Original Article

Alleviation of Hepatotoxicity by Natural Chelators in Lead-induced Poisoning in Rats

Quadri Neha Nausheen, Syed A. Ali

Y.B. Chavan College of Pharmacy, Aurangabad, Maharashtra, India

Abstract

Aims: The study intends to monitor the consequences of lead on the body, its reversal by natural chelators (chitosan and chitosamine), and comparison of monotherapy with the combination using the synthetic ones. **Materials and Methods:** A total of 42 albino Wistar male rats (200–250 g) were divided into seven groups (n = 6). Except for the first group which received sodium acetate 1 g/L (drinking water, vehicle control), all groups received lead acetate 0.4 mg/kg body weight peroral (p.o.). Group II (toxic) received merely lead acetate, whereas the third and fourth groups received 0.2 g/kg (p.o.) of chitosan and chitosamine, respectively. Groups V–VII received ethylenediaminetetraacetic acid (EDTA) 495 mg/kg (p.o.). In addition, the sixth and seventh groups received chitosan and chitosamine (0.2 g/kg) (p.o.), respectively. The hematological, biochemical, oxidative stress parameters, number of porphobilinogen molecules formed/h/mL, and histopathology were assessed. The data obtained were compared using analysis of variance following Tukey's test. **Results:** The results revealed a statistically significant reduction in the hemogram parameters, antioxidant enzymes, porphobilinogen molecules and an increase in oxidative stress, liver biomarkers along with malondialdehyde in the toxic group in comparison with control and treatment groups. The histopathological findings revealed a significant improvement in the chitosan and chitosamine treatment groups when compared with the toxic group, whereas the results obtained from combination therapy with respect to its monotherapy were most significant than the monotherapy alone. **Conclusion:** Chitosan and chitosamine are found to improve hemato- and hepatotoxicity by chelation and can be used as potent detoxifiers in heavy metal toxicities.

Keywords: *Chelation, chitosamine, chitosan, lead toxicity, oxidative stress*

Introduction

Heavy metals possess toxic effects on the human body, out of which the two most common widely studied metal ions are lead and mercury possessing the highest toxicity to the developing central nervous system. According to the World Health Organization, lead ion present in the earth is a highly toxic element present in nature's ecosystem. Its extensive use results in environmental pollution causing significant public health issues owing to human exposure in various corners of the world. Sources of lead are polluted water, air, dust, various food items, or other consumer products. The major treatment involves the removal of lead by chelation therapy.[1] There is indeed a low safety margin between existing occupational blood lead suspension limits and subclinical effects owing to which the parameters for the lead users were set at a low level as per the voluntary Code of Practice observed in some national legislation, specifically Control of Lead at Work Act 2002. Lead being non-biodegradable has devastating effects on the body causing rapid accumulation in the liver, kidney, and other human organs following intestinal absorption.[2] There is not a single organ system in the human frame not influenced by lead poisoning. Unlike the developed countries such as the USA and Canada where the lead is being used meticulously, it is still used intensely in the developing countries.[3] Lead exposure causes various ill effects on the hematopoietic, renal, reproductive, and central nervous system, primarily due to oxidative insults. Blood lead levels exceeding 70 µg/dL are rare, resulting in encephalopathy, coma, and death.[4] These variations play a noticeable role in disease diagnosis and their manifestations.[5] Chelation therapy is suggested in children

How to cite this article: Quadri NN, Ali SA. Alleviation of hepatotoxicity by natural chelators in lead-induced poisoning in rats. J Rep Pharma Sci 2022;11:236-47.

Received: 11 Mar 2022
Accepted: 27 Aug 2022
Published: 23 Dec 2022

Address for correspondence:
Dr. Syed A. Ali,
Y.B. Chavan College of Pharmacy, Aurangabad, Maharashtra, India.
E-mail: ayazpharm@gmail.com

Access this article online

Website: www.jrpsjournal.com

DOI:10.4103/jrptps.JRPTPS_44_22

Quick Response Code:

