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Comparative oxidative stress, metallothionein induction and organ toxicity following chronic exposure to arsenic, lead and mercury in rats

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Abstract

Globally, arsenic, mercury and lead constitutes as the three most hazardous environmental toxicants perturbing imbalance in pro-oxidant and antioxidant homeostasis. Individual toxicity of these environmental toxicants is well known but there is lack of comparative data on variables indicative of oxidative stress. We thus investigated the effects of chronic exposure to sodium arsenite, mercuric chloride and lead acetate on blood and tissue oxidative stress, metal concentration and metallothionein (MT) contents. Male rats were exposed to sodium arsenite, mercuric chloride and lead acetate (0.05 mg/kg each, orally, once daily) for 6 months. Arsenic, mercury and lead exposure led to a significant inhibition of blood δ-aminolevulinic acid dehydratase (ALAD) activity and glutathione level supported by increased thiobarbituric acid reactive substance (TBARS). The level of inhibition was more pronounced in case of lead followed by mercury and arsenic. These metals/ metalloid significantly increased reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS) and glutathione peroxidase (GPx) activity accompanied by a decreased superoxide dismutase (SOD), catalase and reduced and oxidized glutathione (GSH and GSSG) levels in blood and tissues. Mercury alone produced a significant induction of hepatic and renal MT concentrations. Serum transaminases, lactate dehydrogenase and alkaline phosphatase activities increased significantly on exposure to arsenic and mercury exposure suggesting liver injury which was less pronounced in case of lead exposure. These biochemical alterations were supported by increased arsenic, mercury and lead concentrations in blood and soft tissues. The present study suggests that exposure to sodium arsenite and mercuric chloride lead to more pronounced oxidative stress and hepatotoxicity while lead acetate caused significant alterations in haem synthesis pathway compared to two other thiol binding metal/metalloid.

Key words: Sodium arsenite, mercuric chloride, lead acetate, oxidative stress, metallothionein, heme synthesis pathway, organ damage.

Introduction

Industrial applications of metals have increased particularly in the industrialized developing countries and might lead to a situation in which chronic intoxication is common. Metals as environmental and occupational toxicants are gaining concern for their toxic manifestations in humans. Metalloid and metals like arsenic, lead and mercury form one of the most hazardous environmental toxicants posing deleterious health risk in humans due to continuous exposure. Human and animals are at highest risk of exposure to different forms of arsenic through arsenic contaminated water, food and other environmental forms. Agency of toxic substances and disease registry (ATSDR) has placed these toxicants on the list of most perilous substance in the environment (1). Pathology related to chronic arsenic exposure especially carcinogenicity is not well known but implicates with various diseases like skin diseases (hyperkeratosis, hyper-pigmentation, skin cancer), hypertension, gastrointestinal disturbances, diabetes mellitus, neurological disorders, disturbed porphyrin metabolism (2-6). Interest in arsenic poisoning has materialized dramatically in recent years where millions of population is suffering from arsenicosis. Number of countries including Argentina, Taiwan, Mexico, Chile, US, Srilanka, New Zealand but particularly West Bengal region in India and Bangladesh is drinking arsenic infected water from well, which represents a worst impact on human health (7). World Health Organization (WHO) guidelines have reported 10 ppb, the maximum permissible limit of arsenic content in drinking water (8, 9). Numerous reports suggest that arsenic has tendency to bind with sulfhydryl groups and inhibits its associated enzymes. These inhibited enzymes generate reactive oxygen species (ROS) and depletes antioxidant status thereby causes oxidative stress condition (3, 10-12).

Another heavy metal which equally contributes in causing deleterious health effects is lead. Lead remains present in the atmosphere (water, soil or industrial products) because of its persistent nature. Worldwide reports suggest that children are more susceptible to lead as gastrointestinal absorption is higher and blood lead levels lower than 10 µg/dl may also cause serious manifestations like slowed cognitive development and neuropsychological disorders (13-15). Oxidative stress contributes as one of the major mechanism responsible for lead toxicity where in response free radicals and reactive oxygen species are produced. These reactive species interfere with cellular macromolecules and deactivates cellular antioxidant pool (16-17). Lead is known to inhibit heme-biosynthesis pathway by inactivating delta-aminolevulinic acid dehydratase (ALAD) enzyme and also alters calcium dependent pathways as lead mimics calcium with higher affinity (13, 18, 19).

