CaNa$_2$EDTA) or monoisoamyl meso-2,3-dimercaptosuccinic acid (MiADMSA). It could be concluded from our present results that combined therapy with CaNa$_2$EDTA and MiADMSA might be a better treatment protocol than monotherapy with these chelators in lead-induced neurological disorders. We for the first time report the role of Ca$^{2+}$ in regulating neurological dystrophy caused by chronic lead exposure in rats and its recovery with a two-course treatment regime of mono or combination therapy.

Lead poisoning has been a recurrent problem in society for many centuries, and its deleterious effects on central nervous system (CNS) are known as lead encephalopathy or lead neuropathy (Flora et al., 2006). Neurobehavioral impairments, hyperactivity, alterations in brain structure, and learning and cognitive deficits in children have been observed even with low blood lead levels (10–20 μg/dl) (Needleman, 1993). Loss of social function has not only been reported in humans but also in primates and rodents (Bushnell and Bowman, 1979). Although no general hypothesis is known for the mechanism to explain what cellular events underlie the behavioral and cognitive dysfunction in primates and non-primates, the detrimental effects of lead have warranted interest in this area.

One of the reasons for the deleterious effects of lead is its ability to strongly bind to sulfhydryl groups of proteins and to mimic or compete with calcium (Flora et al., 2006). Reports have suggested that lead, even at picomolar concentration, competes with calcium for binding sites on cerebellar phosphokinase C, thereby affecting neuronal signaling and neurotransmitter release (Bressler and Goldstein, 1991), inhibiting calcium entry into cells (Simons, 1993). Apart from targeting the CNS, lead haywires mitochondrial calcium homeostasis, intercellular oxidants levels (Zamzami and Kroemer, 2001), ATP production, and apoptogenic factors (Martinou and Green, 2001).

Erçal et al. (1996) reported that lead induces generation of reactive oxygen species (ROS) in vivo, resulting in systematic mobilization and depletion of intrinsic antioxidant defenses, destabilizing calcium homeostasis by damaging electron transport, ATP depletion, and membrane ion chan-

ABBREVIATIONS: CNS, central nervous system; ROS, reactive oxygen species; CaNa$_2$EDTA, calcium disodium EDTA; DMSA, meso-2,3-dimercaptosuccinic acid; MIADMSA, monoisoamyl meso-2,3-dimercaptosuccinic acid; DCF, 2′,7′-dichlorofluorescin; AM, acetoxymethyl ester; MMP, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetraethylbenzimidazolocarbocyanine iodide; GSH, reduced glutathione; GSSG, glutathione disulfide; TBARS, thiobarbituric acid reactive substances; GPx, glutathione peroxidase; GST, glutathione S-transferase; SOD, superoxide dismutase; AChE, acetyl cholinesterase; MAO, monoamine oxidase; nNOS, neuronal nitric oxide synthetase; 5-HT, 5-hydroxytryptamine; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; bp, base pair(s).
nals disruption (Zughaib et al., 1994), ultimately leading to apoptosis.

Chelation therapy is the only available medical countermeasure to treat lead/metal toxicity. The thiol and amino carboxylic acid metal chelators have been used for the prevention as well as therapy (Saxena and Flora, 2004). The goal of chelation is to enhance lead elimination before irreversible changes occur. Calcium disodium EDTA (CaNa₂EDTA) and 2,3-dimercaprol or British Anti Lewisite have been used conventionally for the treatment of lead intoxication; however, the clinical use of these chelating agents has been under debate (Flora et al., 1995). However, the water-soluble analog of British Anti Lewisite, meso-2,3-dimercaptosuccinic acid (DMSA), was found to be an effective chelator without adverse health effects (Jones, 1994). Clinical human and animal studies have shown that Succimer (trade name of DMSA; Fig. 1) reduces lead levels in blood and other soft tissues (Smith et al., 2000). Among these monoesters, MiADMSA (Fig. 1) has been proved to be effective in gaining intracellular access through various endogenous ligands, thereby having an added advantage over DMSA. Our group has previously reported that combined administration of two structurally different chelators might be a better treatment protocol than monotherapy. However, it is not known whether this treatment protocol (i.e., combined administration of two chelating agents) is equally effective in the recovery of altered neurological disorders.

Thus, we aimed to examine 1) whether chelation therapy in chronic lead-intoxicated rats could circumvent the altered Ca²⁺ homeostasis and prevent neuronal death; 2) whether chelation could be useful in functional recovery in altered behavior, altered neurotransmitter, calcium homeostasis, and apoptotic molecules; 3) the therapeutic efficacy of MiADMSA over DMSA; and 4) the critical molecules that could prevent or revert early neuronal apoptosis.

