Changes in brain biogenic amines and haem biosynthesis and their response to combined administration of succimers and *Centella asiatica* in lead poisoned rats

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Abstract

This study was designed to investigate the therapeutic potential of meso 2,3-dimercaptosuccinic acid (DMSA) and one of its monoesters, monoisoamyl DMSA (MiADMSA), individually or when administered in combination with an extract of *Centella asiatica* against experimental lead intoxication in rats. Biochemical variables indicative of alterations in the central nervous system and haem biosynthesis were investigated to determine the toxicity in male Wistar rats. Thirty five rats were exposed to 0.2% lead acetate for 10 weeks, followed by 10 days of treatment with DMSA and MiADMSA (50 mg kg^{-1}, i.p., once daily) alone and in combination with *C. asiatica* (200 mg kg^{-1}, p.o., once daily). Biochemical variables indicative of oxidative stress and brain biogenic amines, along with lead concentration in blood and brain, were measured. Lead exposure caused a significant depletion of blood and brain δ-aminolevulinic acid dehydratase (ALAD) activity, an important enzyme of the haem biosynthesis pathway, and glutathione (GSH) level. These changes were accompanied by a marked increase in reactive oxygen species (ROS) level, thiobarbituric acid reactive substances (TBARS), δ-aminolevulinic acid synthase (ALAS) and oxidized glutathione (GSSG) activity in blood and brain. Significant depletion of brain noradrenaline (norepinephrine, NE), 5-hydroxytryptamine (5-HT), dopamine (DA) and acetylcholinesterase (AChE) also were observed following lead exposure. Also seen was a significant depletion in brain glutathione peroxidase (GPx), glutathione S-transferase (GST) and monoamine oxidase activity, as well as blood and brain superoxide dismutase (SOD) activity. These biochemical changes were correlated with an increased uptake of lead in blood and brain. Combined administration of MiADMSA and *C. asiatica* was most effective in reducing these alterations, including biogenic amines, besides reducing body lead burden, compared with individual treatment with MiADMSA. Certain other biochemical variables responded favourably to combination therapy and monotherapy with MiADMSA. Thus, supplementation of *C. asiatica* during chelation could be recommended for achieving optimum effects of chelation therapy.

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Introduction

Lead is a metal that has no biological function in man. It adversely affects many organs and systems in animals and in man, where the haematological and nervous systems are among the most important targets. It may be absorbed from the gastro-intestinal tract (children with pica are especially vulnerable) or through the respiratory system. It is transported through blood and causes injury to several organs, including the kidney (renal failure, hypertension), bone marrow (anaemia) and, especially, the central nervous system (CNS). Lead is a highly neurotoxic agent that particularly affects the developing CNS. The mechanism by which lead disrupts normal physiological processes is mainly based on the ability of this metal to mimic the action of calcium. In the brain, lead causes an inappropriate release of neurotransmitters at rest and competes with calcium ions to interfere with evoked neurotransmitter release. The functional states of cholinergic, noradrenergic, dopaminergic and GABAergic neurotransmission are affected. Lead also interferes with excitatory neurotransmission by glutamate, which is the transmitter at more than half the synapses in the brain and is critical for learning (Cory-Slechta 1995).

Lead has a significant effect on haem biosynthesis, causing anaemia at high blood levels (Gurer & Ercal 2000), and is shown to induce changes in the composition of red blood cell membrane proteins and lipids. Lead irreversibly binds to the sulfydryl group of proteins, causing impaired function. The enzymes δ-aminolevulinic acid dehydratase (δ-ALAD), which catalyses the formation of the prophobilinogen ring, and ferrochelatase, which inserts iron into protoporphyrin ring, are both compromised by lead.

The current approved treatment for lead poisoning is to administer a chelating agent that forms an insoluble complex with lead and remove the same from lead burdened tissue. One of the most common chelating agents is calcium disodium edetate ($CaNa_2EDTA$). Unfortunately, chelation with EDTA removes lead from bone and redistributes it to the brain and liver (Flora et al 1995). There is some evidence to suggest that long-term administration of EDTA leads to kidney damage in man. Therefore, alternate drugs have been sought to treat lead-exposed individuals. Chelation therapy using meso 2,3-dimercaptosuccinic acid (DMSA) has been shown to reduce lead levels in blood, brain and tissues (Jones 1994; Pappas et al 1995; Stewart et al 1996). DMSA is one of the least toxic drugs and can be given by the oral route, but the hydrophilic and lipophobic properties of DMSA do not allow it to cross the cell membrane. It was observed that monoesters of DMSA might be a more effective antidote for lead toxicity (Jones et al 1992; Saxena et al 2005). It has been reported that monoisoamyl DMSA (MiADMSA), because of its lipophilic nature, could mobilise brain lead more efficiently than DMSA (Walker et al 1992, Flora et al 2005). Administration of antioxidants during chelation therapy has been found to be beneficial in increasing lead mobilization and providing recovery of altered biochemical variables (Flora 2002; Flora et al 2002; Pande & Flora 2002).

Recently, the clinical importance of herbal drugs has received considerable attention. As many synthetic antioxidants have been shown to have side effects (Musk et al 1994; Nocentini et al 2001), there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing free-radical-induced tissue injury (Koleva et al 2002). Several naturally occurring dietary or non-dietary constituents, as well as part of several species of edible plants with pharmacological activity, influence antioxidant enzymes and provide protection against free-radical-induced damage. *Centella asiatica* (Umbelliferae) syn. *Hydrocotyl asiatica* has been mentioned in ancient Indian literature for its intelligence property (Charak Samhita). It is commonly known as Indian Pennywort in English and Brahmi in Hindi. The plant contains asiaticoside, thakunoside, thankunic acid, asiatic acid, brahmoside, brahminoside, brahmic acid, isobrahmic acid, centoic acid and centilic acid (Srivastava et al 1997). It has been reported to protect against membrane peroxidation (Padma et al 1998) and lipid peroxidation (Chandraprabha et al 1996). The effect of *C. asiatica* on the CNS has been reported and it has been claimed to be beneficial in improving memory and in overcoming the negative effects of fatigue and stress (Nalini et al 1992).

The alcoholic extract of its leaves was found to produce tranquilizing effect in rats (Shukla 1999). The effect of *C. asiatica* has been evaluated in mentally retarded children (Kumar & Gupta 2002) and it was found to increase their IQ level as well as general mental ability and behavioural pattern. Several studies have shown the involvement of neurotransmitters, especially biogenic amines, in learning and memory processes (Nalini et al 1992).

The aim of this study was to investigate the therapeutic efficacy of thiol chelators alone and in combination with *C. asiatica* against lead-induced alterations in the blood and brain besides mobilization of lead in male rats.

Materials and Methods

Chemicals and reagents

Meso 2,3-dimercaptosuccinic acid (DMSA), and δ-aminolevulinic acid (ALA) were procured from Sigma (St Louis, MO, USA) and lead acetate was obtained from BDH Chemicals (Mumbai, India). All other analytical laboratory chemicals and reagents were purchased from Merck (Germany), Sigma or BDH Chemicals. Ultra-pure water prepared using a Millipore apparatus (Millipore Company, New Delhi, India) was used throughout the experiment to avoid metal contamination and for the preparation of reagents and buffers used for various biochemical assays in our study. MiADMSA was synthesized in our synthetic chemistry division, by the controlled esterification of DMSA with isoamyl alcohol in acidic medium (Jones et al 1992). The product was purified (purity 99.9%) and characterized using spectral and analytical methods before experimentation. The chemicals were stored in desiccators at 4°C to avoid oxidation and thermal decomposition. DMSA and MiADMSA were dissolved in 5% sodium bicarbonate solutions, respectively. All solutions of chelating agents were prepared immediately before use. Prof. Y. K. Gupta, Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India, supplied the plant extract of *Centella asiatica*. His group has earlier provided details about the preparation of plant extract, authentication and their correct botanical identity in previous reports (Kumar & Gupta 2002; Gupta et al 2003). The dosing volume amounted to $4\,mL\,kg^{-1}$.

