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Influence of age on arsenic-induced mitochondrial oxidative stress in rat brain regions: Chelation with monoisoamyl 2,3-dimercaptosuccinic acid

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Abstract

Objective: This study was aimed to determine the influence of age on arsenic (As, 10mg/kg body weight given through oral gavage) induced mitochondrial oxidative stress in three different age groups of rats; young (PND21), adult (3 months) and aged (18 months) animals at seven days post-acute exposure. Further, we also evaluated the protective effect of chelating agent, monoisoamyl 2,3-dimercaptosuccinic acid (MiADMSA, 50mg/kg body weight given through oral gavage, 2 h after As administration), against As-induced perturbations in brain regions (cerebral cortex, cerebellum and hippocampus).

Results: Studies conducted at different age points showed significant increase in Malondialdehyde (MDA) and As levels and Glutathione-S-Transferase activity, accompanied by a decrease in the levels of oxidative stress markers such as Superoxide Dismutase isoforms (Mn and Cu/Zn-SOD), Catalase, Glutathione Peroxidase, Glutathione Reductase, Glutathione (GSH) and total Free Sulfhydryl Groups (TSG).

Conclusion: Among the three different age points, aged rats were found to be more vulnerable to the As-induced toxicity. Among the brain regions, As-induced effects were more pronounced in cerebral cortex followed by cerebellum and hippocampus. However, co-administration of MiADMSA along with As elicited significant protection in restoring the altered levels of antioxidant enzymes, towards the control levels. Conclusively, aged animals were found to be most sensitive to the As-induced toxicity as compared to young and adults.

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Introduction

The mechanism underlying aging, an unavoidable biological process which affects most living organisms and age related diseases, such as Alzheimer's, Parkinson's, and diabetes is still an area of significant controversy. One of the most important factors responsible for these diseases includes heavy metals accumulation. Out of all environmental toxic metals, exposure to Arsenic (As) contaminated drinking water represents one of the largest public health poisonings in the history of human civilization, affecting tens of millions of people worldwide [1,2].

As is known to be toxic to different organs, including hepatic, hematopoietic, renal, reproductive, nervous system, etc [3]. Several recent reports worldwide suggest that children and elderly population are more susceptible to the toxic effects of As [4-6]. Differential toxicity of as could be due to differences in absorption, distribution, metabolism and elimination that varies with age [7].

As exposure is associated with risk of impaired cognitive development, subclinical brain dysfunction and behavioral anomalies [8]. The exact mechanisms by which as induces toxicity are still being elucidated [9]. One of the possible molecular mechanism involved in the As-induced neurotoxicity is the disruption of the prooxidant/antioxidant balance, associated with increased generation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) that impair cellular antioxidant defense system and simultaneously damage the critical biomolecules, such as lipids, proteins and DNA [10,11].

Mitochondria have been a central focus of several theories of aging as a result of their critical role in bioenergetics, oxidants generation, and regulation of cell death [12].Several studies have reported alterations in antioxidant enzyme activities such as Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx) which can trigger apoptosis in as treated animals [13,14]. Hence, the present study was focused on mitochondrial oxidative stress marker enzymes.

In spite of many years of research, an effective treatment for As toxicity is still far away. Many metal chelating agents including British Anti-Lewisite (BAL), 2,3-Dimercaptosuccinic Acid (DMSA) and 2,3-Dimercaptopropane Sulfate (DMPS) are being used for treatment [15,16]. However, most of them are known for their limitations and side effects that include low therapeutic index, non-specificity (essential metal loss such as copper and zinc), and inability to penetrate cellular membrane (extracellular nature), metal redistribution, hepatic and renal toxicity [16,17]. In order to improve the efficacy of DMSA, different analogs were synthesized against metal poisoning. Among these analogs, mono-isoamyl ester of DMSA (MiADMSA) was shown to remove heavy metals like As efficiently from acute as well as chronically exposed animals [2,10].

From the above studies, it is very clear that exposure to As induces oxidative stress and the series of tests to evaluate mitochondrial oxidative stress are the antioxidant enzymes. Therefore, the present study was designed to examine the effect of As on the activity levels of antioxidant enzymes and non-enzymatic antioxidants in brain regions (cerebral cortex, cerebellum and hippocampus) at different age groups (young, adult and aged) of rats. Further, we also evaluated the protective effect of a chelating agent, monoisoamyl 2,3-dimercaptosuccinic acid (MiADMSA) against As-induced mitochondrial oxidative damage.

Materials and methods

Procurement and maintenance of animals

The study was conducted to evaluate the influence of age on As induced neurotoxicity at different age group rats. For this, 3 months (adult) and 18 months (aged) old male albino rats (Wistar) were purchased from Sri Venkateswara Traders, Bangalore, whereas PND14 (young) rats were obtained from our laboratory by maintaining pregnant rats. Animals of all age groups were maintained in the animal house of Sri Venkateswara University, Dept. of Zoology, Tirupati. All the rats were kept in well cleaned, sterilized polypropylene cages lined with paddy husk (18" x 10" x 8"). The animals were maintained under a regulated light: dark 12 hr (7:00–19:00) scheduled at 24 $\pm 1^0$C and relative humidity of 55 ± 15%. Rats were provided standard rat chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003) and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India.

Chemicals

The chemicals used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA) and Merck, India. Sodium arsenite ($NaAsO_2$, >99% purity) used in this study was purchased from Sigma–Aldrich (St Louis, MO, USA) and dissolved in sterile distilled water to the desired concentrations. MiADMSA (purity 99.9%) was a gift from Dr. S.J.S. Flora, Division of Regulatory Toxicology, DRDE, Gwalior, India.

Animal exposure

After a week of acclimatization, rats of all age groups (PND21: young; 3 months: adult; 18 months: aged) were randomly divided into 4 groups and were treated for a period of one week as follows:

Group II: MiADMSA (50 mg/kg, once daily, orally through gavage) (n=6)

Group III: Arsenic as sodium arsenite (10 mg/kg, once daily, orally through gavage) (n=6)

Group IV: As (10 mg/kg, once daily, orally) + MiADMSA, (50 mg/kg, once daily, orally, 2 h after As administration) through gavage (n=6).

The control animals which served as group I (n=6), received equivalent volumes of deionized water through oral gavage. The dose of sodium arsenite used in this study was based upon the reports of Itoh et al., [18] and Rodriguez et al., (2001) in rats which represent ¼ of the LD_{50} dose for rats [19]. MiADMSA dose was selected on the basis of earlier published reports by Flora et al. [20,21] ,who reported that 50 mg/kg to be the optimal dose for most effective recoveries in clinical variables as well as reducing arsenic body burden. Hence, we selected this dose for our present study. MiADMSA was dissolved in 5% sodium bicarbonate solution and was prepared immediately before use. The dosing volume amounted to 4 ml/kg body weight. After completion of treatment, animals were sacrificed through cervical dislocation and the brain regions (cerebral cortex, hippocampus and cerebellum) were quickly isolated and stored at --80 ºC for biochemical analysis.

Superoxide Dismutase (SOD) activity

Measurement of total SOD activity was performed according to Misra and Fridovich, [22] based on the inhibition of auto-oxidation of epinephrine. The total reaction mixture contained of 880 µl of 0.05 M carbonate buffer (pH 10.2), 20 µl of 30 mM epinephrine and 100 µl of enzyme source and absorbance was recorded at 480 nm against reagent blank. The enzyme activity was expressed as micromoles of superoxide anion reduced/mg of protein. Mn–SOD activity was determined in the mitochondrial fractions by inhibiting the Cu/Zn–SOD with KCN and subtracting the values from total SOD activity.