### Materials and Methods

#### Animals and Experimental Treatments

All experiments were performed on approximately 6-week-old male Wistar rats weighing 80 ± 10 g. Rats were obtained from animal house facility of Defence Research and Development Establishment (Gwalior, India). All animals received human care in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals. Animal ethical committee of Defence Research and Development Establishment also approved the protocols for the experiments. Before dosing, rats were acclimatized for 7 days to light from 6:00 AM to 6:00 PM alternating with 12 h of darkness. The animals were housed in stainless steel cages in an air-conditioned room with temperature maintained at 25 ± 2 °C. Rats were allowed standard rat chow diet (Amrut Feeds; Pranav Agro, New Delhi, India; metal contents of diet, in ppm dry weight: copper, 10.0; zinc, 45.0; manganese, 55.0; cobalt, 5.0; and iron, 75.0) throughout the experiment. Thirty-five animals were randomized into two groups of five and 30 rats, and they were treated as follows for 6 months: group I, no treatment in drinking water and group II, 0.2% lead acetate (Merck, Mumbai, India) in drinking water.

Lead-exposed animals were divided into six groups of five rats each and given the following treatment for five consecutive days: group IA, no treatment; group IIB, CaNa₂EDTA (50 mg/kg i.p., once daily); group IIC, DMSA (50 mg/kg oral, once daily); group IID, MiADMSA (50 mg/kg oral, once daily); group IIE, DMSA (50 mg/kg oral, once daily) + CaNa₂EDTA (50 mg/kg i.p., once daily); and group IIF, MiADMSA (50 mg/kg oral, once daily) + CaNa₂EDTA (50 mg/kg i.p., once daily). Lead exposure was stopped during chelation therapy to avoid chelation of circulating lead by the chelating agent.

After 5 days of chelation treatment, animals were left without any treatment for 7 days, and then they were given a second course of 5-day treatment. Five animals from each group were sacrificed under light ether anesthesia, 48 h after the last dosing. Blood was collected in heparinized vials. The brain was removed and washed with normal saline. All the extraneous materials were removed before weighing. The brain was kept at ice-cold conditions all times.

#### Preparation of Subcellular Fractions

**Crude Synaptosomal Fraction.** Brain synaptosomes were prepared by homogenizing in 10 volumes (w/v) of 0.32 M sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, and 0.5 mM EDTA, pH 7.4). The homogenate was first centrifuged at 1000g for 10 min at 4°C, and then the supernatant was centrifuged at 12,000g for 20 min. Theuffy layer of pelleted synaptosomes was suspended in a low K⁺-HEPES buffer (125 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 1.2 mM Na₂HPO₄, 1.2 mM MgCl₂, 5 mM NaHCO₃, 10 mM HEPES, and 10 mM glucose, pH 7.4).

**Brain Cytosolic and Mitochondrial Fractions.** The rat brain tissue was mixed and homogenized in 500 µl of buffer A (20 mM HEPES, pH 7.5, 50 mM KCl, 5 mM EGTA, 1 mM EDTA, 2 mM MgCl₂, 220 mM mannitol, 68 mM sucrose, 1 mM leupeptin, 5 µg/ml

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**Fig. 1.** Structures of chelating agents. A, CaNa₂EDTA. B, DMSA. C, MiADMSA.
peptatin A, 5 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride). The homogenate was subjected to differential centrifugation to collect the supernatant (cytosolic fractions) and the pellets (enriched mitochondrial fractions). The cytosolic fractions were frozen at −70 °C until further analysis. Pellets containing mitochondria were treated with the lysis buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 250 mM sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, and protease inhibitor), and they were incubated on ice for 20 min. The lysate was centrifuged at 10,000g at 30 min at 4 °C, and the resulting supernatant was kept as the solubilized mitochondrial-enriched fractions and stored at −70 °C until further use.

Biochemical and Oxidative Stress Enzyme Assay

Reactive Oxygen Species. Amount of ROS in brain was measured using 2′,7′-dichlorofluorescin (DCF)-diacetate, which gets converted into highly fluorescent DCF as described by Socci et al. (1999).

In brief, brain was homogenized in ice-cold 40 mM Tris-HCl buffer, pH 7.4, and further diluted to 0.25% with the buffer. The samples were divided into two equal fractions. In one fraction, 40 μl of 1.25 mM DCF-diacetate in methanol was added for ROS estimation, and the other fraction, 40 μl of methanol was added as a sample control. All samples were incubated for 15 min in a 37 °C water bath. Fluorescence was determined at 488-nm excitation and 525-nm emission using a fluorescence plate reader (LS-55; PerkinElmer Life and Analytical Sciences, Beaconsfield, Buckinghamshire, UK).