Mercury has been associated with several disorders

like renal tubular necrosis, asthma, dermatitis amytrophic lateral sclerosis, Alzheimer's and Parkinson's disease (20, 21). Globally, mercury has emerged as an important concern for its toxic manifestations to humans. Mercury exposure is common in many developing countries like China, Peru and India. Millions of workers are exposed to mercury by working in gold mining (22, 23). In Wanshan China, humans are exposed to the deleterious effects by consuming mercury contaminated rice and this fact was based on the report of the oxidative stress condition induced by mercury- contaminated rice in rats (24). Similar to arsenic and lead, mercury also exerts its toxicity by binding to sulfhydryl groups eventually leading to perturbed enzymatic pathways and metabolism. Free radicals are generated in response to increased production of Reactive Oxygen Species (ROS) and/or a depleted antioxidant system thereby (25). The above discussed three toxic heavy-metals share a common major mechanism of toxicity i.e. through generation of oxidative stress. In the present study, we made an attempt to compare the effects of these metals using same dose, route and duration of exposure and same parameters too were evaluated in rats.

Although the individual effects of arsenic, lead and mercury have been well documented, report on their comparative effects are under current research interest and have not been studied so far in detailed. Interest in multi-metal exposure has been dramatically increased in recent years. The present study thus was planned to investigate the comparative chronic effects of sodium arsenite, mercuric chloride and lead acetate on biochemical variables suggestive of alterations in blood, hepatic and renal oxidative stress in rats. Another highlight of this proposed study was to evaluate the changes in metallothionein content in tissues.

Materials and methods

Chemicals

Sodium m- arsenite ($NaAsO_2$), lead acetate and mercuric chloride were procured from Sigma Chemical (USA) and BDH Chemicals (Mumbai, India). All other analytical laboratory chemicals and reagents were purchased from Merck (Germany), Sigma (USA) or BDH chemicals (Mumbai, India). Ultra pure water prepared by Millipore (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.

Animals and Treatment

Male Wistar rats weighing 110-120 g (between 2-3 months old), were obtained from Defence Research and Development Establishment (DRDE) animal facility and prior to use, were acclimatized for 7 days 12h light/dark cycle. The Animal Ethical Committee of DRDE, Gwalior, India, approved the protocols for the experiments. The animals were housed in stainless steel cages in an air-conditioned room with temperature maintained at 25±2°C. Rats were allowed standard chow diet (Ashirwad Feeds, Chandigarh, India; metal content of diet, in ppm dry weight: Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, Fe 75.0) throughout the experiment and water *ad libitum*. Twenty animals were randomized into four groups of five rats each and were treated as below for 6 months-

Group I: Normal (received normal water)
Group II: Arsenic as sodium arsenite (0.05 mg/kg, orally through gastric intubation)
Group III: Mercury as mercuric chloride (0.05 mg/kg, orally)
Group IV: Lead as lead acetate (0.05 mg/kg, orally)

After 6 months, animals were sacrificed by decapitation at the end of the exposure according to the experimental design. Blood was drawn by retro-orbital plexus and collected in heparinized and non-heparinized treated vials for various biochemical and serum parameters. Liver and kidneys were removed, rinsed in cold saline, blotted, weighed and used for various biochemical variables and metal analysis. Half portion of the liver and kidney from each rat was processed immediately for biochemical estimation and the remaining was stored at –20°C before wet acid digestion with HNO_3 for estimation of arsenic, lead and mercury contents.

Biochemical assays

Separation of red blood cells

To isolate RBCs, whole blood was centrifuged at 1500 x g for 10 min at 4°C. After centrifugation, plasma and buffy coat were removed by aspiration. The obtained RBCs were washed three times in phosphate buffer saline (0.1 M) by centrifuging at 3000 rpm for 10 minutes. The packed cell volume (PCV) obtained after centrifugation was divided into two parts. One part of PCV was diluted with chilled distilled water (100 µl of PCV +1.9 ml of Distilled water) and kept for the analysis of reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH). The other part of PCV was used for the estimation of glutathione peroxidase (GPx). For this, hemoglobin was precipitated in PCV by means of chloroform and ethanol. After centrifugation at 3000 x g for 10 min at 4°C, the supernatant obtained was used for all above mentioned enzymatic assays.

Clinical hematological variables

Level of mean cell volume (MCV), hematocrit (HCT), hemoglobin (HGB), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelets (PLT), red blood cell (RBCs) count and white blood cell (WBCs) count were measured using a Sysmex hematology analyzer (model K4500).

Clinical serum parameters

Lactate dehydrogenase (LDH), aspartate transaminases (AST), alanine transaminases (ALT), alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were measured in serum using Merck (Darmstadt, Germany) kits.

Blood δ-ALA Dehydratase (ALAD)

The activity of blood ALA dehydratase (ALAD) was assayed according to the procedure of Berlin and Schaller (26). Heparinized blood (0.2 mL) was mixed with 1.3 mL of distilled water and incubated for 10 minutes at 37°C for complete hemolysis. After adding 1 mL of standard ALA, the tubes were incubated for 60 minutes

at 37°C. The reaction was stopped after 1 hour by adding 1 mL of 10 % trichloroacetic acid (TCA). After centrifugation (1500 g) of the reaction mixture, an equal volume of Ehrlich reagent was added to the supernatant and the absorbance was recorded at 555 nm after 5 minutes.