Chemical characterization of *Centella asiatica*

Linear-gradient high-performance liquid chromatography (HPLC) was done to achieve the separation of the different compounds of *Centella asiatica*. The column effluent was introduced into a Thermo Finnigan LCQ Advantage mass spectrometer via atmospheric pressure ionisation operated in ESI (electron spray ionisation) mode. Conditions for optimal analysis of *C. asiatica* using ESI conditions were: spray voltage 4 KV; capillary temperature 250°C; sheath gas nitrogen at a flow meter reading of 20. Capillary and tube lens voltages were optimised to give maximum response for the separation of different compounds. Chromatographic separation was performed with

a water–acetonitrile mobile phase and detected with a Finnigan Surveyor PDA detector with UV detection at 220 nm. The retention time (t_R) of each peak was noted (Inamdar et al 1996).

Animals and treatment

All experiments were performed on male Wistar rats, 80 ± 10 g, approximately 8 weeks old (animal house facility of Defence Research and Development Establishment (DRDE), Gwalior). All rats received humane care in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The Animal Ethical Committee of DRDE also approved the protocols for the experiments. Before dosing, rats were acclimatized for 7 days to a 12-h light–dark cycle. The rats were housed in stainless-steel cages in an air-conditioned room with temperature maintained at $25 \pm 2°$C. Rats were allowed standard rat chow diet (metal contents of diet, in ppm dry weight: Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, Fe 75.0; Amrut Feeds, Pranav Agro, New Delhi, India) throughout the experiment.

Thirty-five rats were randomized into two groups of five and thirty rats and were treated as follows for ten weeks: Group I, no treatment (drinking water); Group II, 0.2% lead acetate in drinking water. After ten weeks, lead-exposed rats were divided into six groups of five rats each and given the following treatment for 5 consecutive days: Group IIA, no treatment; Group IIB, DMSA (50 mg kg^{-1}, i.p., once daily); Group IIC, MiADMSA (50 mg kg^{-1}, i.p., once daily); Group IID, *C. asiatica* (200 mg kg^{-1}, p.o., once daily); Group IIE, DMSA + *C. asiatica*; Group IIF, MiADMSA + *C. asiatica*.

Lead exposure was stopped during chelation therapy. Treatment was given in two courses. After five days of chelation treatment, rats were left without any treatment for 7 days and then given a second course of 5 days treatment. Five rats from each group were sacrificed under light ether anaesthesia, 48 h after the last dosing. Blood was collected in heparinized vials. The brain was removed, washed with normal saline and all the extraneous materials were removed before weighing. The brain was kept in ice-cooled conditions at all times.

Biochemical assays

Blood and brain δ-aminolevulinic acid dehydratase (ALAD)

The activity of blood ALAD was assayed according to the procedure of Berlin & Schaller (1974). Heparinized blood (0.2 mL) was mixed with distilled water (1.3 mL) and incubated for 10 min at 37°C for complete haemolysis. For estimation of brain ALAD, 0.5 mL of brain homogenate was mixed with 1 mL of distilled water. After adding 1 mL of standard δ-ALA (δ-aminolevulinic acid), the tubes were incubated for 60 min at 37°C. Enzyme activity was stopped after 1 h by adding 1 mL of 10% trichloroacetic acid (TCA). After centrifugation (1500 g) of reaction mixture, an equal volume of Ehrlich reagent was

added to the supernatant, and the absorbance was recorded at 555 nm after 5 min.

Brain δ-aminolevulinic acid synthase (ALAS)

Brain ALAS was measured by the method of Maines (1980). The reaction mixture contained 0.3 mL brain homogenate 20%, 50 mM glycine, 50 mM sodium citrate, 25 mM sodium monophosphate, 10 mM magnesium chloride, 5 mM pyridoxal 5-phopshate and 10 mM disodium EDTA. Tubes were incubated at 37°C for 1 h, after which the incubation reaction was stopped by adding 1 mL of 10% TCA. The aminoketones formed were converted into pyrroles by condensation with acetyl acetone. Finally, an equal amount of Ehrlich reagent was added and the absorbance was recorded at 555 nm.

Clinical haematological variables

White blood cells (WBC), red blood cells (RBC), haematocrit (Hct), haemoglobin (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were measured on a Sysmex Hematology Analyzer (model K4500).

Blood glutathione (GSH)

Analysis of blood GSH concentration was performed by the method described by Ellman (1959) and modified by Jollow et al (1974). In brief, 0.2 mL of whole blood was added to 1.8 mL of distilled water and incubated for 10 min at 37°C for complete haemolysis. After haemolysis, 3 mL of 4% sulphosalicylic acid was added and tubes were centrifuged at 2500 g for 15 min. Supernatant (0.2 mL) was mixed with 0.4 mL of 10 mM 5,5'dithiobis-(2-nitrobenzoic acid) (DTNB) and 1 mL phosphate buffer (0.1 M, pH 7.4). After 5 min the absorbance was recorded at 412 nm.

Brain reduced (GSH) and oxidized glutathione (GSSG) contents

Brain GSH and GSSG content was measured as described by Hissin & Hilf (1973). Briefly, 4 mL of brain homogenate was precipitated by adding 1 ml of 25% metaphosphoric acid and centrifuged at 100 000 g (Ultracentrifuge, Hitachi, Japan) for 30 min. Supernatant was diluted 20 times with the same buffer and 100 μL of OPT (*o*-pthaldehyde) was added. In addition, for the GSSG assay, 0.5 mL supernatant was incubated at room temperature with 200 μL of 0.04 mol L^{-1} *N*-ethylmaleimide solution for 30 min and to this mixture 4.3 mL of 0.1 mol L^{-1} NaOH was added. A 100-μL sample of this mixture was taken for the measurement of GSSG using the exact procedure described above for the GSH assay, except that 0.1 mol L^{-1} NaOH was used as the diluent instead of phosphate buffer. Samples were incubated at room temperature for 15 min and fluorescence was measured at 350 nm (E_x)/420 nm (E_m) with a Tecan Spectra Fluor Plus instrument (Germany).

Blood and brain superoxide dismutase (SOD) activity

Blood and brain SOD activity was assayed by the method of Kakkar et al (1984). SOD was extracted from purified RBCs

by ethanol–chloroform extraction as described by Winterbourn et al (1975). Reaction mixture contained 1.2 mL of sodium pyrophosphate buffer (0.052 mM, pH 7.0), 0.1 mL^{-1} of phenazine methosulphate (PMS) (186 μM), 0.3 mL of nitro blue tetrazolium (NBT) (300 μM). Twenty microlitres of the supernatant obtained after centrifugation (1500 g, 10 min followed by 10 000 g, 15 min) of 5% haemolysate/homogenate was added to the reaction mixture. Enzyme reaction was initiated by adding 0.2 mL of NADH (780 μM) and stopped precisely after 1 min by adding 1 mL of glacial acetic acid. The amount of chromogen formed was measured by recording the colour intensity at 560 nm.

Blood and brain catalase activity
The catalase activity in purified RBCs and brain was assayed following the procedure of Sinha (1972). Five-percent RBC haemolysate/homogenate was used for catalase estimation. One-hundred microlitres of haemolysate/homogenate was incubated with 0.5 mL of H_2O_2 (0.2 M) at 37°C for 90 s precisely, in the presence of 0.01 M phosphate buffer (pH 7.4). The reaction was stopped by adding 5% dichromate solution. Further samples were incubated at 100°C for 15 min in a boiling water bath. The amount of H_2O_2 consumed was determined by recording the absorbance at 570 nm.