Intracellular Free Calcium. Intrasyntosomal/intracellular calcium was measured according to the method of Meder et al. (1997). The intracellular free calcium was determined by fluorescent calcium indicator dye Fura-2/AM. Fura-2/AM crosses the synaptosomal membrane, and it is hydrolyzed by the esterases to Fura-2 that binds free ionic calcium to give fluorescence, which is proportional to the amount of free calcium. The synaptosomal suspension was incubated with 5 μM Fura-2/AM for 40 min at 37 °C. This was followed by centrifugation at 20,000g for 20 min, and the pellet was washed with Ca2+-free physiological buffer (133 mM NaCl, 4.8 mM KCl, 1.2 mM Na2HPO4, 1.2 mM MgSO4, 10 mM HEPES, and 10 mM glucose, pH 7.4) and recentrifuged. The pellet was resuspended in physiological buffer, and fluorescence (F) at 340- to 380-nm excitation and 510-nm emission was measured, and [Ca2+]i was calculated as follows: [Ca2+]i = (F - Fmin)/(Fmax - F) × Kd.

The Kd value for Ca2+-Fura-2 complex was 225 nm. Maximal fluorescence (Fmax) was measured after lysis of synaptosomes with SDS, and minimal fluorescence (Fmin) was measured in the presence of 5 mM EGTA. The results were expressed as nanomolar of free intracellular calcium.

Mitochondrial Membrane Potential. Mitochondrial membrane potential (MMP) was measured using JC-1 probe (Molecular Probes, Eugene, OR) as described previously with minor modifications (Mehta and Shaha, 2004). In brief, brain homogenate was centrifuged at 800g for 5 min, and the supernatant was collected and recentrifuged at 10,000g for 10 min. An equal number of cells from each group was taken after counting and incubated for 10 min with 10 μM JC-1 at 37 °C. The cells were washed and resuspended in phosphate-buffered saline, and then fluorescence was measured. Subsequently, the changes in fluorescence were monitored at two different wavelengths with excitation at 485 nm and emission at 530 nm and the other excitation at 535 nm and emission at 590 nm. The ratio of the reading at 590 nm to the reading at 530 nm (590/530 ratio) was considered as the relative mitochondrial membrane potential value.

Glutathione and Glutathione Disulfide. Brain GSH and Brain GSSG estimation were performed as described by the method of Saxena and Flora (2004). In brief, 0.25 g of tissue sample was homogenized on ice with 3.75 ml of 0.1 M phosphate-0.005 M EDTA buffer, pH 8.0, and 1 ml of 25% HPO3 that was used as a protein precipitant. The homogenate (4.7 ml) was centrifuged at 100,000g for 30 min at 4 °C. For the GSH assay, 0.5 ml of supernatant and 4.5 ml of phosphate buffer, pH 8.0, were mixed. The final assay mixture (2.0 ml) contained 100 μl of supernatant, 1.8 ml of phosphate-EDTA buffer, and 100 μl of O-pthalaldehyde (1000 μM/ml in absolute methanol, prepared fresh). After mixing, fluorescence was determined at 420 nm with an excitation wavelength of 350 nm using a spectrophuorometer (model RF 5000; Shimadzu, Kyoto, Japan). For the GSSG assay, 0.5 ml of supernatant was incubated at room temperature with 200 μl of 0.04 M N-ethylmaleimide solution for 30 min. To this mixture, 4.3 ml of 0.1 M NaOH was added. A 100-μl sample of this mixture was taken for the measurement of GSSG using the procedure described above for the GSH assay, with the exception that 0.1 M NaOH was used as the diluent instead of phosphate buffer.

Thiobarbituric Acid Reactive Substances. Tissue lipid peroxidation was measured by shaking the 2 ml of whole-brain homogenate [5% homogenate (w/v) in phosphate-EDTA buffer, pH 7.0] in 150 mM KCl, 0.025 M Tris-HCl buffer, pH 7.5, for 30 min at 37 °C and measuring the malondialdehyde formed with the thiobarbituric acid reaction as reported by Saxena and Flora (2004). The incubation was interrupted by adding 0.1 ml of 10% trichloroacetic acid. The reaction mixture (2 ml) was then mixed with 2 ml of 30% trichloroacetic acid, 0.2 ml of 5 M HCl, and 2 ml of 0.75% thiobarbituric acid. The mixture was then kept in a boiling water bath for 15 min to get the red color of thiobarbituric acid-malondialdehyde. After centrifugation, the color was measured at 535 nm. The amount of TBARS was calculated using a molar extinction coefficient of 1.56 × 10^5/M/cm.