Blood glutathione (GSH)

Determination of blood GSH concentration was done by the method of Ellman (27) modified by Jollow et al. (28). 1.8 mL of distilled water was added to 0.2 mL of whole blood and incubated for 10 min at 37ºC for complete hemolysis. After adding 3 mL of sulphosalicylic acid (4%), tubes were centrifuged at 1200g for 15 min .To the supernatant, 200µL of DTNB (10 mM) was added in presence of phosphate buffer (0.1 M pH 7.4). Absorbance recorded at 412 nm was used for calculation of GSH concentration.

Reactive oxygen species (ROS) level in blood and tissues

Amount of ROS in blood and tissue was measured using 2, 7-dichlorofluorescin diacetate (DCFDA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described in the literature by Socci et al. (29). Briefly, 5% RBC hemolysate and 1% tissue homogenate were prepared in ice-cold 40 mM Tris–HCl buffer (pH 7.4), and this was further diluted to 0.25% with the same buffer (40 mM Tris–HCl, pH 7.4) and placed on ice. The samples were divided into two equal fractions (2 mL each). In one fraction, 40 ml of 1.25 mM DCFDA in methanol was added for ROS estimation. Another fraction in which 40 ml of methanol was added served as a control for tissue/hemolysate auto fluorescence. All samples were incubated for 15 min in a water bath at 37ºC. Fluorescence was determined at 488 nm excitation and 525 nm emission wavelength, using a fluorescence plate reader (Perkin-Elmer, LS-55, United Kingdom).

Thiobarbituric reactive substances (TBARS) in blood and tissues

Measurement of blood and tissue lipid peroxidation was carried out following the method described by Ohkawa et al. (30). 0.1 ml of 5% RBC hemolysate was added to 0.2 ml of 8.1% SDS (w/v) and incubated for 10 min. Then 1.5ml of 20% acetic acid (pH 3.5) was added followed by adding 1.5 ml of 0.8% thiobarbituric acid (w/v) and 0.7 ml distilled water and incubation for 1 h in boiling water bath. One milliliter of distilled water was added to the solution after cooling and centrifuged at 6000 rpm for 15 min. The malondialdehyde formation was determined by reading absorbance at 535 nm.

Tissue lipid peroxidation was measured in liver and kidney homogenate [10% tissue homogenate (w/v) in 150 mM KCl] for 30 min at 37°C. The incubation was interrupted by adding 1 ml of 10% trichloroacetic acid. After centrifugation (1 ml) supernatant was then mixed with 1 ml of 0.65% thiobarbituric acid. The mixture was then kept in a boiling water bath for 15 min. The amount of TBARS was calculated using a molar extinction coefficient of 1.56 x 105/M/cm. The absorbance of supernatant was read at 535 nm and the values were expressed as nmoles of MDA/ml blood or µg/gm tissue weight in tissue samples.

Glutathione peroxidase (GPx)

Glutathione peroxidase in tissue was determined by the method of Flohe and Gunzler (31) at 37ºC. Supernatant was obtained after centrifuging 5% tissue homogenate at 1500xg for 10 min followed by 11,000 rpm for 30 min at 4ºC was used for the GPx assay. One milliliter 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 H2O2 (1 mM), and 0.3 ml of tissue homogenate. After incubation at 37ºC for 15 min, reaction was terminated by addition of 0.5 ml of 5% TCA. Tubes were centrifuged at 1500xg for 5 min and supernatant was collected. Of about 0.2 ml phosphate buffer (0.1 M pH 7.4) and 0.7 ml of DTNB (0.4 mg/ml) was added to 0.1 ml of reaction supernatant. After mixing, absorbance was recorded at 420 nm. A molar extinction coefficient of 6.22 x 103 M cm-1 was used to determine the activity.

Tissue reduced (GSH) and oxidized glutathione (GSSG) levels

Liver and kidney were assayed for GSH and GSSG contents according to the method described by Hissin and Hilf. (32). Briefly, 0.25 g of tissue sample was homogenized on ice with 3.75 ml of 0.1M phosphate +0.005M EDTA buffer (pH 8.0) and 1ml of 25% HPO3 which was used as a protein precipitant. The homogenate (4.7 ml) was centrifuged at **100,000×g for** 30 min at 4°C. For the GSH assay, 0.5 ml supernatant and 4.5 ml phosphate buffer (pH 8.0) were mixed. The final assay mixture (2.0 ml) contained 100µl supernatant, 1.8ml phosphate- EDTA buffer and 100µl O-phthaldehyde (OPT; 1000µl/ml in absolute methanol, prepared fresh in dark). After mixing, fluorescence was determined at 350nm excitation wavelength and 420nm emission wavelength using a spectrofluorometer (Model RF 5000 Shimadzu, Japan). For the GSSG assay, 0.5 ml supernatant was incubated at room temperature with 200µl of 0.04mol/l N-ethylmaleimide solution for 30min. To this mixture, 4.3 ml of 0.1mol/l NaOH was added. A 100-µl sample of this mixture was taken for the measurement of GSSG using the procedure described above for GSH assay, except that 0.1mol/l NaOH was used as the diluent instead of phosphate buffer.