when blood lead limits are greater than 40–45 µg/dL.[6] Management of lead toxicity in humans needs the increased excretion of lead from the human body by chelation. Regardless of long years of study, there is no effective treatment available for exposure to heavy metals/metalloids.[7] Moreover, the treatments with synthetic chelators are compromised with several serious adverse effects. The treatment comprises synthetic chelating agents such as 2,3-dimercaptosuccinic acid (Succimer), British anti-Lewisite, and EDTA to minimize consequences of lead toxicity but at the cost of undesired side effects on the body, viz., gastrointestinal disturbances, namely, nausea, vomiting, diarrhea, fatigue, loss of appetite, in some cases thrombocytopenia, weight loss, hepatotoxicity, and so on. Therefore, the search for natural chelating compounds with low or minimum adverse effects, to reduce lead toxicity, is a prerequisite for prophylaxis and is warranted. The widespread account of recent advances in the study of heavy metal poisoning, chiefly concerning the role of ROS generation or oxidative stress in the toxic appearances, indicated that therapies with chelating agents and antioxidants supplementation are more effective than monotherapy with chelators alone in reversing the toxic effects.[8] The use of organic solid waste and by-products obtained from the food industry, viz., chitin and its processed product chitosan, in an effort to recycle and obtain added-value products is a good example of maintaining ecological balance.[9] Chitosan and its derivatives are toxic to several bacteria, fungi, and parasites.[10] Chitosan has a lipid-lowering effect and has clinical use in obesity management.[11] The summative outcomes of the pre-clinical and clinical studies revealed that chitosan proves to be effective in cutting down the total and low-density lipoprotein cholesterol. Moreover, chitosan works at relatively low doses, with as little as 1.2 g/day causing a significant decrease in serum cholesterol.[12] Chitosan plus oat gum lowered liver cholesterol moderately.[13] Being a member of the marine family, chitosan and its derivatives are rich in a large number of essential micronutrients, minerals, vitamins, mainly vitamin D, amino acids, etc.; apart from this, chitosan is used to manage obesity, cut down on high cholesterol and Crohn's disease. It is equally effective in overcoming side effects of dialysis in renal-compromised patients along with conditions such as hypercholesterolemia, hyperphosphatemia "tired blood" (anemia), anorexia, fatigue, and insomnia. Chitosan treats gum inflammation causing tooth loss (periodontitis) and prevents "cavities" (dental caries). Thus in a view to safeguard and detox oneself from inevitable exposure of heavy metals (lead), the study emphasizes to include the natural chelators, viz., chitosan and chitosamine, in our daily diet, being a cheap source with considerable uses and positive physiological effects on the body which not only has health benefits but also increases the quality of life.

Materials and Methods

Chemicals and reagents

Thomas-Baker Chemicals Pvt. limited, Mumbai, India, and S.D. Fine Chem Limited, Mumbai, India, provided chitosan and chitosamine, respectively. ALP (alkaline phosphatase), ALT (alanine transaminase), AST (aspartate aminotransferase), and gamma-glutamyltranspeptidase (GGT) kits were purchased from Biomatic, Mumbai, India. The analytical grade solvents and chemicals were used for experimentation, and chemicals necessary for sensitive biochemical assays were procured from Merck. Analytical kits, viz., CK-MB (creatinine kinase-muscle brain) (Aspen Laboratories, Rapid Diagnostic Pvt. Ltd) and LDH (lactate dehydrogenase) (Crest Biosystems, Goa, India), were used for assessment. All drug compositions were prepared *in situ* in distilled water for daily dosing.

Animals

Animals used in this study are albino Wistar male rats weighing between 200 and 250 g. The animals were kept at 23 ± 2°C temperature with open access to standard rat feed and water. A (12–12 h) light cycle was maintained in the animal house. The care, maintenance, and utilization of experimental treatment animals were performed as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Guidelines, New Delhi, India, as per the Institutional Animal Ethical Committee of Y.B. Chavan College of Pharmacy, Aurangabad, Maharashtra, India, having the approval number (CPCSEA/IAEC/P'col-58/2017-18/139).

Experimental design

Animals were distributed randomly in seven groups ($n = 6$). The treatment was expanded for 24 weeks (6 months).

The dosing treatment was performed every day for 24 weeks at 10:00 am.

At the culmination of the experimental period of 24 weeks, the animals were fasted overnight; anesthetized using a carbon dioxide chamber; and their blood samples were collected in test tubes. All serum specimens from each group were separated and stored at 8°C until they were analyzed.

Blood collection and analysis

A blood cell counter Selenium-380, an automated hematology analyzer (Labsystems Diagnostics, A Trivitron Enterprise, India), was used to assess hematological parameters as erythrogram, viz., hemoglobin (Hb) concentration, red blood cell (RBC) count, mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), packed cell volume (PCV), and leucogram, namely, white blood cells (WBCs), neutrophils, basophils, eosinophils, lymphocytes, monocytes, and the number of platelets.