Glutathione Peroxidase. Brain glutathione peroxidase activity (GPx) was measured by the procedure of Flohé and Gunzler (1984).

Glutathione S-Transferase. Glutathione S-transferase (GST) activity was determined following the procedure of Habig et al. (1974).

Superoxide Dismutase. Superoxide dismutase (SOD) activity in whole brain was assayed spectrophotometrically as described by Kakkar et al. (1984).

Acyethyl Cholinesterase. A 10% brain homogenate (w/v) was prepared in 0.25 M sucrose. Activity of acetylcholinesterase (AChE) in brain was carried out according to the method of Ellman et al. (1961) using acetylcholine as substrate.

Monoamine Oxidase. Brain monoamine (MAO) activity was measured following the procedure described by Wurtman and Axelrod (1963).

Western Blot Analysis

The protein content in both the cytosolic and mitochondrial fractions was determined by the method of Lowry et al. (1951). The cytosolic and mitochondrial fractions were both assessed for cytochrome c, whereas the expression of neuronal nitric-oxide synthase (nNOS), 5-HT, and GABA were assessed in cytosolic fraction only. All samples containing 20 μg of protein were loaded on SDS-PAGE gels (12% SDS-PAGE for nNOS, 5-HT, and GABA and 15% SDS-PAGE for cytochrome c) and subjected to electrophoresis followed by transfer to nitrocellulose membrane. Nitrocellulose membranes were blocked with 5% nonfat dry milk, and then the membranes were incubated with primary antibodies (1:1000) at room temperature for 2 h, followed by washing with phosphate-buffered saline with 0.1% Tween 20 for 30 min and incubation for 1 h at 37 °C with secondary antibody (1:5000) conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The blots were washed with phosphate-buffered saline with 0.1% Tween 20, and bands were revealed by diaminobenzidine tetrahydrochloride system (Sigma, Bangalore, India). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for all membranes as internal control. Bands were quantified by using Scion image analysis software program (Scion Corporation, Frederick, MD).
RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Brain tissue RNA was extracted from differently treated groups using the TRIzol method (Invitrogen). One microgram of RNA was converted into cDNA using Superscript reverse transcriptase. PCR was performed with the initial denaturation cycle at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, followed by final extension of 72 °C for 5 min. The PCR-amplified products were then subjected to electrophoresis on 1.5% agarose gels. The primers used for the study were Bcl2 (sense, 5'-CTTGTGGGAACTGTACGCGCCCAAGCATGC-3'; antisense, 5'-ACAGGGCTCCGCTTTGTTCTATGGAATGAT-3'; 231 bp; accession no. M16506), Bax (sense, 5'-GGGAATCTCAGGTGCGAGAGGTATGATT-3'; antisense, 5'-GCGCCTCCAGGTTGGCATCAGCAAGATGAT-3'; 96 bp; accession no. L22472), and β-actin (sense, 5'-TATGAGAAATTTTGGACGC-3'; antisense, 5'-GTCCAGACGGATGCGCAT-3'; 300 bp; accession no. J00691).

Neurobehavioral Studies

General/Locomotor Activity. General/locomotor activity was evaluated in the open-field test. The open-field behavior of rats was assessed in a box measuring 90 × 90 × 30 cm that was subdivided into 19 equal squares by white lines. Immediately after a rat was placed in the center of the open field, the movements of the rat were scored. The following variables were recorded during a 3-min session: number of squares crossed with all paws (crossings), standing on hind legs and placing forelimbs on the wall (wall rearing), and placing nose against floor (sniffing). These activities were counted in all sessions. Testing was carried out on four consecutive days in 5-min sessions.

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

Learning and Memory. The learning and memory capacities (acquisition and processing of information, decision making, and response-initiating functions) are being assessed by the passive avoidance in a shuttle box. It is a standard, sensitive psychopharmacological test of learning and memory in the rats. The apparatus consisted of two identical chambers, one chamber of which was well lit. The chamber without light was used as a masked compartment. The rats were placed one at a time in the lighted compartment, after which the door separating the two chambers was lifted. As soon as the rat entered the dark compartment within 180 s, the door was shut, and the rat was presented with 50 tones at randomly selected intervals of 10 to 50 s. Each tone lasted 5 s and was followed by a 3-s, 0.1-mA, 40-V footshock. If the rat ran to the other compartment of the box during the tone, the shock scheduled for that trial was canceled. The differences among sessions in the number of shuttle-avoidance responses were interpreted as learning and memory.