Superoxide Dismutase (SOD)

Blood and tissues SOD activity was assayed by the method of Kakkar et al. (33) SOD was extracted from purified RBCs by ethanol chloroform extraction. A 5% tissue homogenate (w/v) was prepared in PBS and used for the determination of SOD and catalase activities. Reaction mixture was prepared which contained 1.2 ml of sodium pyrophosphate, 0.3 ml of PMS, 0.3 ml of NBT, 0.2 ml of hemolysate and tissue homogenate, 0.8 ml of distilled water and 0.2 ml of NADH. The control reaction mixture was prepared and contained 1.2 ml of sodium pyrophosphate, 0.3 ml of PMS, 0.3 ml of NBT, 1ml of distilled water and 0.2 ml of NADH. Both mixtures were incubated at 37ºC for 90 seconds and then 1 ml of Acetic acid was added and the mixture was allowed to stand for 10 min. The absorbance was recorded

at 560nm.

Catalase

Catalase activity in purified erythrocytes and tissues were assayed following the procedure of Sinha et al. (34). Reaction mixture containing 1 ml of phosphate buffer, 0.1ml of hemolysate and tissue homogenate, 0.4 ml of distilled water and 0.2 ml of H2O2 was prepared. The control mixture was prepared; containing 1 ml of phosphate buffer, 0.5ml of distilled water and 0.2 ml of H_2O_2. Both the mixtures were incubated at 37oC for 15 min and reaction was stopped by the addition of 2 ml of acetic acid with dichromate (1:3 ratio of 5% potassium dichromate in distilled water and glacial acetic acid respectively). Above mixture was boiled for 15 min and the mixture was cooled and absorbance was recorded at 570 nm

Metallothionein estimation in tissue

Total metallothionein (MT) concentration in liver and kidneys were measured following the method of Onosaka and Cherian (35). Briefly, 1 g of liver or 0.5 g of kidney was homogenized in 4 volume of 0.25M sucrose and was centrifuged at 18,000 g for 20 minutes at 4°C cytosolic fraction was used for MT. For MT estimation the final volume was adjusted to 3.4 ml with 30 mM tris.HCI buffer, pH 8.0, 1.0 ml cadmium chloride solution (10 ppm Cd) and incubated for 5 minutes at room temperature. The excess of Cd was removed and precipitated by addition of rat RBC hemosylate and heat treatment in a water bath for 1 minute. The denatured Cd-bound haemoglobin was removed by centrifugation at 1000 g for 5 minutes at room temperature. This step was repeated three times. The amount of Cd in the obtained supernatant fraction is a measure of MT-bound Cd and was determined using an atomic absorption spectrophotometer (AAS Perkin Elmer model AAnalyst 100, USA). The concentration of MT in each tissue was calculated assuming that 6 g atom of Cd is bound to each mole of thionein which has a molecular weight of 6050 by amino acid analysis.36

Metal estimation

Arsenic, lead and mercury concentration in blood, liver and kidney were measured after wet acid digestion using a Microwave Digestion System (CEM, USA, model MDS-2100). Arsenic was estimated using a Hydride Vapor Generation System (Perkin Elmer model MHS-10) fitted with an atomic absorption spectrophotometer (AAS, Perkin Elmer model Analyst 100).

Statistical analysis

Data was presented as mean+ SE. Data was also analyzed for statistical comparison using ANOVA followed by student's t-test. $^{a}P<0.001$; $^{b}P<0.01$; $^{cP}<0.05$ compared to normal control as evaluated by Student's t test.

Results

Body weight gain and clinical hematological variables

Body weight decreased marginally in animals exposed to mercuric chloride (non significant) while it decreased significantly animals exposed to lead acetate and sodium arsenite. The weight loss was most prominent in animals exposed to lead followed by arsenic (Table 1). Except for a significant increase in WBC count in mercury exposed and a decrease in HGB level in lead exposed animals, most of the other hematological variables remained unchanged on exposure to lead, arsenic and mercury (Table 2).

Table 1. Comparative effects of arsenic, mercury and lead on body weight gain in rats.

Groups	Body weight (g)
Control	185.17 ± 5.96
Arsenic	165.83 ± 11.67^{c}
Mercury	171.00 ± 15.03
Lead	161.50 ± 9.62^{a}

Body weight in grams. Values are mean±SE; N=5. $^{a}P<0.001$; $^{c}P<0.05$ compared to normal control as evaluated by Student's t test.

Table 2. Comparative effects of arsenic, mercury and lead on some clinical biochemical variables in rats.