Blood and brain thiobarbituric acid reactive substances (TBARS)
Lipid peroxidation in purified RBCs and brain was measured by the method of Onkawa et al (1979). RBC haemolysate/brain homogenate was incubated with 8.1% (w/v) sodium dodecyl sulfate (SDS) for 10 min followed by addition of 20% acetic acid (pH 3.5). The reaction mixture was incubated with 0.6% TBA (w/v) for 1 h in a boiling water bath. A pink chromogen was extracted in butanol–pyridine solution (15:1) and the absorbance was read at 532 nm.

Brain glutathione peroxidase (GPx)
GPx activity was measured by the procedure of Flohe & Gunzler (1984). Supernatant obtained after centrifuging 5% brain homogenate at 1500 g for 10 min followed by 10 000 g for 30 min at 4°C was used for GPx assay. One millilitre of reaction mixture was prepared, which contained 0.3 mL of phosphate buffer (0.1 M, pH 7.4), 0.2 mL of GSH (2 mM), 0.1 mL of sodium azide (10 mM), 0.1 mL of H_2O_2 (1 mM) and 0.3 mL of brain supernatant. After incubation at 37°C for 15 min, the reaction was terminated by addition of 0.5 mL 5% TCA. Tubes were centrifuged at 1500 g for 5 min and the supernatant was collected. Two-hundred microlitres of phosphate buffer (0.1 M pH 7.4) and 0.7 mL of DTNB (0.4 mg mL^{-1}) was added to 0.1 mL of reaction supernatant. After mixing, the absorbance was recorded at 420 nm.

Brain glutathione-S-transferase (GST)
GST activity was determined following the procedure of Habig et al (1974). The reaction mixture contained 0.02 mL of 1-chloro 2,4-dinitro benzene (1 mM), 2.9 mL

of GSH (0.3 mg mL^{-1} in 0.2 M phosphate buffer, pH 7.4) and 30 μL of brain supernatant (as described above), and change in colour was monitored by recording absorbance (340 nm) at 30-s intervals for 3 min.

Blood and brain reactive oxygen species (ROS)
The amount of ROS in brain was measured using 2′,7′-dichlorofluorescin diacetate (DCF-DA), which is converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci et al (1999). Briefly, brain was homogenized (10 mg) in 1 mL of ice-cold 40 mM Tris–HCl buffer (pH 7.4) and further diluted to 0.25% with the same buffer and placed on ice. For estimation of ROS in blood, 5% RBC haemolysate was prepared and diluted to 1.5% with ice-cold 40 mM Tris-HCl buffer (pH 7.4). The samples were divided into two equal fractions. In one fraction, 40 μL of 1.25 mM DCF-DA in methanol was added for ROS estimation. Another fraction in which 40 μL of methanol was added served as a control for tissue/haemolysate autofluorescence. 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 (Tecan Spectra Fluor Plus).

Brain acetylcholinesterase (AChE) activity
A 10% brain homogenate (w/v) was prepared in 0.25 M sucrose. Activity of AChE in brain was determined according to the method of Ellman et al (1961) using acetylthiocholine as substrate. The activity of AChE was measured at 412 nm and its unit is expressed as nmol min^{-1} (mg protein)$^{-1}$.

Brain monoamine oxidase (MAO) activity
MAO activity was studied in brain mitochondrial fraction following the method of Wurtman & Axelrod (1969). Briefly, 1.0 mL of 0.2 M phosphate buffer (pH 7.2) and 0.8 mL distilled water were added to 100 μL of mitochondrial fraction. One-hundred microlitres of benzylamine HCl (0.1 M, pH 7.2) was added to the experimental tubes. The tubes were incubated for 30 min at 37°C and the reaction was stopped by adding 1 mL of 10% perchloric acid. In controls, instead of the mitochondrial fraction, an equal amount of phosphate buffer was added. After centrifugation, the supernatant was diluted with an equal volume of distilled water and read at 250 nm. The enzyme activity was expressed as nM of benzaldehyde formed/min/mg protein.

Brain biogenic amines
The frozen brain tissue samples were weighed and homogenized in acidified butanol. Dopamine (DA), noradrenaline (norepinephrine, NE) and 5-hydroxytryptamine (5-HT) were estimated according to the procedure of Jacobwitz & Richardson (1978). The butanol extracts were re-extracted with phosphate buffer (0.1 M, pH 6.5), a part of which was derivatized by adding sodium EDTA and iodine solution. The reaction was terminated by alkaline sulphite and neutralized with acetic acid (5 N).

Fluorescence was read by excitation at 385 nm and emission at 485 nm for NE, and 320 nm and 385 nm, respectively, for DA. To determine 5-HT, the butanol layer was extracted with 0.1 N HCl in the presence of *n*-heptane and the acid layer was mixed with *o*-phthaldehyde. After boiling and cooling, fluorescence was read by excitation at 360 nm and emission at 470 nm.

Elemental analysis
Lead, zinc and copper concentrations in blood and brain were measured after wet acid digestion using a Microwave Digestion System (model MDS-2100; CEM, USA). Samples were brought to a constant volume and determination of blood and tissue lead was performed using an auto sampler (AS-72) and graphite furnace (MH) fitted with an atomic absorption spectrophotometer (AAS, Perkin Elmer model Analyst 100) (Yeager et al 1971). Zinc and copper contents were also measured in the digested tissue samples using AAS (Parker et al 1967).

Statistical analysis

Data are expressed as means $\pm$ s.e.m. Data comparisons were carried out using one-way analysis of variance followed by Bonferroni test to compare means between the different treatment groups. Differences between all possible pair-wise comparisons were tested and $P < 0.05$ was considered significant.

Results

Effect on ALAD and ALAS activity in blood

Table 1 represents some lead-sensitive markers of haem biosynthesis and their response to chelating agents either alone or in combination with *C. asiatica*. Lead exposure caused significant inhibition of blood δ-ALAD and increased ALAS activity, suggesting an altered haem-biosynthesis pathway. Inhibited ALAD activity responded

favourably to the treatment with MiADMSA but the best protection was achieved following combined treatment with MiADMSA and *C. asiatica*. The two values, however, were not statistically different from each other. Altered blood ALAS activity showed significant improvement following combined treatment with chelators and *C. asiatica* compared with single chelator treatment.

Effect on biochemical variables indicative of oxidative stress in red blood cells

Biochemical variables indicative of lead induced oxidative injury to red blood cells and their response to treatment with chelating agents either alone and in combination with *C. asiatica* are shown in Table 1. A significant increase in blood ROS and TBARS level was noted on exposure to lead. Elevated ROS level showed significant protection to MiADMSA administration individually and in combination with *C. asiatica*, although it remained insensitive to treatment with DMSA. Interestingly, when *C. asiatica* was administered in combination with DMSA, it led to a significant improvement in altered ROS level. Blood TBARS level, on the other hand, remained unchanged by any other treatment (Table 1).

Effect on enzymes of antioxidant defence system in blood

Alterations in antioxidant enzymes like SOD, catalase and concentration of antioxidant molecule GSH following lead exposure and on chelation treatment in red blood cells are shown in Table 1. There was a significant depletion in the activity of SOD and catalase in RBCs, while GSH level showed a marginal depletion on lead exposure. Blood GSH level was near the normal value on combined treatments with DMSA/MiADMSA and *C. asiatica*. Inhibited activity of SOD and catalase showed maximum protection on combined treatment with DMSA plus *C. asiatica* compared with other treatment.