Catalase (CAT) activity

CAT activity was measured by following the method of Chance and Maehly [23]. The reaction mixture contained 1.9 ml reagent grade water, 1.0 ml of 0.059 M hydrogen peroxide (H_2O_2) in buffer. The reaction mixture was incubated in spectrophotometer for 4–5 min to achieve temperature equilibration and to establish blank rate if any. 0.1 ml of mitochondrial fraction was added and decrease in absorbance was recorded at 240 nm for 2-3 min. ΔA_{240}/min from the initial (45s) linear portion of the curve was calculated. The enzyme activity was expressed as µmoles of H_2O_2 decomposed/mg protein/min.

Glutathione Peroxidase (GPx) activity

GPx activity was determined by following the method of Rotruck et al., [24]. Briefly, the reaction mixture contained 0.5 ml of 0.4 mol/l sodium phosphate buffer (pH 7.0), 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.1 ml H_2O_2, and 0.5 ml of 1:10 diluted aliquot of the mitochondrial fraction and the total volume was made up to 2 ml with distilled water. The tubes were incubated at 37 ºC for 3 min and the reaction was terminated with 0.5 ml 10% TCA and centrifuged at 4 ºC for 10 min at 1500 rpm. The supernatant was collected and to this 4 ml of 0.3 mol/l disodium hydrogen phosphate and 1 ml DTNB (dithionitro benzoic acid; 0.004%) reagent were added. The color developed was read at 412 nm against the reagent blank containing only phosphate solution and DTNB reagent. The enzyme activity was expressed as µmoles of GSH oxidized/mg of protein/min.

Glutathione Reductase (GR) activity

GR activity in the mitochondrial fraction of brain regions was assayed as described by Staal et al., [25]. The reaction mixture in a final volume of 3.0 ml contained 1.0 ml of 0.3 M Sodium phosphate buffer (pH-6.8), 0.5 ml of 250 mM EDTA, 0.5 ml of 12.5 mM GSSG, 0.7 ml of distilled water, 0.2 ml of 30 mM NADPH and 0.1 ml of mitochondrial fraction. Changes in absorbance were recorded at 340 nm in a spectrophotometer. The enzyme activity was expressed as µmoles of NADPH oxidized/mg protein/min.

Glutathione-S-Transferase (GST) activity

GST activity in the mitochondrial fraction of brain regions was assayed by using 1-chloro-2, 4-dinitro benzene (CDNB) (at 340 nm) as described by Habig et al., [26]. The reaction mixture in a final volume of 3.0 ml contained; 150 mM phosphate buffer (pH 7.5), 1 mM CDNB, 5 mM Glutathione (GSH) and an appropriate amount of mitochondrial fraction. The reaction was initiated by the addition of GSH and incubated at 37 ºC. The formation of a thioether by the conjugation of CDNB to GSH was monitored at 340 nm in a spectrophotometer (Hitachi model, U-2001). Thioether concentration was determined from the slopes of initial reaction rates. The activity was expressed as µmoles of thioether formed/mg protein/min.

Glutathione (GSH) content

GSH content was determined according to the method of Ellman, [27]. Here, the mitochondrial fraction was doubled diluted and trichloroacetic acid (5%) was added to precipitate the protein content. After centrifugation at 10000 g for 5 min, the supernatant was collected. To the supernatant, 5,5-dithiobis [2-nitrobenzoic acid solution (Ellman's reagent)] was added and the absorbance was measured at 412 nm. The values were expressed as millimoles/gm tissue.

Total Sulfhydryl Group (TSG) content

TSG content was determined by using the method of Sedlak and Lindsay, [28]. Briefly, to the mitochondrial fraction (100 µl), 1 ml of tris-EDTA buffer (0.2 mol/L, pH 8.2) and 0.9 ml of EDTA solution (0.02 mol/L, pH 4.7) was added followed by the addition of 20 µl of Ellman's reagent. After 30 min of incubation at room temperature, samples were centrifuged and the absorbance was read at 412 nm in spectrophotometer.

Lipid Peroxidation (LPx)

The lipid peroxides were determined by following the method of Ohkawa et al., [29]. The reaction mixture contained 0.1 ml of mitochondrial fraction, 0.2 ml of 8.1% Sodium Dodecyl Sulphate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% aqueous solution of Thiobarbituric Acid (TBA). The pH was adjusted to 3.5. The mixture was finally made up to 4.0 ml with distilled water and heated at 95 ºC for 60 min. After cooling under tap water, 0.1 ml of distilled water and 5.0 ml of a mixture of n-butanol and pyridine (15:1, V/V) were added and the mixture was shaken vigorously on a vortex mixture. After centrifugation at 2200 x g for 5 min the upper organic layer was separated and the absorbance was read at 532 nm in a spectrophotometer. The rate of lipid peroxidation was expressed as µ moles of malondialdehyde formed/gm wet wt. of tissue/hr.

Evaluation of As concentration in brain regions

Brain regional As levels were estimated according to the method of Ballentine and Burford, [30]. To 100 mg of tissues, 1 ml of concentrated nitric acid was added, followed by 1 ml of perchloric acid. The sample was then digested over a sand bath until the solution turns yellow in color. If the color of the digest was brown, 0.5 ml each of nitric acid and perchloric acid were added and the oxidation was repeated. The digest was made up to 2 ml volume with deionized water. Aliquots of this were used to estimate As by using atomic absorption spectrophotometer (AAS, Perkin Elmer model AAnalyst 100).

Statistical analysis of the data

Significance of each age group among different treatments was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test using GraphpadPrism 3.11 program software to compare the effects among various groups. The 0.05 level of probability was used as the criterion for significance.

Results

Our results showed that mitochondrial antioxidant enzymatic activities such as SOD isoforms (Mn-SOD and Cu/Zn-SOD), Catalase (CAT), Glutathione Reductase (GR), Glutathione peroxidase (GPx), non-enzymatic antioxidant levels of Glutathione (GSH) and total free sulfhydryl groups (TSG) were decreased with concomitant increase in lipid peroxidation and GST in different

brain regions (cerebral cortex, cerebellum and hippocampus) of As exposed rats of all the three different age groups (young: PND21; adult: 3 months; aged: 18 months).

Effect of As exposure on SOD isoforms

In control rats, the activities of Mn-SOD and Cu/Zn-SOD were found to be maximum in adults followed by young and aged rats. Maximum activities of Mn-SOD and Cu/Zn-SOD were observed in cortex followed by cerebellum and hippocampus in adult rats. Exposure to As significantly decreased the Mn-SOD and Cu/Zn-SOD activities and the decrease in enzyme activities was found to be greater in aged rats followed by young rats and adult rats. Among all the brain regions studied maximum decrease was observed in cerebral cortex followed by cerebellum and hippocampus (Figures 1 and 2).

Effect of As exposure on CAT

Similar to SOD isoforms, the CAT activity in control rats was found to be maximum in adult rats compared to young and aged rats. Among brain regions, cortex showed maximum CAT activity followed by cerebellum and hippocampus (Figure 3) at all the age points studied. Exposure to As resulted in significant decrease in the specific activities of CAT in all the three brain regions and the decrease in activity was found to be greater in aged rats followed by young rats and adult rats. Cerebral cortex documented maximum decrease in enzyme activity than cerebellum and hippocampus (Figure 3).