Groups	Control	Arsenic	Mercury	Lead
		BLOOD		
RBC	8.28±0.19	8.33±0.26	7.68±0.66	7.66±0.49
WBC	15.86±1.68	20.24±2.4	38.63±5.7^{a}	20.6±1.52
HGB	14.01±0.65	15.06±0.28	14.57±0.57	11.4±.0.27^{a}
MCHC	30.71±0.72	32.36±0.10	30.53±0.70	31.66±0.62
HCT	45.6±1.55	46.52±0.95	39.96±3.99	43.64±3.51
MCV	55±1.29	55.8±0.55	55.43±0.90	56.28±1.20
MCH	16.9±0.57	18.08±0.25	16.93±0.51	17.85±0.7
PLT	887.3±100.5	927.5±43.2	693.8±69.5	813.2±55.8

RBC-Red blood cells as $\times10^{6}/\mu l$; WBC-White blood cells as $\times10^{3}/\mu l$; HGB-Hemoglobin as g/dL; HCT-Hematocrit as %; MCV-Mean cell volume as fL; MCH-Mean cell haemoglobin as pg; MCHC-Mean cell haemoglobin concentration as g/dl; PLT-Platelet as $\times10^{6}/\mu l$. Values are mean±SE; N=5. $^{a}P<0.001$; compared to normal control as evaluated by Student's t test.

Table 3. Comparative effects of arsenic, mercury and lead on delta aminolevulinic acid dehydratase and biochemical variables of oxidative stress in blood of rats.

Groups	ROS	GSH	TBARS	ALAD	SOD	Catalase
Control	16.5±0.76	0.53±0.002	434.7±36.5	6.88±0.31	0.88±0.009	1.22±0.15
Arsenic	26.3±1.02[a]	0.39±0.01[a]	540.1±11.3[a]	4.07±0.37[a]	0.56±0.006[a]	1.02±0.13
Mercury	33.1±4.05[a]	0.44±0.011[a]	480.9±14.2	3.24±0.40[a]	0.80±0.008	1.99±0.21[a]
Lead	19.5±0.96[b]	0.31±0.008[a]	530.9±7.70[a]	0.76±.29[a]	0.57±0.005[a]	1.48±0.15[b]

ROS, reactive oxygen species as FIU; GSH, reduced glutathione as mg/ml; TBARS, thiobarbituric reactive substances as mg/ml; ALAD, δ-aminolevulinic acid dehydratase as nmol $min^{-1}ml^{-1}$ erythrocytes; SOD, superoxide dismutase as U/mg protein/min; Catalase as µmol H_2O_2 degraded /min/ mg protein. Values are mean±SE; N=5. [a]P<0.001; [b]P<0.05 compared to normal control as evaluated by Student's t test.

Table 4. Comparative effects of arsenic, mercury and lead on serum parameters in rats.

Groups	AST	ALT	ACP	ALP	LDH
Control	35.5±2.50	32.6±2.70	13.0±0.17	353.3±52.3	482.0±35.4
Arsenic	65.7± 3.30[c]	45.5±2.70[c]	13.7±0.77	474.5±28.0[a]	601.6±40.1[a]
Mercury	55.4±2.10[c]	37.5±3.30	11.25±0.4	494.9±14.40[a]	582.8±22.1[b]
Lead	38.6±4.30	38.1±3.50	9.85±0.29[a]	659.3±32.60[a]	505.1±31.9

AST-Aspartate aminotransferases as U/L; ALT-Alanine aminotransferases as U/L; ACP, acid phosphatase as U/L; ALP, alkaline phosphatase as U/L; LDH-Lactate dehydrogenase as U/L. Values are mean±SE; N=5. [a]P<0.001; [b]P<0.01; [cP]<0.05 compared to normal control as evaluated by Student's t test.

Blood oxidative stress variables

Table 3 shows the toxic effects of sodium arsenite, mercuric chloride and lead acetate on delta aminolevulinic acid dehydratase and biochemical variables indicative of oxidative stress in blood of rats. All the three metals produced a significant increase in ROS and TBARS levels accompanied by a depletion of reduced GSH level and SOD activity suggesting oxidative stress. While, the induction of ROS was more prominent in mercury and arsenic, GSH depletion was more noticeable in lead acetate treated animals. SOD activity significantly more inhibited in arsenic and lead treated animals. Catalase activity showed more pronounced increase in mercuric chloride treated animals followed by lead acetate. On the other hand lead acetate was more effective in reducing ALAD activity followed by mercuric chloride and sodium arsenite.

Serum biochemical parameters

Serum AST activity increased significantly on exposure to sodium arsenite and mercuric chloride suggesting liver injury. No significant change was observed in ALT activity was noted on exposure to lead and mercury while it showed an increase on arsenic exposure. On the other hand, exposure to arsenic, mercury and lead resulted in a pronounced increase in ALP and LDH activity compared to control animals. ACP activity on the other hand, decreased significantly in lead exposed group as compared to other exposed group (Table 4).