Table 1 Effect of chelation therapy individually or in combination with *C. asiatica* on some lead-sensitive blood variables in lead-exposed rats

	Normal	Lead	DMSA	MiADMSA	*C. asiatica*	DMSA + *C. asiatica*	MiADMSA + *C. asiatica*
ALAD	6.33 ± 0.15*	1.31 ± 0.08†	1.68 ± 0.12†	2.32 ± 0.16‡	1.26 ± 0.24†	1.92 ± 0.04†	2.85 ± 0.21‡
ALAS	7.81 ± 0.22*	10.87 ± 0.23†	9.42 ± 0.68†	9.18 ± 0.45†	10.68 ± 0.10†	8.01 ± 0.28*	7.91 ± 0.23*
ROS	4.22 ± 0.22*	6.01 ± 0.31†	6.65 ± 0.08†	4.23 ± 0.12*	5.13 ± 0.32*	4.52 ± 0.63*	4.19 ± 0.25*
TBARS	7.81 ± 0.22*	10.87 ± 0.93†	9.42 ± 0.68†	9.18 ± 0.45†	10.68 ± 0.10†	10.01 ± 0.28†	9.91 ± 0.23†
GSH	2.81 ± 0.09*	1.84 ± 0.05†	2.11 ± 0.04†	2.11 ± 0.07†	2.02 ± 0.07†	2.91 ± 0.09*	2.77 ± 0.04*
Catalase	15.40 ± 0.12*	9.70 ± 1.42†	10.10 ± 0.79†	9.20 ± 1.02†	10.78 ± 0.09†	14.90 ± 0.51*	11.02 ± 0.63†
SOD	9.70 ± 0.12*	5.21 ± 0.82†	7.10 ± 0.29†	6.99 ± 1.02†	6.02 ± 0.63†	9.27 ± 0.06*	7.22 ± 0.59†

ALAD, δ-aminolevulinic acid dehydratase (μmol min^{-1} (mL erythrocytes)$^{-1}$); ALAS, δ-aminolevulinic acid synthase (μmol min^{-1} (mL erythrocytes)$^{-1}$); ROS, reactive oxygen species (μmol min^{-1}(mL of RBC)$^{-1}$); TBARS, thiobarbituric acid reactive substances (μg mL^{-1}); GSH, reduced glutathione (mg mL^{-1}); catalase (μmol min^{-1} mL^{-1}); SOD, superoxide dismutase (U min^{-1} (mg haemoglobin)$^{-1}$). Values are mean ± s.e.m., n = 5. *, †, ‡, Differences between values with matching symbol notations within each row are not statistically significant at 5% level of probability.

Effect on clinical haematological variables

Table 2 shows the clinical haematological indicators of lead poisoning and their response to chelation therapy alone and in combination with *C. asiatica*. The haemoglobin content and RBC counts decreased significantly following lead exposure, while no changes were noted in WBC, Hct, MCH, MCHC levels or platelet counts. No recovery was seen in these variables either during monotherapy with DMSA, MiADMSA or *C. asiatica*, or after combination treatment.

Effect on ALAD and ALAS activity in rat brain

The two enzymes involved in haem biosynthesis were also studied in rat brain and the data is shown in Table 3. Lead exposure led to a significant fall in δ-ALAD activity, while ALAS activity showed significant increase on lead exposure. Following two courses of 5 days treatment, inhibited δ-ALAD activity showed marginal recovery on DMSA and MiADMSA administration. Treatment with MiADMSA alone provided significant recovery of the elevated ALAS activity. The best protection in ALAD activity was achieved on combined administration of thiol chelators and *C. asiatica*, while combination treatment had no added advantage over the individual effect of chelators on ALAS activity.

Effect on brain ROS and TBARS levels

Lead exposure caused significant elevation of ROS and TBARS in rat whole brain (Table 3). None of the chelating agents when administered alone were able to reduce TBARS, while increased ROS levels responded favourably to treatment with thiol chelators and *C. asiatica* when administered individually. TBARS levels remained unchanged following treatment with DMSA, MiADMSA or *C. asiatica* alone. Combined treatment with MiADMSA and *C. asiatica*, however, was able to reduce TBARS levels to the normal value.

Table 2 Effect of chelation therapy individually or in combination with *C. asiatica* on some clinical haematological variables in lead-exposed rats

	Normal	Lead	DMSA	MiADMSA	*C. asiatica*	DMSA + *C. asiatica*	MiADMSA + *C. asiatica*
Hb	11.8 ± 0.08*	9.9 ± 0.19†	10.1 ± 0.95†	10.7 ± 0.14†	10.7 ± 0.52†	10.8 ± 0.21†	10.1 ± 0.23†
MCV	52.7 ± 0.98*	52.9 ± 0.64*	51.3 ± 0.70*	53.6 ± 1.10*	52.0 ± 1.53*	53.4 ± 0.43*	53.2 ± 0.76*
MCH	17.7 ± 0.68*	18.2 ± 0.17*	17.1 ± 0.23*	18.1 ± 0.32*	17.4 ± 0.62*	18.0 ± 0.19*	17.5 ± 0.10*
MCHC	33.5 ± 0.82*	34.5 ± 0.20*	33.4 ± 0.58*	33.7 ± 0.12*	33.4 ± 0.31*	33.7 ± 0.18*	33.2 ± 0.08*
RBC	7.57 ± 0.20*	5.80 ± 0.20†	5.67 ± 0.48†	7.22 ± 0.16†	6.5 ± 0.26†	6.99 ± 0.17†	7.01 ± 0.19†

Hb, haemoglobin (g dL^{-1}); MCV, mean cell volume (fL); MCH, mean cell haemoglobin (pg); MCHC, mean cell haemoglobin concentration (g dL^{-1}); RBC, red blood cells as ($10^6 \mu$L^{-1}). Values are mean ± s.e.m.; n = 5. *, †, Differences between values with matching symbol notations within each row are not statistically significant at 5% level of probability.

Table 3 Effect of chelation therapy individually or in combination with *C. asiatica* on some lead-sensitive biomarkers in brain of lead-exposed rats

	Normal	Lead	DMSA	MiADMSA	*C. asiatica*	DMSA + *C. asiatica*	MiADMSA + *C. asiatica*
ALAD	1.33 ± 0.02*	0.55 ± 0.03†	0.92 ± 0.09‡	0.93 ± 0.03‡	0.72 ± 0.02†	1.16 ± 0.05*	1.19 ± 0.04*
ALAS	5.81 ± 0.22*	8.87 ± 0.93†	7.42 ± 0.18†	6.18 ± 0.15*	8.18 ± 0.10†	7.35 ± 0.28†	5.78 ± 0.23*
ROS	0.54 ± 0.11*	2.62 ± 0.44†	1.01 ± 0.15‡	1.50 ± 0.36‡	0.97 ± 0.22‡	1.03 ± 0.18‡	0.92 ± 0.32‡
TBARS	12.70 ± 0.58*	17.80 ± 0.69†	18.00 ± 1.58†	17.50 ± 0.48†	16.20 ± 0.61†	17.20 ± 1.33†	12.50 ± 0.59*
GSH:GSSG	1.77 ± 0.13*	0.72 ± 0.06†	0.84 ± 0.05†	1.02 ± 0.10†	0.91 ± 0.04†	1.09 ± 0.11†	1.37 ± 0.02‡
Catalase	11.40 ± 0.22*	6.70 ± 0.82†	7.10 ± 0.79†	9.20 ± 0.79†	9.00 ± 0.63†	8.10 ± 0.51†	10.80 ± 0.19‡
SOD	9.77 ± 1.42*	5.41 ± 0.43†	6.87 ± 0.17†	6.93 ± 0.18†	6.37 ± 0.49†	5.84 ± 0.44†	7.28 ± 0.64†
GPx	1.82 ± 0.12*	0.69 ± 0.02†	0.92 ± 0.01‡	0.92 ± 0.03‡	0.88 ± 0.06‡	1.09 ± 0.08‡	1.57 ± 0.21*
GST	30.8 ± 1.27*	16.3 ± 1.02†	21.7 ± 0.90‡	21.4 ± 1.02‡	19.4 ± 0.94‡	17.5 ± 1.19†	24.3 ± 1.15‡