Effect of As exposure on GPx

In control rats, the GPx activity was found to be maximum in adults followed by young and aged rats. Among the three brain regions, cortex showed maximum GPx activity followed by cerebellum and hippocampus (Figure 4) at all the age points studied. Exposure to As exhibited significant decrease in the GPx activity and the decrease was found to be greater in aged rats followed by young rats and adult rats. From the data, it is evident that cerebral cortex exhibited maximum decrease in enzyme activity than cerebellum and hippocampus (Figure 4).

Effect of As exposure on GR

Similar to GPx, GR activity was found to be maximum in adults followed by young and aged control rats with cortex documenting maximum GR activity followed by cerebellum and hippocampus (Figure 5). Exposure to As exhibited significant decrease in the specific activity of brain regional GR and the decrease in activity was found to be greater in aged rats followed by young and adult rats. Cerebral cortex exhibited maximum decrease in enzyme activity than cerebellum and hippocampus following As exposure (Figure 5).

Effect of As exposure on GST

Exposure to As exhibited significant increase in the brain regional GST activity levels and the increase in levels was found to be greater in aged rats followed by young rats and adult rats. From the results, it is clear that cerebral cortex exhibited maximum increase in GST activity than cerebellum and hippocampus (Figure 6).

Effect of As exposure on GSH and TSG

Similar to enzymatic antioxidants; the non-enzymatic antioxidants, GSH and TSG levels in control rats were found to greater in adult rats followed by young and aged rats. Among the three brain regions, cortex showed maximum levels followed by cerebellum and hippocampus (Table 1) at all the age points studied. Exposure to As exhibited significant decrease in the levels of brain regional GSH, TSG levels and the decrease was found to be maximum in aged rats followed by young rats and adult rats. Cerebral cortex exhibited maximum decrease in GSH and TSG levels than cerebellum and hippocampus (Table 1).

Effect of As exposure on LPx

In the present investigation, Malondialdehyde (MDA) formation was recorded as a measure of lipid peroxidation in both control and experimental rat brain regions. The MDA content increased with age in control brain regions with the cortex documenting the highest MDA levels followed by cerebellum and hippocampus in aged rats. Exposure to As exhibited significant increase in the brain regional MDA levels and the increase in levels was found to be greater in aged rats followed by young rats and adult rats. From the results, it was found that cerebral cortex exhibited maximum increase in MDA levels than cerebellum and hippocampus (Table 2).

Brain regional As levels

Brain regional accumulation of As at different age points was shown in Table 2. As exposure produced a significant increase in As content in all the three brain regions at all age points. Maximum increase in As content was observed in young rats followed by aged and adult rats. Among the brain regions maximum increase in As levels was found in cerebral cortex compared to cerebellum and hippocampus (Table 2).

Protective effect of MiADMSA

We also evaluated the protective effect of MiADMSA, new thiol-chelator against As-induced alterations in mitochondrial oxidative stress markers in the brain regions of different age groups of rats. Chelation with MiADMSA was able to elicit significant protection to the As-induced alterations in all selected enzymatic antioxidant activities (Mn-SOD, Cu/Zn-SOD, Cat, GPx, GR, GST), non-enzymatic antioxidant levels (GSH and TSG), MDA and As levels in all brain regions (cerebral cortex hippocampus and cerebellum) of young, adult and aged rats (Figures 1 to 6 and Tables 1 and 2). These observed reversal effects in the levels of enzymatic and non-enzymatic antioxidants and MDA levels were significant at $P < 0.05$ (Figures 1 to 6 and Tables 1 and 2).

Discussion

The current study was aimed to evaluate the influence of age on As-induced alterations in brain regional (cerebral cortex, hippocampus, cerebellum) mitochondrial antioxidant system at different age group rats and further to evaluate the protective effect of MiADMSA against As-induced neurotoxicity. Increased As levels in rat brain regions exposed to As indicate that As and it's metabolites are able to cross the Blood Brain Barrier (BBB), which was consistent with the results reported by Zheng et al. [31].

Among different antioxidative mechanisms in the body, SOD is found to be one of the major enzymes which protects against tissue damage caused by the potentially cytotoxic radicals [32,33]. In our study, As exposure lead to a significant decrease in the activity of SOD isoforms in the brain regions of all age groups compared to their respective controls. The decrease in SOD isoforms may be attributed to enhanced superoxide radical production during As metabolism [34,35]. The effect of As on SOD has also been attributed to the following three reasons; (i) altered SOD expression (ii) modification in cellular antioxidant uptake, GSH and vitamin depletion or (iii) alteration in antioxi-

dant activities by affecting their structure (oxidation/reduction of thiol group and displacement of essential metals)

Among the three different age groups studied, the activity levels of SOD-isoforms were significantly decreased in aged rats as compared to young and adult rats. This age-related decrease in SOD-isoforms activity in brain specific regions observed in the present study was in corroboration with Carrillo et al., [33] investigations who reported that the activity levels of antioxidant enzymes, total SOD, and Mn-SOD were decreased with age in the cerebral cortex followed by cerebellum. The possible mechanism suggested was that, an increase in arachidonic acid turnover (e.g. increase in prostaglandin synthase activity) may play a role in the increased oxygen radical load [37]. Sawada and Carlson, [38] reported that superoxide radical formation increases along with age, therefore a decreased protection against toxic radicals may have deleterious consequences for the aging brain.

CAT has been shown to be responsible for the detoxification of significant amounts of hydrogen peroxide (H_2O_2). From the experimental results, it is evident that As exposure significantly decreased CAT activity at all age groups compared to their respective controls. In general, CAT requires NADPH for its regeneration from its inactive form. The activity of glucose-6-phosphate dehydrogenase (G6PDH) decreases with advancing age. As the NADPH level depends on that of G6PDH, a decrease in the activity of latter affects the levels of former. The paucity in NADPH production along with increased superoxide radicals during As exposure also decreases catalase activity [39,40]. The gradual decrease in catalase with increasing age appears to be due to an age-dependent effect in the expression of their genes [41].

Glutathione-related enzymes, such as glutathione peroxidase (GPx) and glutathione reductase (GR), function either directly or indirectly as antioxidants, whereas glutathione S transferase (GST) plays a major role in metabolic detoxification. GPx metabolizes peroxides like H_2O_2 and protects cell membranes against lipid peroxidation. In the present study, a significant reduction in GPx activity has been observed upon exposure to As at all age groups studied. As may directly interacts with Se and form insoluble and inactive As–Se complex [42,43] rendering it unavailable and resulting in the inhibition of GPx activity or alter the expression and synthesis of selenoproteins such as GPx [44,45].

In the present study, in aged rats, the activity of GPx was lower in brain regions than young and adult controls. Our results are in agreement with Brannan et al. [46], who observed a similar decline in the activity of GPx as well as SOD during aging. In addition, on aging, considerable increase in the production of H_2O_2 and a decrease in the activity of GPx were reported. Decreased GPx activity with age may also be attributed due to the decline in GSH concentration.

GR is the key enzyme for maintaining the intracellular concentration of reduced glutathione. From the results, it is evident that, As exposure significantly decreased GR activity in all age groups compared to their respective controls. Arsenite and its methylated metabolites have been known to be potent inhibitors of GR *in vitro* [47-49]; thus, exposure to arsenicals would compromise the antioxidant mechanisms by consuming GSH and inhibiting the enzyme responsible for its recycling eventually leading to cell death.

Our data on decreased GR gets further support from the fact that trivalent arsenicals are potent inhibitors of GR [48] which can cause inhibition by the interaction of As with critical thiol groups in these enzymes [50]. However, in the present study, a significant decrease in GR activity was observed in the brain regions of aged rats compared to the young and adult rats. Thus the significant reduction observed in GR of aged rats may be associated with age-related decrease of GSH in different brain regions [51].