Metal Analysis

Lead, zinc, and copper concentrations in brain were measured after wet acid digestion using a microwave digestion system (model MDS-2100; CEM, Matthews, NC). Samples were brought to a constant volume, and determination of blood and tissue lead was performed using an autosampler (AS-72) and graphite furnace (model MHS-10; PerkinElmer Life and Analytical Sciences) fitted with an atomic absorption spectrophotometer (model AAnalyst 100; PerkinElmer Life and Analytical Sciences). Essential metals (zinc, copper, calcium, and iron) contents were also measured in the digested tissue samples using atomic absorption spectrophotometer.

Statistical Analysis

Data are expressed as means ± S.E.M. Data comparisons were carried out using one-way analysis of variance followed by Tukey’s post hoc test, except for the data presented in Fig. 3C, which was analyzed using two-way analysis of variance to compare means between the different treatment groups. Differences between all possible pairwise comparisons were tested, and a p value <0.05 was considered significant.

Results

Reversal of Elevated Brain Oxidative Stress Variables [Ca2+], Levels by Chelation Therapy. Lead exposure caused significant increase in ROS levels in rat brain compared with the normal animals, suggesting oxidative stress (Fig. 2A). Among the three chelating agents studied, administration of CaNa2EDTA and DMSA alone did not show any beneficial effects on ROS; however, monotherapy with MiADMSA and in combination with CaNa2EDTA reduced lead-induced ROS levels by 85 to 90%. Lead exposure also significant altered the levels of GSH, GSSG, and TBARS...
Lead-induced changes in brain oxidative stress parameters and their response to combined administration of CaNa₂EDTA, DMSA, and MiADMSA either alone or in combination

<table>
<thead>
<tr>
<th>GSH</th>
<th>GSSG</th>
<th>GPx</th>
<th>GST</th>
<th>SOD</th>
<th>TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/g tissue</td>
<td>μg/min/mg protein</td>
<td>nmol conjugate/min/mg protein</td>
<td>U/min/mg protein</td>
<td>μg/g tissue</td>
<td></td>
</tr>
<tr>
<td>Normal animals</td>
<td>5.23 ± 0.11*</td>
<td>0.25 ± 0.02*</td>
<td>3.4 ± 0.11*</td>
<td>2.9 ± 0.5*</td>
<td>5.1 ± 0.5*</td>
</tr>
<tr>
<td>Lead (control)</td>
<td>1.25 ± 0.14†</td>
<td>0.43 ± 0.02†</td>
<td>1.4 ± 0.02†</td>
<td>10.5 ± 0.7†</td>
<td>2.5 ± 0.4†</td>
</tr>
<tr>
<td>CaNa₂EDTA</td>
<td>1.26 ± 0.11†</td>
<td>0.46 ± 0.03†</td>
<td>2.5 ± 0.01†</td>
<td>9.5 ± 0.5†</td>
<td>2.2 ± 0.4†</td>
</tr>
<tr>
<td>DMSA</td>
<td>4.23 ± 0.10‡</td>
<td>0.34 ± 0.02‡</td>
<td>1.6 ± 0.01‡</td>
<td>5.7 ± 0.2‡</td>
<td>2.7 ± 0.5‡</td>
</tr>
<tr>
<td>MiADMSA</td>
<td>4.87 ± 0.22*</td>
<td>0.29 ± 0.01*</td>
<td>1.8 ± 0.02*</td>
<td>5.3 ± 0.5*</td>
<td>3.6 ± 0.2*</td>
</tr>
<tr>
<td>CaNa₂EDTA + DMSA</td>
<td>2.11 ± 0.26*</td>
<td>0.35 ± 0.01†</td>
<td>1.6 ± 0.02†</td>
<td>5.4 ± 0.1†</td>
<td>3.1 ± 0.3†</td>
</tr>
<tr>
<td>CaNa₂EDTA + MiADMSA</td>
<td>4.43 ± 0.2*</td>
<td>0.25 ± 0.01*</td>
<td>2.5 ± 0.02*</td>
<td>3.2 ± 0.2*</td>
<td>3.8 ± 0.3†</td>
</tr>
</tbody>
</table>

* † ‡ Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability.

along with the enzymes suggestive of oxidative stress, such as SOD, GPx, and GST (Table 1). Among the three chelators, monotherapy with MiADMSA was notably effective in the recovery of the above-mentioned variables, except for GPx. Combined administration of two chelators was unable to provide further beneficial effect on the above-mentioned parameters compared with the effects of these chelators individually (Table 1). Chronic exposure to lead resulted in a remarkable increase in [Ca²⁺] levels (Fig. 2B). Monotherapy with MiADMSA was effective in reducing elevated [Ca²⁺] levels, but the recovery was more pronounced following combined administration of MiADMSA and CaNa₂EDTA compared with the effects after monotherapy with these chelators (Fig. 2B).