Tissue metallothionein (MT)

Among the three metals only mercuric chloride exposure led to a significant increase in liver and kidney MT concentration (Fig. 1). No effect of sodium arsenite or lead acetate at the dose used in this study was able to affect liver or kidney MT concentration.

Tissue oxidative stress variables

Significant increase in hepatic ROS and TBARS levels was observed on sodium arsenite, mercuric chloride and lead acetate exposed animals (Table 5). No change in hepatic GSH, GSSG levels and GPx and catalase activities was noted while hepatic SOD activity decreased on arsenic and lead exposed animals (Table 5).

Kidney ROS and TBARS levels increased in mercu-

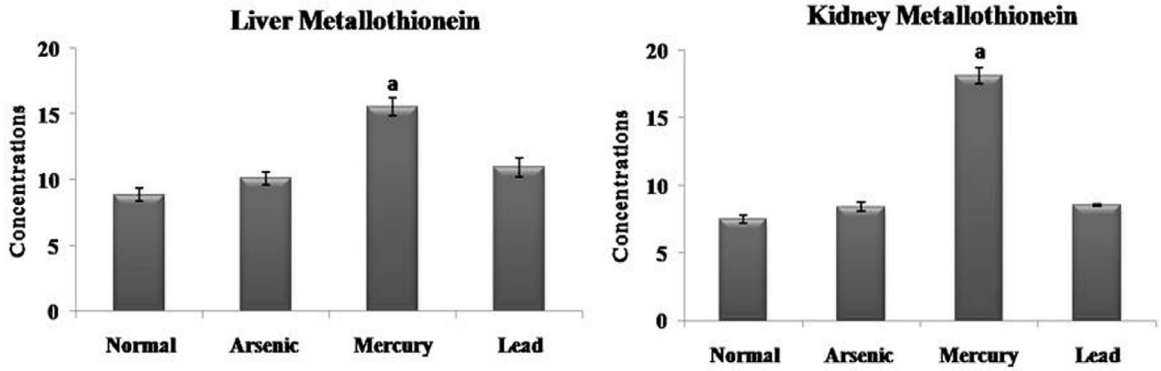

Figure 1. Comparative effects of arsenic, mercury and lead on tissue metallothionein concentration in liver and kidney of rats**.** MT, metallothionein as µg/gm. Values are mean ±SE; N=5. [a]P<0.001; compared to normal control as evaluated by Student's t test.

Table 5. Comparative effects of arsenic, mercury and lead on tissue biochemical variables of oxidative stress in liver and kidney of rats.

Groups	Control	Arsenic	Mercury	Lead
		LIVER		
ROS	340.8±4.87	442.3±10.21[a]	469.8±9.41	**442.4±16.36**
TBARS	3.38±0.12	4.95±0.58[a]	3.98±0.33	**4.21±0.18[b]**
GSH	2.28±0.02	2.31±0.03	2.41±0.03	**2.38±.0.05**
GSSG	1.09±0.01	1.06±0.01	1.15±0.02	**1.16±0.02**
SOD	0.77±0.11	0.65±0.04	0.47±0.10[a]	**0.41±0.05[a]**
Catalase	26.03±1.89	22.1±0.8[c]	28.99±0.87	**27.63±3.34**
GPx	0.42±0.04	0.54±0.04	0.49±0.10	**0.52±0.06**
		KIDNEY		
ROS	227.2±4.54	243.0±0.99	278.4±5.30[a]	**260.6±5.09**
TBARS	9.81±0.91	15.05±2.27[a]	15.5±1.04[a]	**14.06±1.14[a]**
GSH	6.24±0.21	5.25±0.31[c]	6.72±0.15	**6.62±0.25**
GSSG	2.84±0.08	2.72±0.05	2.68±0.05	**2.61±0.06**
SOD	0.51±0.04	0.30±0.02[a]	0.43±0.05	**0.30±0.03[a]**
Catalase	41.84±3.04	36.1±3.32[b]	20.29±1.75[a]	**18.31±2.94[a]**
GPx	0.37±0.04	0.55±0.07[a]	0.53±0.06[a]	**0.61±0.07[a]**

ROS, reactive oxygen species as FIU; TBARS, thiobarbituric acid reactive substance as µg/gm; GSH, reduced glutathione as mg/gm; GSSG, oxidized glutathione as mg/gm; SOD, superoxide dismutase as units/min/mg protein; catalase as unit/min/mg protein; GPx, glutathione peroxidase as µg/min/mg protein. Values are mean±SE; N=5. [a]P<0.001; [b]P<0.01; [c]P<0.05 compared to normal control as evaluated by Student's t test.

Table 6. Administration of arsenic, lead, and mercury on blood and tissue metal concentration.