As exposure increased GST activity in all the brain regions of all the age groups studied. Increased GST activity following As exposure suggests a counteracting mechanism adopted by system to eliminate As and its metabolites. Kim et al., (2003), reported that the GST activity was increased by 1.7 fold in the brains of 9-month-old rats compared to those of 5-week-old rats. In our current study, GST was increased significantly in all the three brain regions of aged animals. Enhanced GST activity is assumed to respond to aging as an adaptive mechanism secondary to increases in oxidative stress [10].

Reduced glutathione (GSH), a non-protein thiol, plays an important role in the detoxification via facilitating As removal from the cellular sites and stimulating excretion of methylated As [52], which is responsible for As-induced cytotoxicity. From our experimental results, it is evident that As exposure significantly depleted GSH levels at all age groups compared with their respective controls. Among the different age groups, As exposure lead to a significant GSH levels reduction in aged rats, followed by young and adult rats. As-induced GSH depletion may be attributed due to its direct binding with GSH due to its high binding affinity towards thiol groups and also electrophillic nature of GSH. Indirect depletion of GSH by As may also due to its utilization in scavenging of As-induced free radicals generation. This could be attributed due to the utilization of GSH as electron donor for As metabolism or direct binding due to thiol preference. An age-associated decline in GSH level has been reported in the brain tissue of various species [53,54], which are consistent with the present findings. Maiti and Chatterjee, [55] also reported that depletion of GSH may also be caused due to the GPx mediated excess utilization of GSH.

Glutathione and total thiols (sulfhydryl groups) non-enzymatically reduce peroxides and/or prevent occurrence of peroxidation. In the present study, a significant decrease in these sulfhydryl groups was observed in aged rats. The recycling of GSH from GSSG (oxidized glutathione) is catalyzed by glutathione reductase using NADPH as a cofactor. NADPH is generated in a pathway involving G6PDH. Age-related decline in the activity of G6PDH leading to diminished production of NADPH may be the cause for the observed decrease in sulfhydryl groups.

LPx measured as MDA levels in the present study forms the most common indices to assess mitochondrial oxidative stress. The peroxidation products of lipids, usually Polyunsaturated Fatty Acids (PUFA) are susceptible to the attack of free radicals forming an important biomarker [56]. In the current study, a significant increase in LPx was observed in young and aged rats, compared to the adult rats. Increased lipid peroxidation in response to As is thought to be the consequences of oxidative stress, which occurs when the dynamic balance between prooxidant and antioxidant mechanism is altered. The present results also support the correlation of increased MDA levels with the decreased antioxidant defense system due to As toxicity.

In this study the basal control levels were increased with age from young to adult and decreased in aged animals. The average increase was 29.2%, 28.45% and 26.42% in cerebral cortex, cerebellum and hippocampus respectively from young to adult and

the average decrease was 40.76%, 42.82% and 42.56% in cerebral cortex, cerebellum and hippocampus respectively from adult to aged animals. As treated groups showed significant decrease in all ages. However, the young and aged showed greater decrease compared to adults. This could be due to the fact that developing and differentiating brain with under-developed blood-brain barrier is more vulnerable to toxic insult than fully developed adult brain [1,57,58]. Similarly aged brain is more vulnerable to toxic insult because of increased reactive oxygen species and the primary mechanism of As toxicity is mediated by oxidative damage [1]. The brain regions also respond differently to As due to the differences in their formation and transmitter synthesizing neuronal system and also due to differences in absorption, distribution, metabolism and elimination that varies with age [7].

Among the brain regions studied, the activity levels of oxidative stress marker enzymes were found to be altered to a greater extent in cortex, followed by cerebellum and hippocampus, indicating that these regions are highly sensitive to oxidative stress. Certain brain regions such as cortex and striatum are highly enriched with non-heme iron, which is catalytically involved in free radicals generation [59]. The relative sensitivity of cortex when compared to cerebellum and hippocampus may be attributed to the differences in the iron content which influence the ROS generation [10,60].

One of the primary factors determining the effectiveness of chelation therapy against metal induced toxicity is the ability of chelating agents to reach the intracellular site. Moreover, for chelation therapy, it is mandatory to know whether the chelator has any toxicity, prooxidant, or antioxidant effect that could supplement its chelating properties. It is hypothesized that the protective efficacy and toxicity may be different in different age groups of subjects. Hence, the present study was designed to evaluate the protective effect of MiADMSA against As-induced oxidative stress in young, adult and aged animals.

The co-administration of MiADMSA along with As proved significantly effective in restoring the altered activities of brain regional antioxidant enzymes towards its normal values in all the ages studied. However, co-administration of MiADMSA was found to produce greater protective changes in young animals compared to the adult and aged animals. This could be attributed due to its lipophilic nature, which allows to cross cell membranes more effectively and to chelate intracellular As from its storage sites by forming an adduct utilizing the sulfhydryl and carboxyl groups in its structure. It would be interesting to speculate that, apart from the ability of MiADMSA to remove As from the brain, presence of two vicinal thiol (sulfhydryl) groups in the structure may also be responsible for these reversals. MiADMSA, an ester derivative of DMSA, might be more lipophilic in nature than its parent compound but still retains antioxidant properties of the parent compound, DMSA [61]. However, we, in this current study, were not able to provide enough experimental evidence to prove this hypothesis and hence recommend future studies in the area.

These results are in agreement with Flora et al., (2007) who reported similar observations during aging upon administration with MiADMSA. Our results with MiADMSA are consistent with our previously published reports [10]. Based on the findings of the present study, it is suggested that MiADMSA has the best reversal effect among the current available choices of drugs for As poisoning.