ALAD, δ-aminolevulinic acid dehydratase as (μmol min^{-1} (mg protein)$^{-1}$); ALAS, δ-aminolevulinic acid synthase as (μmol min^{-1} (mg protein)$^{-1}$; ROS, reactive oxygen species (μmol min^{-1} (mg protein)$^{-1}$); TBARS, thiobarbituric acid reactive substances (μg (g tissue)$^{-1}$); GSH, reduced glutathione (mg (g tissue)$^{-1}$); GSSG, oxidized glutathione (mg (g tissue)$^{-1}$); Catalase (μmol min^{-1} (mg protein)$^{-1}$); SOD, superoxide dismutase (U min^{-1} (mg protein)$^{-1}$; GPx, glutathione peroxidase (μg min^{-1} (mg protein)$^{-1}$; GST, glutathione S-transferase as (μmol min^{-1} (mg protein)$^{-1}$). Values are mean ± s.e.m., n = 5. *, †, ‡, Differences between values with matching symbol notations within each row are not statistically significant at 5% level of probability.

Effect on enzymes of antioxidant defence system in brain

There was a small but significant depletion of SOD and catalase activity after lead exposure (Table 3). Brain GPx, GST activity and GSH:GSSG ratio showed significant depletion on lead exposure, suggesting impaired antioxidant defence system. SOD activity remained unaffected on chelation therapy, while combined treatment with MiADMSA and *C. asiatica* was able to reduce depletion of catalase activity and GSH:GSSG ratio. Inhibited brain GPx activity showed significant protection following combined treatment with *C. asiatica* and MiADMSA. GST activity recovered significantly following monotherapy with DMSA and MiADMSA and, to a lesser extent, with *C. asiatica*. The best protection was achieved after combined administration of MiADMSA and *C. asiatica* but the value was not statistically different to any of the monotherapy groups.

Effect on brain biogenic amines

The effects of lead on whole brain biogenic amines like NE, 5-HT, DA and the activity of AChE and MAO and their response to treatment with thiol chelators either individually or in combination with *C. asiatica* are shown in Figures 1 and 2. Levels of NE, 5-HT, DA and the activity of AChE and MAO decreased significantly on lead exposure. All the treatments except combined administration of MiADMSA and *C. asiatica* were ineffective in influencing NE level, while MiADMSA alone was effective in reducing alteration in 5-HT levels. The DA level remained unchanged on monotherapy with thiols or *C. asiatica*. On the other hand, combined administration of MiADMSA and *C. asiatica* provided significant recovery in DA, NE and 5-HT levels. Alteration in AChE and MAO activity also responded favourably to the combined administration of DMSA/MiADMSA and *C. asiatica* and DMSA and *C. asiatica*, respectively.

Effect on blood and brain lead and essential metal concentration

Figure 3 concentrates on the effects of chelation therapy on the mobilization of lead from blood and brain. Exposure to lead resulted in a significant increase in lead concentration of blood and brain. No significant effect of
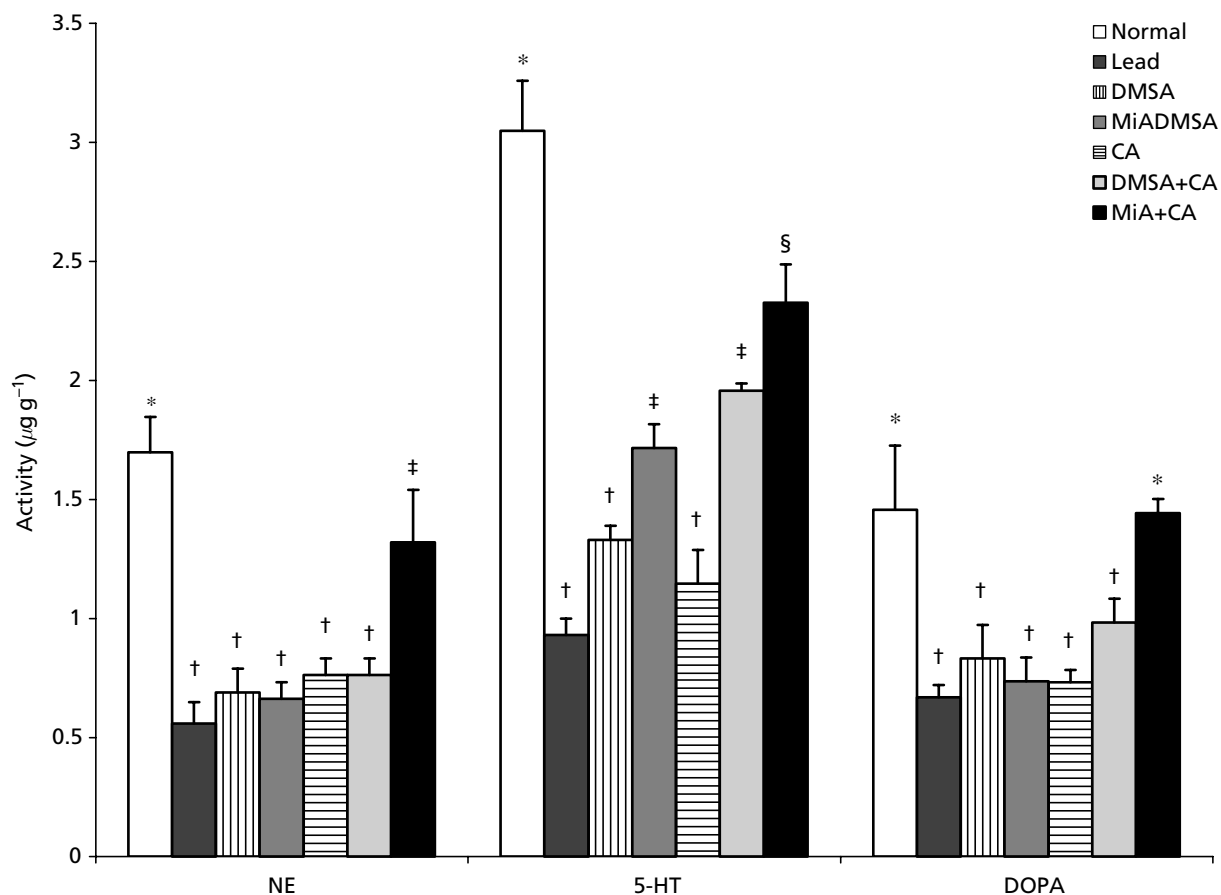