	Normal animal	Lead	Arsenic	Mercury
Blood				
Lead, µg/dl	1.13±0.45	26.2±5.01[a]	1.63±0.33	2.43±0.56
Arsenic, ng/dl	1.13±0.45	1.22±0.11	11.45±0.32[a]	0.83±0.06
Mercury µg/dl	N.D.	N.D.	N.D.	2.15±0.23
Liver				
Lead µg/g	1.56±0.45	7.26±1.22[a]	1.63±0.33	1.43±0.56
Arsenic, ng/g	1.13±0.45	1.22±0.05	16.89±0.25[a]	1.09±0.16
Mercury µg/g	N.D.	N.D.	N.D.	5.15±0.67
Kidney				
Lead µg/g	2.03±0.33	11.2±1.58[a]	1.63±0.33	1.43±0.56
Arsenic	1.53±0.15	1.26±0.21	13.5±0.38[a]	0.93±0.16
Mercury µg/g	N.D.	N.D.	N.D.	10.15±1.68

Values are mean±SE; N=5. [a]P<0.001 compared to normal control as evaluated by Student's t test.

ric chloride and lead acetate while only TBARS level increased in animals exposed to sodium arsenite (Table 5). These changes were accompanied by a decrease in kidney GSH and an increase in GSSG levels. Kidney SOD and catalase activities decreased while GPx activity showed an increase on exposure to these metals.

Metal Estimation

Concentration of sodium arsenite, lead acetate and mercuric chloride increased in blood, liver and kidneys on exposure to these metals (Table 6).

Discussion

The results suggest that oral exposure to a relatively low dose of sodium arsenite, mercuric chloride and lead acetate produced significant changes in the hematopoietic system, blood and tissue oxidative stress and organ damage. We further observed that at the doses used in this study, i) lead exhibited more pronounced deleterious effects on haem synthesis pathway than mercury and arsenic; ii) arsenic is more effective in inducing oxidative stress in blood and soft tissues and, iii) liver is the major target organ in case of arsenic while, mercury has a more pronounced toxic effects on kidneys.

Arsenic, mercury and lead are well known toxicants and particularly known for their thiol binding ability, alterations in the heme synthesis pathway and inducing oxidative stress. It was thus of interest to investigate which one among the three are more toxic than other two. We were also interested to know which toxic metal is more potent in inducing metallothionein, a thiol containing low molecular weight protein. These observations eventually showed that which heavy-metal is comparatively more perilous than other in exerting toxic manifestations. To rank or compare these toxicants, we carried out number of parameters which are

known to acts as biomarkers for the above mentioned systemic injury.

δ- aminolevulinic acid dehydratase, is the second enzyme in the heme biosynthetic pathway which catalyses the condensation of two molecules of ALA to form one molecule of porphobilinogen. Erythrocyte ALAD activity is well known to get rapidly inhibited by lead exposure. Determination of ALAD activity in erythrocytes is one of the most sensitive and specific marker for evaluating early lead exposure. In the present study among the three toxic metals, the inhibition of ALAD was more pronounced in case of lead than arsenic and mercury. Plasma protein provides additional binding sites for lead, arsenic and mercury and it has been shown (37) that amino-acids in the plasma protein have a greater affinity for lead than for arsenic and mercury. This is because of the fact that the activity is extremely sensitive to specific for blood lead concentration.

The synthesis of low-molecular-weight, cysteine-rich metal-binding proteins, known as metallothioneins (MTs), is induced by exposure to some essential and non-essential metals, especially cadmium, and mercuric chloride. MTs play important roles in the regulation, storage, and detoxification of metals and, as a consequence, influence the bioavailability of metals through the trophic chain. Mercury (Hg), a highly toxic metal is known to bind to cellular organelles once it enters the cell. Inorganic salts of mercury are reported to be poorly absorbed from the gastrointestinal tract and they rapidly tend to accumulate in the kidney within hours. The kidney is a critical target organ following the ingestion of inorganic mercury compounds. If patients survive GI tract damage following exposure to mercury salts, oliguria, anuria, necrosis of the proximal tubule epithelium and acute renal failure may occur within 24 hours of the ingestion of mercuric chloride prior to death (38). Morcillo and Santamaria (39) reported that even at the lower doses in rats mercuric chloride (0.5 mg/Hg/kg) may induce metallothionein predominantly in kidney. Among the three metals, only mercuric chloride was able to induce metallothionein prominently in kidneys suggesting MT to be capable of binding and storing mercury in this organ (40) compared to arsenic and lead. It has been reported that metallothionein induction attenuates depletion in the protein–SH binding on mercury exposure, however it (metallothionein) increases thiol groups pool in the cytosol eliminating oxygen radicals and inhibiting lipid peroxidation. The depletion in the proteins –SH including glutathione leads to an imbalance of cellular redox homeostasis due to the decrease in antioxidant defences (41). We also noted a significant increase in free radicals generation in blood, liver and kidney on mercuric chloride and lead acetate as compared to sodium arsenite. Reactive oxygen species (ROS) are implicated as an important pathologic mediators in many disorders. Recent studies indicated that arsenic might generate ROS causing cellular toxicity and carcinogenesis (3). Enhanced production of free radicals leads to membrane disruption, elicited by the oxidation of poly unsaturated fatty acids of the bilayer known as lipid peroxidation. The results are in agreement with number of previous study with arsenic and mercury (41, 42). Mercuric chloride and sodium arsenite notably increased TBARS levels in blood and kidney suggesting increased lipid peroxidation which can be attributed to increased cellular oxidative stress as a result of depletion of antioxidant scavenger system. The present study further confirms that all the three toxic metals (sodium arsenite, mercuric chloride and lead acetate) increase free radical production and/or decreases the anti-oxidative enzymes like superoxide dismutase (SOD) and catalase (CAT) and GPx which probably make the tissue more susceptible to biochemical injury. GPx reduces lipid hydro peroxides into lipid alcohols in the presence of GSH. The decrease in the level of GSH and increase in the level of GSSG and lipid peroxidation (LPO) result in a decrease GPx activity on arsenic and mercury exposure. Glutathione peroxidase plays an important role in the redox cycling of GSH to GSSG that is necessary for maintainance of the thiol content of the cell. GP_X activity requires GSH as a cofactor to exert its function; the lower GSH will limit the maximum activity of GP_X. Arsenic induced decrease in the liver GPx activity may also be an outcome of impaired functional group such as GSH and NADPH or selenium mediated detoxification of toxic metals (43).