Figures

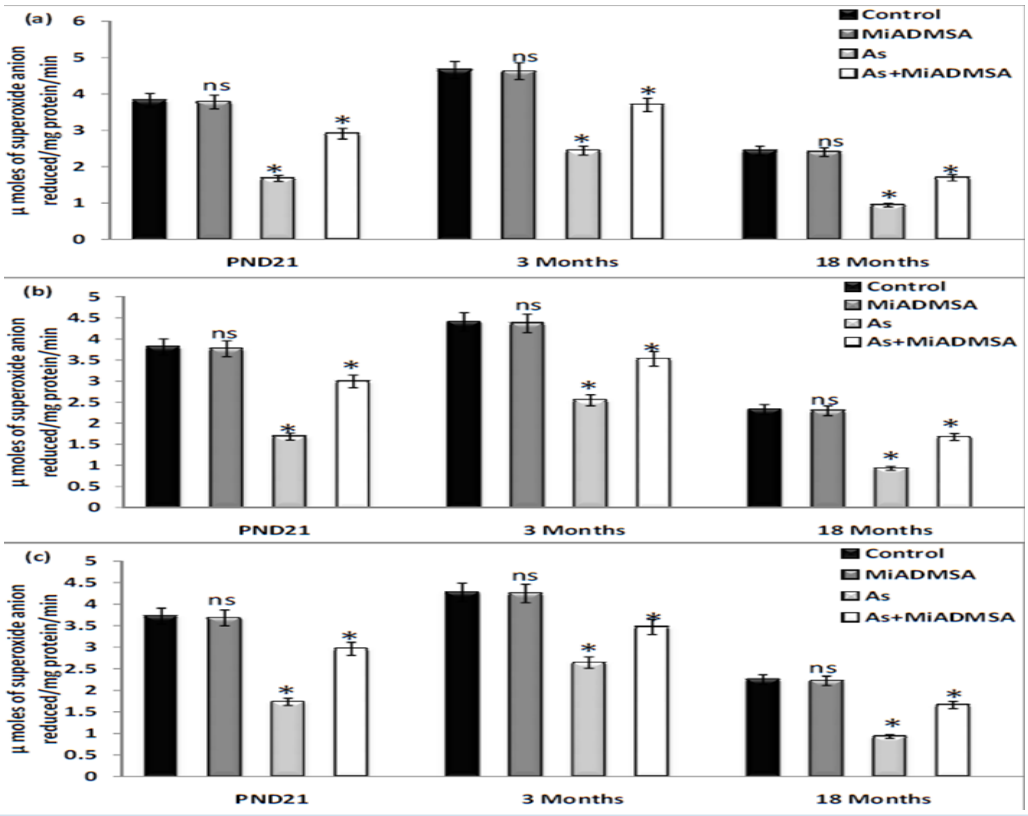

Figure 1: Influence of age on arsenic-induced alterations in Mn-SOD activity in three different brain regions [(a) cerebral cortex, (b) cerebellum and (c) hippocampus] and its reversal by MiADMSA. Rats were treated with normal saline, arsenic (10 mg/kg orally), chelated with MiADMSA (50 mg/kg orally) for a period of 1 week. Each bar represents mean ± SD (n = 6). The values marked with asterisk (*) are significant at $P < 0.001$ with their respective control groups, whereas the non-significant values are designated as ns ($P > 0.05$).

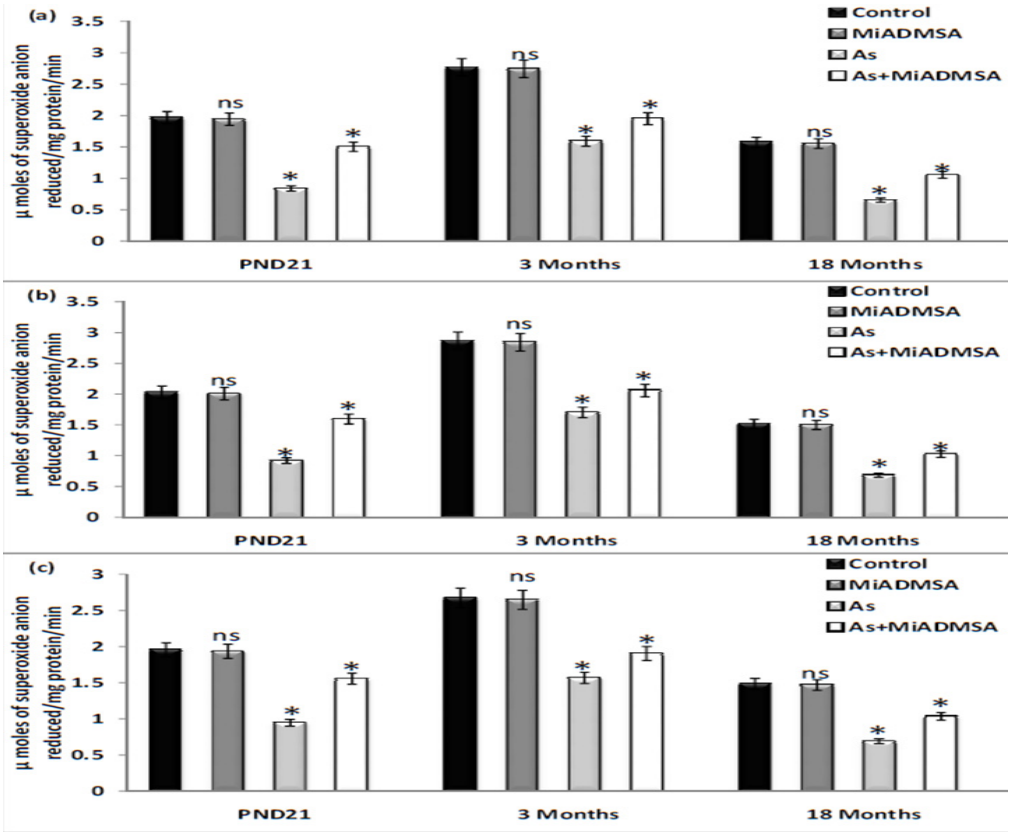

Figure 2: Influence of age on arsenic-induced alterations in Cu/Zn-SOD activity in three different brain regions [(a) cerebral cortex, (b) cerebellum and (c) hippocampus] and its reversal by MiADMSA. Rats were treated with normal saline, arsenic (10 mg/kg orally), chelated with MiADMSA (50 mg/kg orally) for a period of 1 week. Each bar represents mean ± SD (n = 6). The values marked with asterisk (*) are significant at $P < 0.001$ with their respective control groups, whereas the non-significant values are designated as ns ($P > 0.05$).

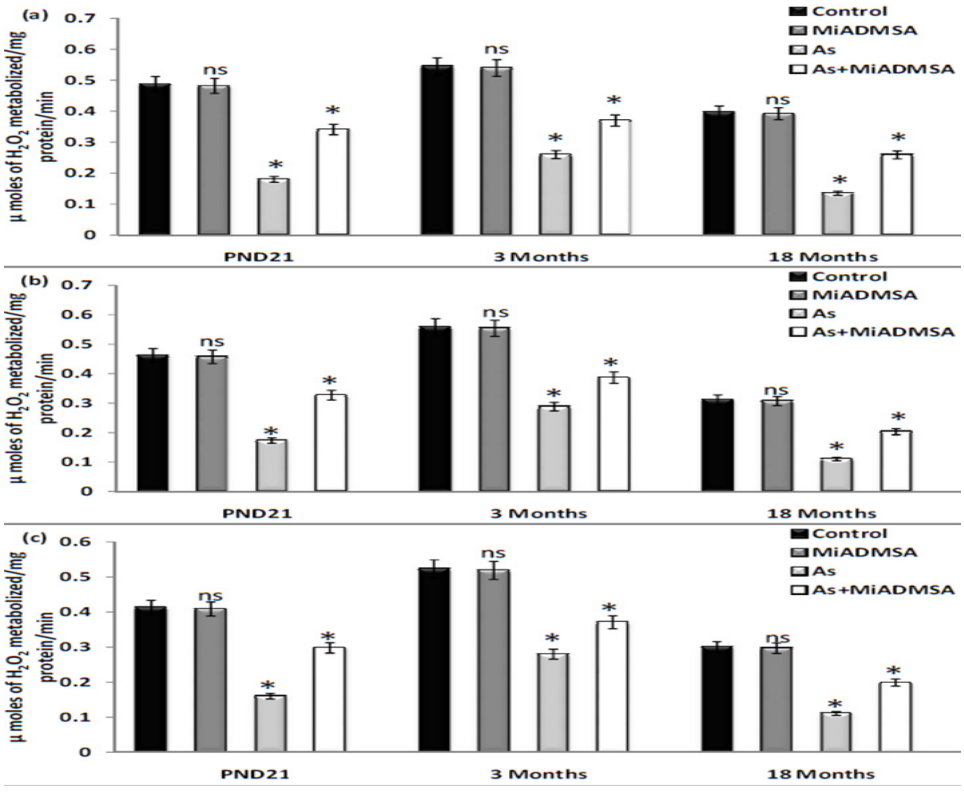

Figure 3: Influence of age on arsenic-induced alterations in CAT activity in three different brain regions [(a) cerebral cortex, (b) cerebellum and (c) hippocampus] and its reversal by MiADMSA. Rats were treated with normal saline, arsenic (10 mg/kg orally), chelated with MiADMSA (50 mg/kg orally) for a period of 1 week. Each bar represents mean ± SD (n = 6). The values marked with asterisk (*) are significant at $P < 0.001$ with their respective control groups, whereas the non-significant values are designated as ns ($P > 0.05$).