**MiADMSA Alone or in Combination with CaNa₂EDTA Reduced nNOS Expression.** nNOS expression due to lead exposure was examined by Western blot. Lead exposure increased nNOS expression appreciably in brain, which could not be influenced by treatment with DMSA or CaNa₂EDTA alone or in combination (Fig. 2C). However, administration of MiADMSA alone provided significant recovery (45%) as depicted with a decrease in the nNOS expression, but, coadministration with MiADMSA and CaNa₂EDTA provided more pronounced decrease (90%) in nNOS expression. GAPDH was used as an internal control to calculate the changes in nNOS levels.

**Lead Affects Neurotransmitters Levels and Behavior in Rats.** Four brain biochemical parameters, namely, 5-HT, GABA, MAO, and AChE were studied. We observed a notable depletion in 5-HT (65–70%) and GABA (90–95%) expression following lead exposure (Fig. 3A). Treatment with CaNa₂EDTA alone showed remarkable recovery (50%) in 5-HT, whereas administration of both the thiol chelators individually showed no recovery. Conversely, depleted GABA level showed notable recovery following monotherapy with the three chelators, with CaNa₂EDTA being the most effective followed by MiADMSA, whereas CaNa₂EDTA with MiADMSA was effective in the recovery of decreased 5-HT levels (Fig. 3A). Lead markedly altered rat brain MAO and AChE activities, which showed considerable improvement following combination therapy of MiADMSA with CaNa₂EDTA (Table 2).

The general behavior, crossing was assessed through open field test (Fig. 2B). Chronic exposure of lead significantly decreased the total general behavior, which included crossing (control, 39 ± 1.64 versus lead-treated, 30 ± 1.23), rearing (control, 15 ± 0.75 versus lead-treated, 11 ± 0.23), and sniffing (control, 18 ± 0.89 versus lead-treated, 12 ± 0.45). However, no major difference in wall rearing behavior was noted (data not shown). Monotherapy with these chelators had no effect on these variables, except for crossing (Fig. 3B), but...
combination therapy with MiADMSA and CaNa2EDTA notably provided significant recovery in the general behavior in terms of crossing (37 ± 0.78) and rearing (14 ± 0.49) and to some extent sniffing (16 ± 0.35).

Learning and memory were assessed using shuttle box (Fig. 3C). Lead-treated animals showed lack of learning and memory compared with normal animals, because these animals showed increased number of avoidance trials. In the groups treated with chelating agents individually and in combination and following continued training, there was a marked improvement in the performance of the animals. Combined administration of MiADMSA and CaNa2EDTA proved to be most beneficial compared with other treatments, whereas lead-exposed animals continued to perform poorly (Fig. 3C).

Table 3 demonstrates the exploratory behavior in animals exposed to chelation therapy following lead treatment. The animals exposed to lead exhibited diminished head dip and head-dipping duration behavior in the holeboard compared with the normal animals. Monotherapy with CaNa2EDTA and DMSA was ineffective in recovering the exploratory behavior of animals, whereas treatment with MiADMSA showed promising effects. Combined administration of CaNa2EDTA with both the thiol chelators showed recovery in the altered behavior (Table 3).

**Chronic Lead Exposure Induces Apoptosis in Brain via Mitochondria.** MMP was performed using a fluorescent probe JC-1, a potentiometer dye that accumulates in the mitochondria in proportion to MMP and ATP-generating capacity. Lead caused a significant disruption of MMP (Fig. 4A).

| TABLE 3 |
|-----------------|-----------------|
| Individual and combined therapeutic efficacy of CaNa2EDTA and thiol chelators (DMSA and MiADMSA) on exploratory behavior in lead-exposed rats. |

<table>
<thead>
<tr>
<th>Head Dip</th>
<th>Head-Dipping Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>count</td>
<td>s</td>
</tr>
<tr>
<td>Normal</td>
<td>10.90 ± 0.75*</td>
</tr>
<tr>
<td>Lead</td>
<td>6.20 ± 0.61†</td>
</tr>
<tr>
<td>CaNa2EDTA</td>
<td>6.53 ± 0.62‡</td>
</tr>
<tr>
<td>DMSA</td>
<td>5.94 ± 0.76§</td>
</tr>
<tr>
<td>MiADMSA</td>
<td>9.16 ± 0.52‡</td>
</tr>
<tr>
<td>CaNa2EDTA + DMSA</td>
<td>10.01 ± 0.54*</td>
</tr>
<tr>
<td>CaNa2EDTA + MiADMSA</td>
<td>10.30 ± 0.61*</td>
</tr>
</tbody>
</table>

* † ‡ Differences between values with matching symbol notations within each row are not statistically significant at 5% level of probability.