Liver is an imperative organ for metabolism and detoxification. The determination of the patho-physiological enzymes like transaminases, phosphatases and lactate dehydrogenase are the common indicator of liver damage. In the present study we also observed significant increase in the activities of above indicators on sodium arsenite and mercuric chloride exposure and to a lesser extend lead, suggesting liver damage in rats which are supported by an elevated concentration of arsenic and lead in blood, kidney and liver. Most of the hematological variables remained insensitive to three metals except for a significant increase in WBC count in mercury exposed and a decrease in HGB level in lead exposed animals which might be due to a relatively low dose of metal exposure.

The proposed mechanism behind the metal salts induced oxidative stress might be due to following mentioned events inside the cell:

a) Generation of free radical might be via a Fenton-type/Haber-Weiss reaction in which ferrous iron(II) is oxidized by hydrogen peroxide to ferric iron(III), a hydroxyl radical, and a hydroxyl anion (44). Besides the Fenton-type and Haber-Weiss-type mechanisms these metal salts (sodium arsenite, mercuric chloride and lead acetate) might be directly reacting with cellular molecules to generate free radicals such as ROS that may attack polyunsaturated fatty acids present in the biological membranes. Catalase, superoxide dismutase and glutathione peroxidase might be scavenging these radicals. Catalase and glutathione peroxidase, a selenium-containing enzyme requires the presence of reduced GSH for its action; catalyse the conversion of hydrogen peroxide to water.

b) These metals salts are without redox potential (45) which impairs the antioxidant defences especially the thiol containing enzymes like GSH-dependent enzymes (glutathione peroxidase). This might results in the depletion of GSH which alters the redox balance (GSH/GSSG) in the cell.

c) Another important event which takes place for the detoxification of these metal ions is through the induction of metallothionein (MT) protein in kidney and liver. MT

possesses numerous cysteine residues and has the capacity to bind various metals, including inorganic mercury, cadmium, zinc, copper etc. Interaction of metallothionein with other essential proteins e.g. ferritin of the body causes a redox reaction. The redox function of MT is also referred to GSH/GSSG which modulates transfer of metal between MT and metal-binding proteins. Due to the high affinity of mercuric ions than other two metals, for binding thiols naturally suggests that following depletion of intracellular thiols (especially glutathione) either directly or indirectly causes, or predisposes, proximal tubular cells to oxidative stress (44).

The study thus provides some interesting observations for the relatively low level exposure to these metals and the related toxic manifestations. We recommend future studies particularly with different routes of exposure (like inhalation, dermal etc), doses durations (like low level long term exposure) and also the effects following combined exposure to these metals on these sensitive biomarkers.

In conclusion, the present study reports that the involvement of metal salts in the induction of oxidative damage is multi-faceted. These metals share common mechanisms underlying their toxicities, including production of oxidative stress, reaction with sulfhydryl groups and impairment in the activities of the antioxidant enzymes. Interestingly, metallothionein induction in kidney and liver also validates the hypothesis, which in turn suggests oxidative stress to be the major mechanism involved in the toxic manifestations in rats. The study also highlights the need to investigate i) the effects of multi-metals exposure in the workplace and, ii) risk assessment issues in humans from high dose animals to low dose human exposure and route to route extrapolation remain open. The data collected from this study will be useful while studying interactions of metal/metalloid mixtures in animals.

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