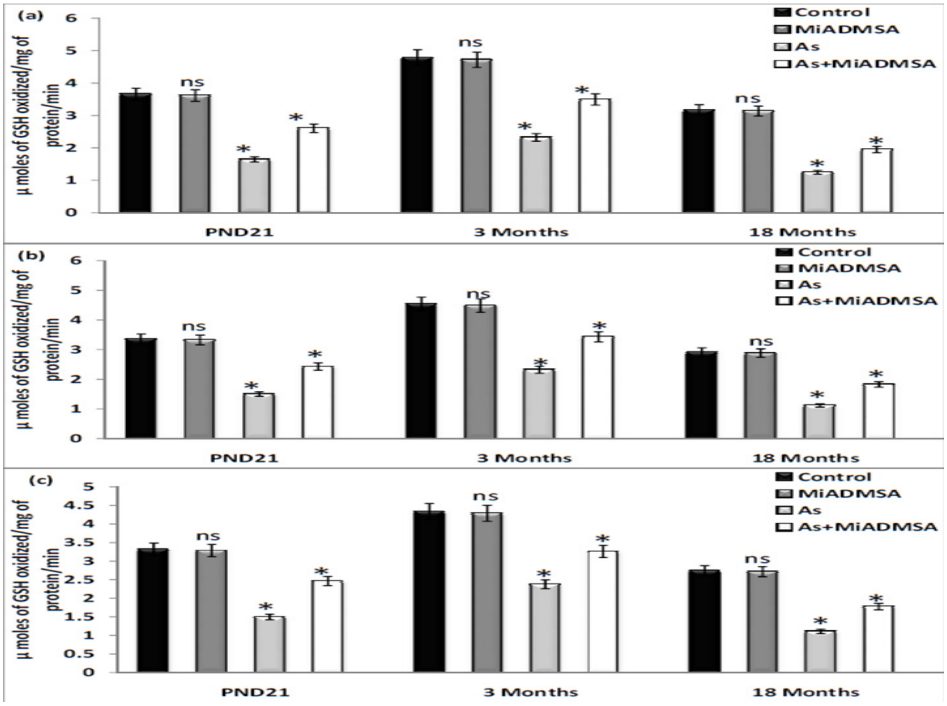

Figure 4: Influence of age on arsenic-induced alterations in GPx activity in three different brain regions [(a) cerebral cortex, (b) cerebellum and (c) hippocampus] and its reversal by MiADMSA. Rats were treated with normal saline, arsenic (10 mg/kg orally), chelated with MiADMSA (50 mg/kg orally) for a period of 1 week. Each bar represents mean ± SD (n = 6). The values marked with asterisk (*) are significant at P < 0.001 with their respective control groups, whereas the non-significant values are designated as ns (P > 0.05).

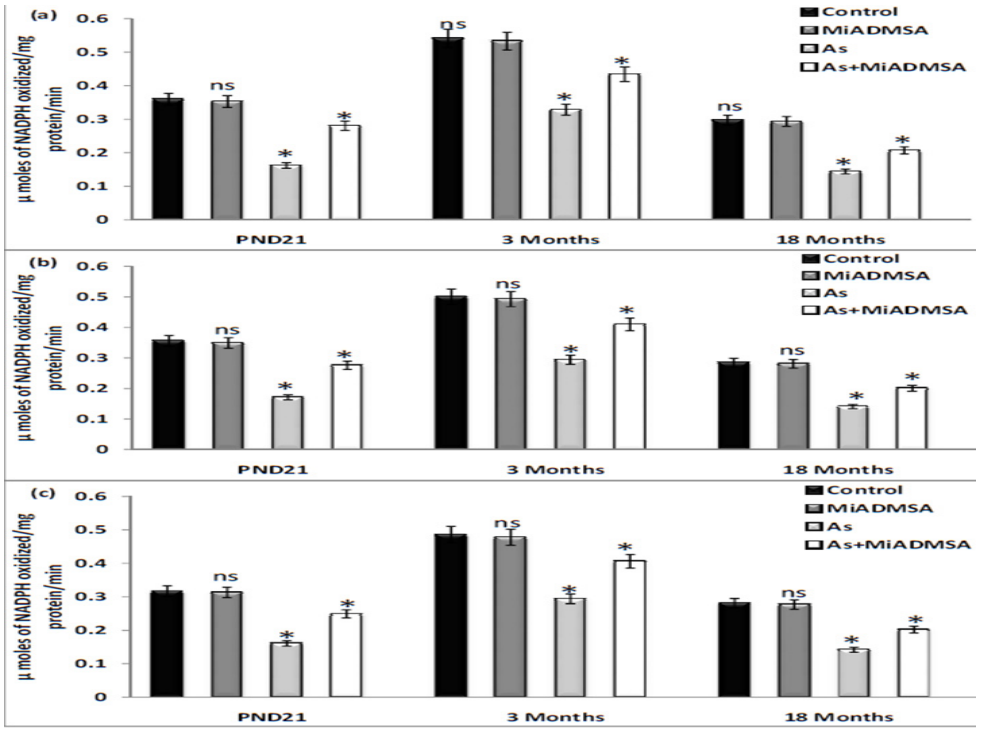

Figure 5: Influence of age on arsenic-induced alterations in GR activity in three different brain regions [(a) cerebral cortex, (b) cerebellum and (c) hippocampus] and its reversal by MiADMSA. Rats were treated with normal saline, arsenic (10 mg/kg orally), chelated with MiADMSA (50 mg/kg orally) for a period of 1 week. Each bar represents mean ± SD (n = 6). The values marked with asterisk (*) are significant at P < 0.001 with their respective control groups, whereas the non-significant values are designated as ns (P > 0.05).

Tables

Table 1: Influence of age on arsenic-induced alterations in GSH and TSG content in three different brain regions [CC (cerebral cortex), CBM (cerebellum) and Hippo (hippocampus)] and its reversal by MiADMSA. Rats were treated with normal saline, arsenic (10 mg/kg orally), chelated with MiADMSA (50 mg/kg orally) for a period of 1 week. Each value represents mean ± SD (*n* = 6). The values marked with asterisk (*) are significant at *P* < 0.001 with their respective control groups, whereas the non-significant values are designated as ns (*P* > 0.05).

	GSH Content				TSG Content			
	Control	MiADMSA	As	As+MiADMSA	Control	MiADMSA	As	As+MiADMSA
Young								
CC	0.879±0.0473	0.866±0.0481^{ns}	0.367±0.0196*	0.671±0.0460*	1.574±0.0860	1.543±0.0796^{ns}	0.701±0.0383*	1.118±0.0583*
CBM	0.856±0.0550	0.844±0.0501^{ns}	0.381±0.0265*	0.681±0.0396*	1.428±0.0828	1.411±0.0749^{ns}	0.667±0.0335*	1.032±0.0587*
Hippo	0.832±0.0430	0.820±0.0464^{ns}	0.382±0.0196*	0.672±0.0490*	1.231±0.0660	1.221±0.0682^{ns}	0.588±0.0322*	0.911±0.0613*
Adult								
CC	0.927±0.0677	0.914±0.0514^{ns}	0.532±0.0367*	0.701±0.0377*	1.986±0.1215	1.966±0.1384^{ns}	1.125±0.0693*	1.424±0.0865*
CBM	0.917±0.0503	0.904±0.0508^{ns}	0.536±0.0348*	0.704±0.0393*	1.857±0.1064	1.841±0.1494^{ns}	1.074±0.0783*	1.359±0.0825*
Hippo	0.915±0.0496	0.903±0.0571^{ns}	0.539±0.0280*	0.709±0.0522*	1.811±0.1354	1.792±0.1063^{ns}	1.069±0.0747*	1.355±0.0758*
Aged								
CC	0.734±0.0408	0.727±0.0385^{ns}	0.286±0.0168*	0.469±0.0256*	1.325±0.0748	1.307±0.0887^{ns}	0.509±0.0304*	0.776±0.0502*
CBM	0.722±0.0368	0.715±0.0383^{ns}	0.290±0.0146*	0.478±0.0255*	1.246±0.0946	1.231±0.0620^{ns}	0.507±0.0295*	0.769±0.0395*
Hippo	0.710±0.0409	0.703±0.0382^{ns}	0.302±0.0157*	0.480±0.0271*	1.183±0.0668	1.159±0.0617^{ns}	0.496±0.0300*	0.744±0.0386*