Biochemical analysis

Preparation of serum: light CO_2 anesthesia was used to withdraw the blood from the retro-orbital plexus from the inner canthus of the eye by glass capillary tubes. The serum was separated for 15 min at 3000 rpm in an R-24 research centrifuge (Remi Instruments Ltd, Mumbai, India).[18] Serum was used for the analysis of CK-MB and LDH using CK-MB kit (Aspen Laboratories, Rapid Diagnostic Pvt. Ltd) and LDH kit (Crest Biosystems, Goa, India), respectively, using Biochemical Autoanalyzer (Preitest, Robonik). Serum ALT, AST, ALP, and GGT levels were determined by enzymatic methods (Biomatic, Mumbai, India) on Biochemical Autoanalyzer (Preitest, Robonik).

Measurement of prothrombin time[19]

Blood is withdrawn into a test tube that contains liquid sodium citrate acting as an anticoagulant to bind the calcium ions in a sample. The blood is mixed and centrifuged for 10 min at 2000 rpm using a refrigerated centrifuge that separates plasma from whole blood (as prothrombin time is most commonly measured using blood plasma). A sample of the plasma is extracted from the test tube and placed into a measuring test tube. Next is the addition of an excess of calcium (in a phospholipid suspension) to reverse anticoagulation due to citrate causing the blood to coagulate again. Finally, the tissue factor (factor III) is added to the process to activate the extrinsic cascade pathway to measure optically the time taken for the sample to coagulate. The prothrombin ratio, i.e., international normalized ratio (INR), is a quantitative measure of comparison of test prothrombin time concerning its control plasma raised to the power of the ISI value for the analytical system being used.[20]

Measurement of bleeding time

The test for bleeding time estimation is a method to determine coagulation abnormalities.[21] Nevertheless, it is the most reliable way to assess clinical bleeding in patients with uremia and to assess the platelet activation conditions if any.[22] The bleeding time procedure was carried out by the Dukes method.[23]

Measurement of clotting time

The clotting time is determined using the capillary tube method. Blood calcium ion levels and a variety of underlying disorders have an impact on it. Clotting time is usually measured in minutes and ranges from 8 to 15 min. Blood is collected in a glass test tube at 37°C, and the time required for the blood to clot is monitored to estimate clotting time using the test tube method.

Preparation of tissue homogenate

Euthanasia is practiced to sacrifice the animals under carbon dioxide anesthesia followed by the cutting of the carotid artery. The livers were swiftly removed, washed in ice-cold saline dried up using a filter paper, and weighed. A 10% homogenate was prepared in 0.15 M potassium chloride (KCl) for the assessment of tissue malondialdehyde, and the homogenate for the tissue glutathione was primed in 0.02 M EDTA.

Estimation of tissue glutathione[24,25]

A liver tissue sample (100–150 mg) was homogenized in 5 mL of EDTA (0.02 M), 4 mL of cold distilled water was added, and 1 mL of trichloroacetic acid (50%) was mixed in a vortex mixer and agitated intermittently for 10 min. After 10 min, the contents were transferred to centrifuge tubes (which had been washed with EDTA) and centrifuged for 15 min at 4°C at 6000 rpm. An aliquot of 2 mL supernatant was combined with 4 mL of tris buffer after centrifugation (0.4 M, pH 8.9). The solution was thoroughly mixed before adding 0.1 mL of DTNB (0.01 M). Within 5 min after adding DTNB at 412 nm against an acceptable blank, the absorbance values were estimated.

Measurement of tissue malondialdehyde (MDA)[26]

Measurement of tissue MDA: measurement of lipid peroxidation by determination of liver malondialdehyde content was performed by the thiobarbituric acid (TBA) method. About 10% of liver homogenate sample was used in buffered 0.9% KCl pH 7.4 for estimating tissue MDA. An aliquot of 0.5 mL trichloroacetic acid (30%) and 0.5 ml thiobarbituric acid (0.8%) was added to 1 mL homogenate and agitated for 5 min. The tubes were then heated in a water bath at 80°C for 30 min, cooled in ice cold water for 10 min, and then centrifuged at 5000 rpm for 15 min. Using an adequate blank, the absorbance of the clear supernatant was determined at 540 nm. Calculation: nmol MDA = $V \times$ O.D. 540/0.152.

where E is the extinction coefficient (1.52×105)

V the final volume of test solution (2 mL)

OD the optical density at 540 nm.

Preparation of post-mitochondrial supernatant (PMS)

Preparation of post-mitochondrial supernatant (PMS): Remi homogenizer was used to homogenize the tissues in the chilled buffer of potassium phosphate (50 mM, pH 7.4). The PMS is obtained for various biochemical assays by centrifuging it for 20 min at 4°C in a chilled centrifuge (10,500 rpm) for evaluation of enzymes such as catalase and superoxide dismutase.