Figure 1 Effect of chelation therapy individually or in combination with *C. asiatica* (CA) on altered neurotransmitters levels in brain of lead-exposed rats. NE, noradrenaline (norepinephrine) (μg (g tissue)$^{-1}$); 5-HT, 5-hydroxytryptamine (μg (g tissue)$^{-1}$); DA, dopamine (μg (g tissue)$^{-1}$). Values are mean $\pm$ s.e.m., n = 5. *, †, ‡, §, Differences between values with matching symbol notations within each block of columns are not statistically significant at 5% level of probability.
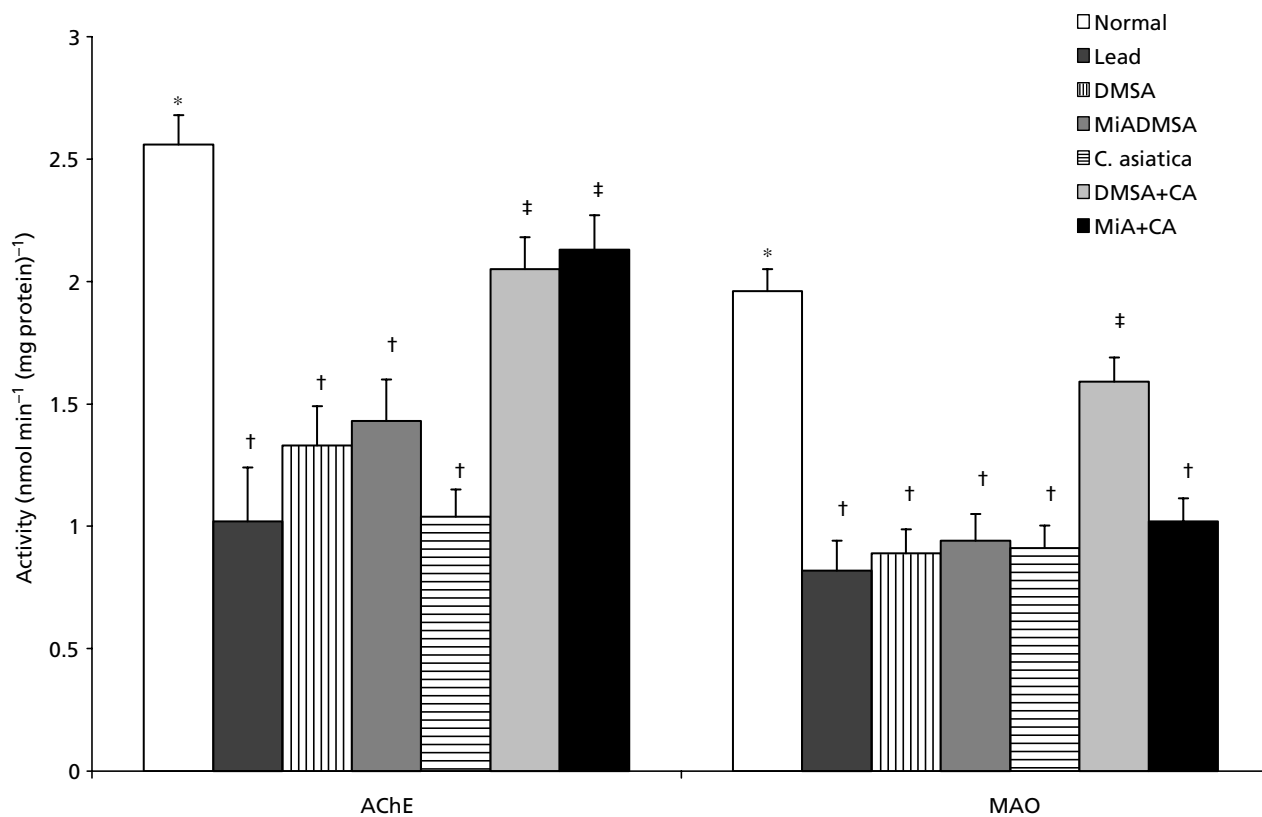
Figure 2   Effect of chelation therapy individually or in combination with *C. asiatica* (CA) on altered neurotransmitters levels in brain of lead-exposed rats. AchE, acetyl cholinesterase (nmol min^{-1} (mg protein)$^{-1}$); MAO, monoamine oxidase (nmol min^{-1} (mg protein)$^{-1}$). Values are mean ± s.e.m., n = 5. *, †, ‡, Differences between values with matching symbol notations within each block of columns are not statistically significant at 5% level of probability.

monotherapy with chelating agents on blood and brain lead concentration was noted. Administration of *C. asiatica* with MiADMSA, however, proved to be significantly effective in the depletion of brain lead concentration compared with all other treatments.

Lead exposure had no effect on zinc and copper concentration in blood and brain. There was, however, a significant depletion of zinc on monotherapy with DMSA, while copper level decreased on MiADMSA treatment. No adverse effects of *C. asiatica* either alone or in combination with the chelators were noted on the concentration of these essential metals (data not shown).

Discussion

This work provides interesting data about the use of *C. asiatica* during chelation treatment with two potent thiol chelators, in providing better recovery in altered biochemical variables and mobilization of lead from blood and brain post lead exposure in rat. *C. asiatica* is known for its strong antioxidant property and ability to cross the blood–brain barrier. Two courses of 5 consecutive days of chelation treatment were given as we earlier reported that a standard single five-day course of chelation

treatment may not be sufficient to the achieve the desired effect (Saxena & Flora 2004).

The effects of lead on the haematopoietic system are well known and include decreased haem synthesis and anaemia (Waldron 1966). The effects on RBC membranes, in particular, have been intensely studied because RBCs have a high affinity for lead and contain the majority of lead found in the blood stream and thus are known to be more vulnerable to oxidative damage than a number of other cells (Rice-Evans 1990). Waldron (1966) reported increased osmotic and mechanic susceptibilities of RBCs in lead toxicity accompanied by shortened lifespan of RBCs. Decreased RBC count and anaemia noted in this study supports the above observations. After RBCs, the brain is the next most vulnerable organ to oxidative damage due to the high utilization of oxygen, the large amount of easily oxidizable polyunsaturated fatty acids, the abundance of redox-active transition metal ions and the relative dearth of antioxidant defence system.

Multiple mechanisms may be involved in lead-induced toxicity, such as disruption of calcium homoeostasis and inhibition of thiol-containing enzymes (Bressler & Goldstein 1991; Gurer & Ercal 2000). The inability of the few known mechanisms of lead toxicity to explain some of the recent observations has led to investigation
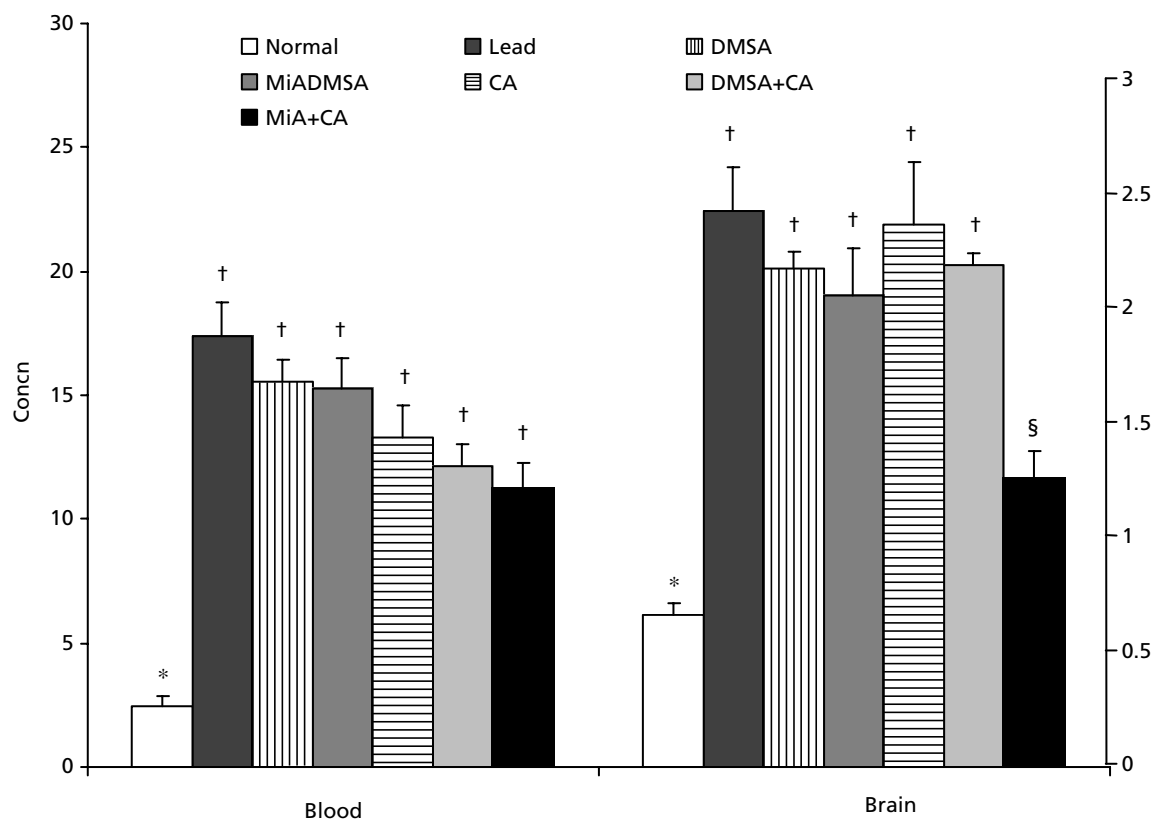
Figure 3 Effect of chelation therapy individually or in combination with *C. asiatica* (CA) on lead concentration in blood and brain of lead-exposed rats. Blood lead is expressed as $\mu g\,dL^{-1}$; brain lead is expressed as $(\mu g\,(g\,wet\,tissue)^{-1}$. Values are mean $\pm$ s.e.m., n = 5. *, †, §, Differences between values with matching symbol notations within each block of columns are not statistically significant at 5% level of probability.