Table 2: Influence of age on arsenic-induced alterations in lipid peroxidation and tissue As levels in three different brain regions [CC (cerebral cortex), CBM (cerebellum) and Hippo (hippocampus)] and its reversal by MiADMSA. Rats were treated with normal saline, arsenic (10 mg/kg orally), chelated with MiADMSA (50 mg/kg orally) for a period of 1 week. Each value represents mean ± SD (n = 6). The values marked with asterisk (*) are significant at P < 0.001 with their respective control groups, whereas the non-significant values are designated as ns (P > 0.05).

	Lipid Peroxidation				Tissue As Levels			
	Control	MiADMSA	As	As+MiADMSA	Control	MiADMSA	As	As+MiADMSA
Young								
CC	0.970±0.0623	0.985±0.0605^{ns}	1.688±0.1174*	1.270±0.0739*	0.015±0.005	0.019±0.002^{ns}	0.248±0.0133*	0.143±0.0082*
CBM	0.810±0.0418	0.821±0.0486^{ns}	1.395±0.0716*	1.072±0.0781*	0.013±0.001	0.015±0.006^{ns}	0.208±0.0125*	0.119±0.0077*
Hippo	0.620±0.0334	0.628±0.0349^{ns}	1.067±0.0569*	0.810±0.0555*	0.014±0.003	0.016±0.004^{ns}	0.218±0.0113*	0.126±0.0079*
Adult								
CC	1.316±0.0667	1.334±0.0693^{ns}	1.989±0.1196*	1.652±0.0853*	0.017±0.0015	0.019±0.0014^{ns}	0.169±0.0125*	0.103±0.0065*
CBM	1.253±0.0636	1.268±0.0751^{ns}	1.881±0.1298*	1.541±0.0838*	0.015±0.004	0.016±0.003^{ns}	0.145±0.0078*	0.088±0.0072*
Hippo	1.17±0.0815	1.130±0.0635^{ns}	1.684±0.1162*	1.360±0.0732*	0.012±0.003	0.015±0.004^{ns}	0.113±0.0083*	0.069±0.0048*
Aged								
CC	2.575±0.1619	2.615±0.1732^{ns}	4.594±0.287*	3.653±0.2407*	0.015±0.002	0.018±0.0014^{ns}	0.189±0.0151*	0.116±0.0067*
CBM	2.309±0.1693	2.339±0.1643^{ns}	4.112±0.3141*	3.239±0.2271*	0.014±0.0013	0.017±0.0014^{ns}	0.170±0.0093*	0.103±0.0071*
Hippo	2.198±0.1679	2.227±0.1620^{ns}	3.888±0.314*	3.067±0.2303*	0.013±0.006	0.015±0.011^{ns}	0.153±0.0088*	0.098±0.0064*

References

1. Jain A, Flora SJS. Age dependent changes in arsenic and nicotine induced oxidative stress in male rat. Interventional Medicine and Applied Science. 2011; 3: 195-202.
2. Jain A, Flora GJS, Bhargava R, et al. Influence of age on arsenic-induced oxidative stress in rat. Biological Trace Element Research. 2012; 149: 382-390.
3. Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological profile for arsenic. US Department of Health and Human Services. Public Health Service. 2000; 428.
4. Wright RO, Amarasiriwardena C, Woolf AD, et al. Neuropsychological correlates of hair arsenic, manganese, and cadmium levels in school-age children residing near a hazardous waste site. Neurotoxicology. 2006; 27: 210-216.
5. Rosado JL, Ronquillo D, Kordas K, et al. Arsenic exposure and cognitive performance in Mexican school children. Environmental Health Perspectives. 2007; 155: 1371-1375.
6. vonEhrenstein OS, Poddar S, Yuan Y, et al. Children's intellectual function in relation to arsenic exposure. Epidemiology. 2007; 18: 44-51.
7. Chouhan S, Flora SJS. Arsenic and fluoride: two major ground water pollutants. Indian Journal of Experimental Biology. 2010; 48: 666-678.
8. Tsai SY, Chou HY, The HW, et al. The effects of chronic arsenic exposure from drinking water on the neurobehavioral development in adolescence. Neurotoxicology. 2003; 24: 747-753.
9. Wang G, Fowler BA. Roles of biomarkers in evaluating interactions among mixtures of lead, cadmium and arsenic. Toxicology and Applied Pharmacology. 2008; 233: 92-99.
10. Kumar MR, Flora SJS, Reddy GR. Monoisoamyl 2,3-dimercaptosuccinic acid attenuates arsenic induced toxicity: Behavioral and neurochemical approach. Environ. Toxicol. Pharmacol. 2013; 36: 231-242.
11. Pachauri V, Mehta A, Mishra D, et al. Arsenic induced neuronal apoptosis in guinea pigs is Ca^{2+} dependent and abrogated by chelation therapy: Role of voltage gated calcium channels. Neurotoxicology. 2013; 35: 137-145.
12. Basha DC, Basha SS, Reddy GR. Lead-induced cardiac and hematological alterations in aging Wistar male rats: alleviating effects of nutrient metal mixture. Biogerontology. 2012; 13: 359-368.
13. Ercal N, Gurer-Orhan H, Aykin-Burns N. Toxic metals and oxidative stress. Part 1. Mechanisms involved in metal-induced oxidative damage. Current Topics in Medicinal Chemistry. 2001; 1: 529-539.
14. Kadeyala PK, Sannadi S, Gottipolu GR. Alterations in apoptotic caspases and antioxidant enzymes in arsenic exposed rat rain regions: reversal effect of essential metals and a chelating agent. Environmental Toxicology and Pharmacology. 2013; 36: 1150-1166.
15. Aposhian HV and Aposhian MM Meso 2,3-dimercaptosuccinic acid: chemical, pharmacological and toxicological properties of an orally effective metal chelating agent. Annual Review of Pharmacology and Toxicology. 1990; 30: 279-306.
16. Flora SJS, Pachauri V. Chelation in metal intoxication. International Journal of Environmental Research and Public Health. 2010; 7: 2745-2788.
17. Flora SJS. Arsenic-induced oxidative stress and its reversibility. Free Radical Biology and Medicine. 2001; 51: 257-281.
18. Itoh T, Zhang YF, Murai S, et al. The effect of arsenic trioxide on brain monoamine metabolism and locomotor activity of mice. Toxicology Letters. 1990; 54: 345-353.
19. Brown, Kitchin KT. Arsenite, but not cadmium, induces ornitine decarboxylase and heme oxygenase activity in rat liver: relevance to arsenic carcinogenesis. Cancer Letters. 1996; 98: 227-231.
20. Flora SJS, Mehta A, Gautam P, et al. Essential metal status, prooxidant/antioxidant effects of MiADMSA in male rats: age-related effects. Biological Trace Element Research. 2007; 120: 235-247.
21. Flora SJS, Bhatt K, Mehta A. Arsenic moiety in gallium arsenide is responsible for neuronal apoptosis and behavioral alterations in rats. Toxicology and Applied Pharmacology. 2009; 240: 236-244.
22. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. Journal of Biological Chemistry. 1972; 247: 3170-3175.
23. Chance B, Maehly AC. Assay of catalase and peroxidases. Methods in Enzymology I. 1955; 11: 764-775.
24. Rotruck JT, Pope AL, Ganther HE. Selenium: biochemical role as a component of glutathione purification and assay. Science. 1973; 179: 588–590.
25. Staal GE, Visser J, Veeger C. Purification and properties of glutathione reductase of human erythrocytes. Biochimica et Biophysica Acta. 1969; 185: 39-48.
26. Habig C, Di Giulio RT, Abou-Donia MB. Comparative properties of channel cat fish (Ictalururspunctatus) and blue crab (Callinectssapidus) acetylcholinesterases. Comparative Biochemistry and Physiology. 1988; 91: 293-300.
27. Ellman GL. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics . 1959; 82: 70-77.
28. Sedlak J, Lindsay RH. Estimation of total, protein bound, and non-protein sulfhydryl groups in tissue by Ellman's reagent. Analytical Biochemistry. 1968; 25: 192-208.
29. Ohkawa H, Ohisi , Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 1979; 95: 351-358.
30. Ballentine R, Burford DD. Determination of metals. Methods in Enzymology. 1957; 3: 1002-1035.
31. Zheng W, Perry DF, Nelson DL, et al. Choroid plexus protects cerebrospinal fluid against toxic metals. FASEB Journal. 1991; 5: 2188-2193.
32. McCord JM, Fridovich I. Superoxide dismutase. An enzymatic function for erythrocuprein. Journal of Biological Chemistry. 1969; 244: 6049-6055.
33. Carrillo MC, Kanai S, Sato Y, et al. Age-related changes in antioxidant enzyme activities are region and organ, as well as sex, selective in the rat. Mechanisms of Ageing and Development. 1992; 65: 187-198.
34. Yamanaka K, Hasegawa A, Sawamura R, et al. Dimethylated arsenics induce DNA strand breaks in lung via the production of active oxygen in mice. Biochemical and Biophysical Research Communications. 1989; 165: 43-50.
35. Yamanka K, Hoshino M, Okamoto M, et al. Induction of DNA damage by dimethylarsine, a main metabolite of inorganic arsenics, is for the major part due to its peroxyl radical. Biochemical and Biophysical Research Communications. 1990; 168: 58-64.
36. deVizcaya-Ruiz A, Barbier O, Ruiz-Ramos R, et al. Biomarkers of oxidative stress and damage in human population exposed to