Assessment of catalase (CAT)[27]

Principle: The assay of catalase was based on the ability of catalase to initiate the breakdown of H_2O_2.

$$H_2O_2\,(\text{Abs at } 240\,\text{nm}) \xrightarrow{\text{Catalase}} 2H_2O + O_2\,(\text{No Abs at } 240\,\text{nm})$$

Catalase is a protective enzyme that catalyzes the following reactions:

1. Decomposition of H_2O_2 to give H_2O and O_2.
2. Oxidation of H^+ donors, e.g., methanol, ethanol, and formic acid with the consumption of 1-mole of peroxide (peroxides) activity.

The breakdown of H_2O_2 or the liberation of O_2 can be used to determine catalase activity. The method of choice for biological material is the UV spectrophotometric approach.

Reagents

Potassium dihydrogen phosphate (KH_2PO)

Disodium hydrogen phosphate (Na_2HPO_4)

Hydrogen peroxide (H_2O_2) 19 mM

Preparation of solutions

Potassium phosphate buffer 50 mM (pH 7.4)

A. 6.81 g of KH_2PO_4 was dissolved in distilled water and made up to 1000 mL.

B. 18.9 g of Na_2HPO_4 was dissolved in distilled water and made up to 1000 mL.

Solutions A and B were mixed in a proportion of 1:1.55.

Hydrogen peroxide (19 mM)

0.187 mL of 30% H_2O_2 was dissolved in 100 mL of 50 mM potassium phosphate buffer, pH 7.4.

Procedure

The cytosolic supernatant (50 µL) was added to the cuvette containing 2.95 mL of hydrogen peroxide (19 mM) solution prepared in potassium phosphate buffer (50 mM, pH 7.4). The variation in absorbance was read at 240 nm on the Shimadzu spectrophotometer for 3 min with a 1 min interval.

Calculation: Catalase activity was calculated as follows:

nmol H_2O_2 consumed/min/mg protein = Δ Optical density min^{-1} × Volume of assay /0.081 × volume of PMS × mg protein,

where
ΔO.D. min^{-1} = Change in absorbance in 1 min;
The volume of assay = (3 mL);
0.081= Extinction coefficient;

Volume of PMS= 0.05 mL;

mg of protein amount of protein in the sample.

Estimation of superoxide dismutase (SOD) assay[28]

Principle: The assay of SOD was based on the ability of SOD to inhibit the spontaneous oxidation of pyrogallol.

Reagents

Tris–HCl buffer (50 mM)

EDTA (1 mM)

Pyrogallol (24 mM)

Preparation of reagents

Tris–HCl buffer (50 mM)

788 mg of Tris–HCl buffer and 186 mg of EDTA were dissolved in 100 mL of distilled water and then adjusted to pH 8.5

Pyrogallol (24 mM)

15.1 mg of pyrogallol was dissolved in 5 mL of 10 mM HCl

For the assay, a freshly prepared solution was used *in situ*.

Procedure

The supernatant was assayed for the assessment of the inhibition of pyrogallol auto-oxidation. An aliquot of 100 µL of cytosolic supernatant was mixed with Tris–HCl buffer, pH 8.5, to which the volume was adjusted to 3 mL; 25 µL of pyrogallol was mixed and variations in absorbance at 420 nm were noted for 3 min with a 1-min interval. The presence of SOD inhibits the increase in absorbance at 420 nm after the addition of pyrogallol.

Calculation

A unit of SOD is defined as the quantity of enzyme required to inhibit pyrogallol autoxidation by 50% in 3 mL of assay mixture, as calculated by the following formula:

Unit of SOD per mL of sample = $[A-B/A \times 50] \times 100$,

where A is the deviation in absorbance per minute in the control sample;

B the deviation in absorbance per minute in the test sample.

Data are presented as SOD units per milligram protein (SOD/mg protein).

Estimation of body and organs weight

In each group, the body weights of rats were noted before and after treatment. Isolated livers were weighed after keeping them in chilled saline and squeezing out the blood.

Estimation of lead toxicity marker

(a) Porphobilinogen molecules formed[29]:

Delta-aminolevulinic acid dehydratase (δ-ALAD) activity

A spectroscopic approach was used to calculate delta-ALAD activity. Blood samples were taken from rats by cardiac puncture and placed in heparinized vials before being held at 4°C until analysis. Water was used to hemolyze the blood for 10 min at 37°C. The blood sample was incubated at 37°C for 90 min with potassium phosphate buffer and ALA (12 mM). The enzymatic activity was stopped after incubation by adding 10% TCA (10 mM $HgCl_2$). After centrifuging the samples at 6000 rpm for 10 min, 1 mL of Ehrlich reagent was added to the supernatant and incubated for 20 min. To the incubated sample, 0.5 mL of distilled

water was mixed and read at 555 nm in a spectrophotometer (Rayleigh, UV9200). The data obtained were stated as porphobilinogen (PPB) nanomoles formed per hour per milliliter.