of alternative mechanisms for lead intoxication. Among the most recent and important mechanisms suggested for lead toxicity include disruption in the pro-oxidant/antioxidant balance leading to oxidative stress in cells (Hermes-Lima et al 1991; Monteiro et al 1995). Generation of highly reactive oxygen species (ROS), such as hydroxyl radical (OH^{-}), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$) and lipid peroxides (LPO^{-}), after lead exposure has been reported (Bressler & Goldstein 1991).

The most obvious toxic effects of lead appear in haem biosynthesis and the CNS (WHO 1987; Skerfving et al 1998). ALAD has been suggested as a sensitive index of the effect of lead exposure on haem biosynthesis. ALAD is the second enzyme in the haem biosynthesis pathway and catalyses the condensation of two molecules of ALA to the prophobilinogen. ALAD possesses thiol (SH) groups, which are essential for its activity. Lead, because of its affinity for SH groups, is known to inhibit ALAD resulting in the accumulation of ALA (Goyer & Clarkson 2001). This accumulated ALA has been shown to be involved in lead-induced oxidative damage by causing formation of ROS. In this study, the ALAD activity decreased significantly in blood and brain, while the ALAS activity increased significantly. This implies that ALAD activity might be a promising indicator of lead-induced oxidative damage in blood and brain. Several

antioxidant enzymes and molecules have been used to evaluate lead-induced oxidative stress in blood and brain in rats. In this study, there was a significant increase in the MDA level after lead exposure; it could be because MDA content is evaluated as an end-point indicative of the extent of lipid peroxidation, since MDA is known to be one of the most abundant aldehydes formed as a by-product of lipid peroxidation. SOD and catalase activity are evaluated as key components of cellular defence against oxidative stress. The SOD converts $O_2^{\cdot-}$ to H_2O_2 and catalase converts H_2O_2 to oxygen and water. In this study, both SOD and catalase activity decreased significantly, which could be explained by the fact that the oxidative stress induced could accelerate pro-oxidant formation and reduce the antioxidant defence of cells (Pande & Flora 2002). Inhibition of antioxidant enzymes viz. glutathione peroxidase and glutathione-S-transferase in the brain of lead-exposed animals also points to disturbed antioxidant defence system of cells, which might lead to generation of oxidative stress. Further, the tripeptide glutathione (GSH) acts both as a direct scavenger of ROS and as a cofactor in metabolic detoxification (Griffith 1999). It is found in-vivo as both the reduced thiol (GSH) and oxidized disulphide (GSSG) forms. In the presence of reactive species, GSH is oxidized rapidly to GSSG resulting in a decrease in GSH and increase in

GSSG content. Therefore, decreased GSH/GSSG ratio is used as an indicator of oxidative stress. This may further lead to disturbed brain functioning and severe neurological complications, as observed in neuropathy. As described in the results, inhibition of neurotransmitters (NE, 5-HT, DA, AChE) was observed in the rat brain. The mechanism by which lead produces neuronal dysfunction constitutes the basis of numerous studies (Struzynska et al 1997; Dabrowska-Bouta et al 1999). Many of the biological dysfunctions produced by lead appear to be associated with the metal's ability to mimic or inhibit the action of calcium. In the nervous system, calcium aids in the conduction of a nerve impulse across a synapse by invoking the release of neurotransmitters. In the CNS, lead increases the permeability of the blood–brain barrier, which produces brain oedema. Lead, even at low concentrations, has the ability to increase the basal release of neurotransmitters from presynaptic nerve endings. This can occur both in the peripheral nervous system and CNS. Micromolar concentrations of lead can cause the spontaneous depletion of dopamine and acetylcholine (ACh). Control movement and emotional response are some of the brain processes that are affected by dopamine. An acetylcholine receptor is responsible for transducting nerve impulses to muscular contraction. At low concentrations, lead appears to increase the basal release of neurotransmitter from a presynaptic nerve ending. This phenomenon occurs both in the peripheral and the central nervous systems. Lead also has the ability to block the release of neurotransmitters during the normal action potential. The increased neuronal activity induced by lead exposure can inhibit this process and have lasting adverse effects on the synaptic anatomy and function of the brain. This may be one of the underlying causes of learning and behavioural problems in young children (Bellinger 1995; Shi et al 1995).

The current therapeutic approach to lead poisoning is to increase the excretion of lead by chelation. Although chelation has been shown to reduce blood lead levels, the safety and efficacy of the various chelators may be questioned. It is well known that the chelation therapy with many synthetic chelating agents is compromised by the number of side effects (Flora & Kumar 1993; Flora et al 1995; Flora 2002). The most widely used chelating agents are calcium disodium ethylenediamine tetra acetic acid (CaNa$_2$EDTA), D-penicillamine and British anti-lewisite (BAL). CaNa$_2$EDTA is the most promising drug for treating lead toxicity. For example, at high doses it might cause cell death due to necrosis (Weeden et al 1983), while redistribution of lead from the hard tissue deposits to soft organs too has been reported (Flora et al 1995). Meso 2,3-dimercaptosuccinic acid (DMSA), an analogue of BAL, possesses a thiol group, has higher therapeutic index, water solubility (Graziano et al 1985), and can be administered orally (Miller 1998). It is known to be a non-toxic agent that can reduce the body burden of several toxic metals (Graziano et al 1985; Aposhian & Aposhian 1990; Jones 1991) and is already in clinical use. Recently, monoesters of DMSA with more lipophilic properties have been developed and tried as therapeutic agents for

metal poisoning in mice and rats (Kreppel et al 1993; Kostial et al 1995; Flora et al 2004). A number of recent studies support the hypothesis that the monoisoamyl ester of DMSA could be a potential drug for the treatment of heavy metal poisoning.