arsenic. Mutation Research. 2009; 674: 85-92.

37. Arivazhagan P, Shila S, Kumaran S, et al. Effect of DL-α- Lipoic acid on the status of lipid peroxidation and antioxidant enzymes in various brain regions of aged rats. Experimental Gerontology. 2002; 37: 803-811.

38. Sawada M, Carlson JC. Changes in superoxide radical and lipid peroxide formation in the brain, heart and liver during the life time of the rat. Mechanisms of Ageing and Development. 1987; 41: 125-137.

39. Kirkman MN, Gactani GF. Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH. Proceedings of the National Academy of Sciences. 1984; 81: 4343-4647.

40. Kono Y, Fridovich I. Superoxide radicals inhibit catalase. Journal of Biological Chemistry. 1982; 257: 5751-5754.

41. Sohal RS, Mockett RJ and Orr WC. Current issues concerning the role of oxidative stress in aging: a perspective. Results and Problems in Cell Differentiation. 2000; 29: 45-66.

42. Flora SJS, Kannan GM, Kumar P. Selenium effects on gallium arsenide induced biochemical and immunological changes in male rats. Chemico-Biological Interactions. 1999; 122: 1-13.

43. Zeng H, Uthus EO and Combs GF Mechanistic aspects of the interaction between selenium and arsenic. Journal of Inorganic Biochemistry. 2005; 99: 1269-1274.

44. Ganyc D, Talbot S, Konate F, et al. Impact of trivalent arsenicals on selenoprotein synthesis. Environmental Health Perspectives. 2007; 115: 346-353.

45. Meno SR, Nelson R, Hintze KJ, et al. Exposure to monomethylarsonous acid (MMA(III)) leads to altered selenoprotein synthesis in a primary human lung cell model. Toxicology and Applied Pharmacology. 2009; 239: 130-136.

46. Brannan TS, Maker HS, Weiss C, et al. Regional distribution of glutathione Peroxidase in the adult rat brain. Journal of Neurochemistry. 1990; 35: 1013-1014.

47. Styblo M, Thomas DJ. In vitro inhibition of glutathione reductase by arsenotriglutathione. Biochemical Pharmacology. 1995; 49: 971-974.

48. Styblo M, Serves SV, Cullen WR, et al. Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. Chemical Research in Toxicology. 1997; 10: 27-33.

49. Chouchane S, Snow ET. In vitro effect of arsenical compounds on glutathione-related enzymes. Chemical Research in Toxicology. 2001; 14: 517-522.

50. Hughes MF. Arsenic toxicity and potential mechanism of action. Toxicol. Lett. 2002; 133: 1-16.

51. Ravindranath V, Shivakumar BR, Anandatheerthavarada HK, Low glutathione levels in brain regions of aged rats. Neuroscience Letters. 1989; 101: 187-190.

52. Vahter M. Mechanism of arsenic biotransformation. Toxicology. 2002; 181/182: 211–217.

53. Liu J, Atamna H, Kuratsune H, et al. Delaying brain mitochondrial decay and aging with mitochondrial antioxidants and metabolites. Annals of the New York Academy of Sciences. 2002; 959: 133-166.

54. Kim HG, Hong SM, Kim SJ, et al. Age related changes in the activity of antioxidant and redox enzymes in rats. Molecules and Cells. 2003; 16: 278-284.

55. Maiti S, Chatterjee AK. Effects on levels of glutathione and some related enzymes in tissues after an acute arsenic exposure in rats and their relationship to dietary protein deficiency. Archives of Toxicology. 2001; 75: 531-537.

56. Dotan Y, Lichtenberg D, Pinchuk I. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Progress in Lipid Research. 2004; 43: 200-227.

57. Lindgren A, Danielsson BRG, Dencker L, et al. Embryotoxicity of arsenite and arsenate: distribution in pregnant mice and monkeys and effects on embryonic cell in vitro. Acta Pharmacologica et Toxicologica. 1984; 54: 311-320.

58. Willhite CC, Ferm VH. Prenatal and developmental toxicology of arsenicals. Advances in Experimental Medicine and Biology. 1984; 177: 205-228.

59. Hill JM, Switzer RC. The regional distribution and cellular localization of iron in the rat brain. Neuroscience. 1984; 11: 595-603.

60. Kumar MR, Reddy GR. Protective effects of zinc and vitamin-E for arsenic induced mitochondrial oxidative damage in rat brain. Current Trends in Biotechnology and Pharmacy. 2017; 11: 67-83.

61. Ercal N, Treratphan P, Hammond TC, et al. In vivo indices of oxidative stress in lead exposed C57BL/6 mice are reduced by treatment with meso-2,3-dimercaptosuccinic acid or N-acetyl cysteine. Free Radical Biology and Medicine. 1996; 21: 157-161.