Discussion

Lead remains a serious toxic agent, particularly for children, despite reduction in the cases of exposure and the preventive measures taken worldwide. One of the factors associated with lead toxicity is pro- and antioxidant imbalance, which is associated with increased production of free radicals and perturbation of intracellular redox equilibrium, resulting in oxidative stress (Gurer and Ercal, 2000). The brain in particular is more vulnerable to oxidative damage in rat brain. Chelation with MiADMSA was beneficial, because it increased the reduced MMP levels. Combination therapies with CaNa2EDTA and DMSA/MiADMSA were effective in reversing the fall in MMP. As a consequence of drop in MMP levels, cytochrome c release, a known marker for apoptotic cell death, was evaluated in the mitochondrial fraction as well as in the cytosolic fraction. Western blot analysis (Fig. 4B, a) revealed a decreased expression of cytochrome c in the brain mitochondrial fraction (Fig. 4B, b) accompanied by an increased expression in the cytosolic fraction (Fig. 4B, c) following chronic lead exposure, indicating the translocation of cytochrome c from the mitochondria to the cytosol. Administration of chelating agents showed recovery in mitochondrial as well as cytoplasmic levels. Combined administration of CaNa2EDTA with DMSA or MiADMSA was equally effective in the recovery of altered cytochrome c release level in mitochondrial fraction. However, combination therapy did not have any additional beneficial effects on cytochrome c release in cytosolic fraction.

Because there was a translocation of cytochrome c from the mitochondria to the cytosol, which is considered as an indication of apoptosis, we confirmed the same by observing gene expression changes in bcl2 and bax levels (Fig. 4C, a), because these are one of the major regulators of apoptosis. An increase (17-fold) in bax/bcl2 ratio was observed following lead exposure, which showed significant recovery following chelation therapy (Fig. 4C, b). Interestingly, the best recovery (2.3- versus 17-fold in lead-treated group) was observed with combined administration of CaNa2EDTA and MiADMSA, followed by treatment with MiADMSA alone (4.3-fold) (Fig. 4C, b).

**Chelation Therapy Recovers Altered Brain Lead and Calcium Levels.** Although we assumed that concentration of lead and calcium was important to determine their relationship with the above-mentioned biochemical variables, we also determined the level of other trace metals, such as zinc, iron, and copper by atomic absorption spectrophotometer. Figure 5A depicts the effect of chelating agents alone and in combination on lead concentration in the brain of exposed rats. Brain lead concentration increased significantly following chronic exposure to lead. Administration of CaNa2EDTA or DMSA marginally (nonsignificant) depleted the brain lead concentration, whereas treatment with MiADMSA either alone or in combination with CaNa2EDTA led to a significant fall in brain lead concentration. Decreased calcium levels following lead treatment did not recover after monotherapy with these chelators; however, it increased notably following treatment with CaNa2EDTA and MiADMSA in combination (Fig. 5B). No significant changes were observed in copper, iron, and zinc concentration in brain following lead exposure and treatment with chelating agents (data not shown).
due to its high use of inspired oxygen. Two mechanisms have been proposed for lead-induced neurotoxic effects: 1) disruption of pro/antioxidant balance and 2) cell apoptosis (He et al., 2000). The present study confirms both of the mechanisms, and in addition it suggests that combined chelation therapy using an amino- and a thiol-based chelator could be a better remedial measure for clinical recoveries in lead-induced neuropathy than monotherapy with these chelators.