We have recently reported on the toxicological safety of MiADMSA in male and female rats, as well as its comparison with other metal chelators (Mehta & Flora 2001; Mehta et al 2002; Flora & Mehta 2003), and found that the chelator does not produce any adverse effects on the tissues, except for a mild hepatotoxicity and copper loss. Effective chelation therapy for intoxication by certain heavy metals depends on whether the chelating agents are able to reach the intracellular site where the heavy metal is firmly bound. A number of strategies have been suggested to minimize the numerous problems. One important approach has been the use of combination therapy (Flora et al 1995). Recently, a monoester of DMSA, mono-isoamyl DMSA, was found to be effective in chelating intracellularly bound metals (Jones 1994) as opposed to its parent compound DMSA, which does not have intracellular access.

As induction of ROS by lead and depletion of antioxidant cell defences results in tissue injury via oxidative damage to critical biomolecules, a therapeutic strategy to increase the antioxidant capacity of cells is required to enhance the long-term effective treatment of lead poisoning. This may be accomplished either by reducing blood and tissue lead levels viz. chelation, thereby reducing the possibility of lead interacting with critical biomolecules and inducing oxidative damage, or by bolstering the cell's antioxidant defences through exogenous supplementation of antioxidant molecules. This prompted us to explore whether the co-administration of an antioxidant during the removal of lead by a chelating agent could provide any additional advantage (i.e., abatement of the toxic effects of lead). Earlier, we reported an interesting treatment protocol of using thiol chelators in combination with vitamins C and E (naturally occurring antioxidants) for achieving a better therapeutic efficacy against chronic lead intoxication (Flora et al 2003). Co-administration of vitamins C and E during chelation with DMSA or MiADMSA proved to be beneficial in the effective reduction of the body lead burden in addition to reversal of altered biochemical variables and oxidative stress (Flora et al 2003). Similarly, in this study, the major hypothesis behind using combination therapy of chelating agents with naturally occurring antioxidant C. asiatica is that: firstly, C. asiatica is reported to cross the blood–brain barrier and recover the altered neurotransmitters levels (Nalini et al 1992) and because of its ability to restore impaired pro-oxidant/antioxidant balance will accelerate clinical/biochemical recovery; and, secondly, MiADMSA, because of its lipophilic nature, will have intracellular access and hence would be more effective in the mobilization of metal out from the body.

In this study, two courses of combination treatment with the thiol chelator and the natural antioxidant C. asiatica proved to be beneficial in the recovery from lead-induced oxidative stress, including the level of

biogenic amines and body lead burden, as compared with the monotherapy. Further, administration of *C. asiatica* during chelation provided more pronounced effects, particularly in the recovery of oxidative stress parameters, suggesting that with the removal of lead from the target tissue, this antioxidant provides effective reversal in the altered parameters indicative of oxidative stress. There are some observations with the combined administration of *C. asiatica* and chelators that are worth noting. For example, concomitant administration of *C. asiatica* and MiADMSA was able to restore the altered levels of neurotransmitters (NE, DA and AChE) to normal as compared with the effects of chelators individually. It is therefore possible that restoration of altered biochemical variables after combined administration of *C. asiatica* may be responsible for the decreased oxidative stress, and the recovery in neurotransmitter levels in particular. A study by Kumar & Gupta (2002) also supports our results, wherein they have reported that the aqueous extract of whole plant of *C. asiatica* has two pronounced effects – improving the learning and memory and the antioxidant property by decreasing lipid peroxidation and augmenting endogenous antioxidant enzymes in brain. The bioactive components that could be responsible for the antioxidant property of *C. asiatica*, as reported by Inamdar et al (1996), are terpene acids, asiatic acid, madecassic acid and their respective glycosides, asiaticoside and madecassoside (Figure 4).

MiADMSA is among the series of newly synthesized monoesters of DMSA found to be highly effective against cadmium and mercury poisoning (Jones et al 1992; Kostial

et al 1995). Walker et al (1992) and Saxena et al (2005) also reported superior efficacy of monoesters to DMSA in mobilizing brain lead. The greater effectiveness of MiADMSA compared with DMSA in mobilizing lead from the body and reducing its toxic effects may be attributed to its ability to gain intracellular access to various endogenous ligands (i.e., its lipophilic nature) (Figure 4). Thus, it certainly has the advantage over chelators like DMSA that its distribution is restricted to extracellular sites only. Our report provides evidence of its ability to produce turnover in a number of altered lead-induced biochemical variables besides lead depletion both individually and in combination with *C. asiatica*.

Conclusion

This work provided evidence of beneficial effects of combination chelator/*C. asiatica* treatment against subchronic lead poisoning in rats. However, it is recommended that more detailed studies against acute and chronic lead-poisoned experimental animals must be conducted to obtain a final recommendation.

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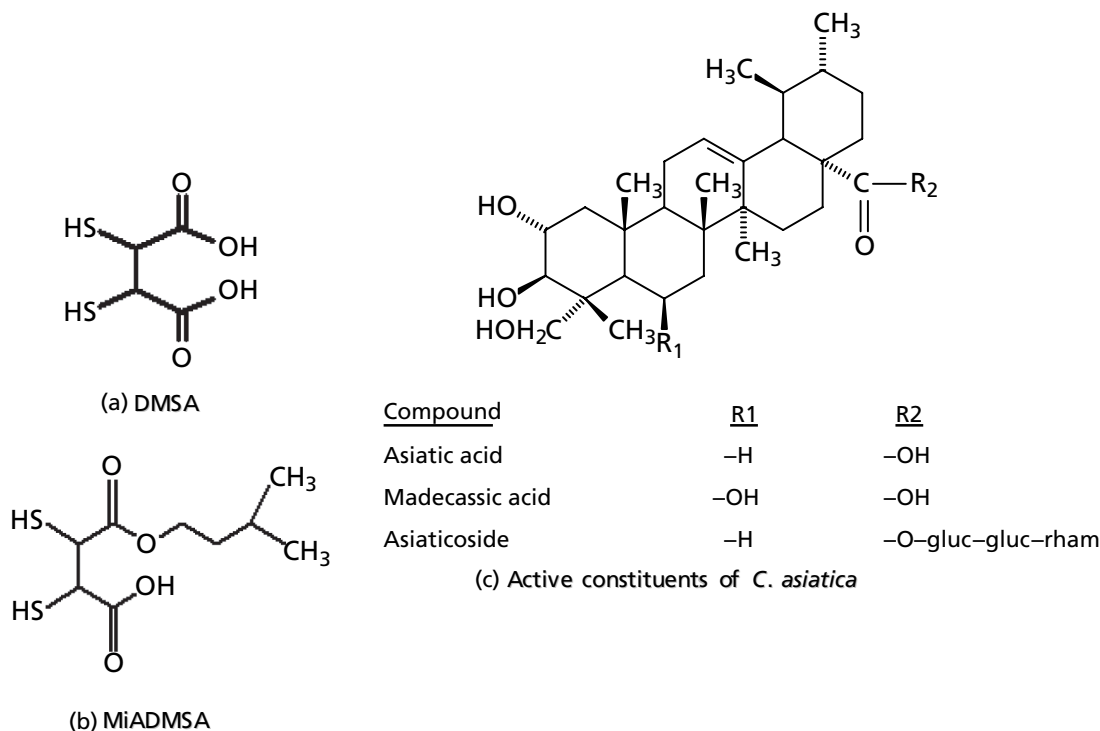

Compound	R1	R2
Asiatic acid	–H	–OH
Madecassic acid	–OH	–OH
Asiaticoside	–H	–O–gluc–gluc–rham

(a) DMSA

(b) MiADMSA

(c) Active constituents of *C. asiatica*

Figure 4 Chemical structures of chelating agents and some active constituents of *C. asiatica*.

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