Results show that lead causes oxidative imbalance and leads to excessive ROS production. Increased ROS levels cause an imbalance in Ca²⁺/H⁺ regulation, because of compromised cellular lipid bilayer. Reports have shown that ROS and Ca²⁺/H⁺ regulation is tightly bound and that disruption in either could affect the other (Bressler and Goldstein, 1991; Mehta and Shaha, 2004). Compromised cellular membranes are indicated by loss in MMP, which in turn causes an imbalance in anti- and proapoptotic molecules, such as bcl2 and bax (He et al., 2003). Altered bcl2/bax ratio induces the release of cytochrome c for the activation of terminal cascade of apoptosis (Antonsson et al., 2000, Hengartner, 2000). Our data are in accordance with the concept. Once cytochrome c is released, the cell is programmed for apoptosis (Ferri and Kroemer, 2001), and it cannot survive; we, too, noted the same, because combination therapy was unable to decrease the cytosolic levels of cytochrome c effectively. However, chelation therapy was significantly effective in reversing the initial changes, such as MMP, ROS, and bcl2 levels, further emphasizing that initial steps of mitochondrial-driven apoptosis could be reversed, but not the latter.

Ca²⁺ ion plays a key role as a regulator of numerous cellular functions, whereas lead mimics calcium in many ways in the CNS. It accumulates in the same mitochondrial compartment as Ca²⁺, and it has been linked to disruptions in intracellular calcium metabolism (Bressler and Goldstein, 1991). Increased intracellular calcium levels cause release of excitatory neurotransmitters and oxidative stress in CNS cells (Naarala et al., 1997). Results show a significant alter-
vation in all the neurotransmitter studied. Lead exposure affected 5-HT and GABA expressions, which are responsible for learning and memory (File, 1995; Ma et al., 1999). Acetylcholinesterase plays a crucial role in signaling and coordination. These altered neurotransmitters also caused an impact on the functional status (neurobehavioral) in the study. Behavior, which is a net output of sensory, motor, and cognitive functions in the nervous system, makes it a potentially sensitive endpoint of lead-induced neurotoxicity (File et al., 2000; Moreira et al., 2001). Reduced head dips and decreased shuttle-avoidance response suggest hyperactivity, decreased exploratory behavior, and impairment in learning and memory. These impairments in the present study may be attributed to the increased hyperactivity in the rats (Altmann et al., 1993). These alterations in behavior may also be due to the direct inhibitory effect of lead on neurotransmitters in the adult brain (Ma et al., 1999).

For a potential chelating agent targeting metal-induced neurological conditions, the blood-brain barrier must be overcome (Cory-Slechta and Weiss, 1989; Smith et al., 1998; Seaton et al., 1999). One of the primary factors in determining the effectiveness of chelation therapy against chronic toxicity is the ability of chelating agents to reach the intracellular site. In this study, three chelators were used either alone or in combination. We have reported previously that combination of two chemically and structurally different chelating agents—a soft tissue mobilizer (DMSA) with a bone lead mobilizer (CaNa₂EDTA)—may prevent redistribution or redepensation of lead mobilized from bone while depleting soft tissue lead (Flora et al., 1995). However, whether this combination would help in providing clinical recoveries in neurological disturbances was under question. Regardless, the beneficial effects of combination therapy (using two different chelating agents) include 1) reduction in the dose of potentially toxic chelating agents, such as CaNa₂EDTA (Cory-Slechta et al., 1987; Flora et al., 2006), particularly when prolonged treatment is required; and 2) more effective and rapid elimination of body lead store.

In the current study, coadministration of CaNa₂EDTA with DMSA or MiADMSA showed remarkable recoveries in most of the variables studied. Interestingly, the recoveries were generally more prominent when CaNa₂EDTA was coadministered with MiADMSA not only on biochemical variables but also on variables indicative of CNS toxicity, and to some extent, on markers for apoptosis. Lipophilicity and molecular size of MiADMSA might be the important factors responsible for the removal of lead (Kreppel et al., 1995) from the binding sites, possibly leading to better therapeutic efficacy. Although a number of studies have reported the beneficial effects of combination therapy against lead-induced oxidative stress, no information is available to treat the lead-induced neurological disorders.

In conclusion, this was the first attempt made in our knowledge where combined administration of thiol chelators DMSA or MiADMSA has been investigated with the conventional chelating agent CaNa₂EDTA to study and treat neurological malfunctioning in rats chronically exposed to lead. The study suggests that 1) combination therapy could reverse altered neurological functioning better than monotherapy, 2) the use of two structurally different chelators could remove lead more effectively, 3) altered neurotransmitters and behaviors changes reverted toward normalcy, and 4) to a certain extent neuronal cell death could be prevented following combinational therapy. However, it would be interesting to evaluate the changes in the signaling cascade, because calcium acts as a second messenger, too. Although results indicate that the combined treatment with chelating agents could be effective and could be recommended for treatment for reverting back from the lead-induced neurological disorders, more extensive studies are required in this important area before a final conclusion can be drawn.

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