(b) Lactate dehydrogenase (LDH) assay[30]:

LDH is an enzyme of cytoplasm distributed extensively in the body. It is present in organs like heart, liver, kidney, etc. It is one of the cardiac panel enzymes and is a valuable detection aid for the onset of coronary heart disease.

Principle: *NAD to NADH*. In this test, the enzyme activity is proportional to the increase in the absorbance of the test solution due to the reduction of NAD.

$$\text{L-Lactate} + \text{NAD}^{+} \xrightarrow{\text{Lactate Dehydrogenase}} \text{Pyruvic} + \text{NADH} + \text{proton.}$$

Procedure: Serum samples (25 µL) were mixed with 500 µL of reagent and were incubated for 60 s at 37°C and then at the same time two additional absorbances were taken at 340 nm with 1 min interval. The mean absorbance change per min, i.e., ΔA/min, was calculated. LDH was calculated using the following formula:

$$LDH\left(\frac{U}{L}\right) = \frac{\Delta A}{min} \times 8095.$$

(c) Creatine kinase-MB (CK-MB) assay[31]:

Principle: The sample was incubated in the CK-MB reagent, which includes the anti-CK-MB antibody. The non-inhibited CK-B activity was then determined utilizing the following reaction:

$$\text{ADP} + \text{Creatinine Phosphate} \xrightarrow{\text{CK}} \text{Creatinine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose-6-Phosphate}$$

$$\text{G-6-P} + \text{NAD}^{+} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate} + \text{NADH} + \text{H}^{+}$$

Procedure: The serum sample (50 µl) was mixed with 1000 µL of reagent and was incubated for 300 s at 37°C after which absorbance was taken. At the same time, two additional absorbances at 1 min of interval at 340 nm were noted. The mean absorbance change per min was calculated, i.e., ΔA/min. CK-B was calculated using the following formula:

$$CK-B\left(\frac{U}{L}\right) = \frac{\Delta A}{min} \times 3333.$$

Histopathological studies[32,33]

The liver of rats from each group was collected, washed, and rinsed thoroughly with cold saline water, squeezing the excess blood out. The samples were then fixed in 10% formalin, processed for standard procedures, and embedded in paraffin wax. The blocks were then sectioned rendering the hematoxylin and eosin methods. Under a light microscope, the sections were viewed, and images were taken at a magnification of 10×.

Statistics

The values were calculated as mean ± SEM values/group. One-way analysis of variance following Tukey Kramer's multiple comparison tests was utilized for statistical analysis. Values of $P < 0.001$, $P < 0.01$, and $P < 0.05$ were considered statistically significant. The complete statistical analysis was accomplished using the statistical package, GraphPad Instat Version 8.0 (GraphPad Software Inc.).

Results

Hematological indices

The results showed a highly significant decrease in the erythrogram values ($P < 0.001$) in the toxic group when compared with the control group. Hemoglobin, RBC, MCHC, MCV, and PCV parameters were found to be increased statistically ($P < 0.001$) in all treatment groups when compared with the toxic control. A marked decrease was observed in the levels of leucogram in rats of the toxic group relative to the healthy control rats ($P < 0.001$) and the treatment groups ($P < 0.001$). In addition, the clotting factors, viz., prothrombin time, bleeding time, and clotting time, were found to be increased in the group merely exposed to lead when compared with the control group. The treatment groups III, IV, and VI showed $P < 0.05$ level of significance, whereas groups V and VII showed $P < 0.01$ level of significance. Tukey's test helped to display the results that the EDTA + chitosan-treated group was found to be more significant than EDTA + chitosamine in the above respective parameters. The findings also reflected that the chelation effect of chitosan was more significant ($P < 0.001$, $P < 0.01$) when compared with that of chitosamine in hematological and clotting factors parameters. Moreover, it also showed that the results obtained from the comparison of natural and synthetic chelators have nearly the same level of significance values ($P < 0.001$) [Table 2].

Liver enzymes

The toxic control group led to a significantly increased ALT, AST, ALP, GGT enzymes along with total bilirubin in comparison with the vehicle-treated group ($P > 0.001$). The mean concentration of ALT, AST, ALP, and GGT enzymes showed no significant difference in all treatment groups in comparison with the control group ($P > 0.01$). Moreover, a significant decrease was recorded in total bilirubin levels in treatment groups when compared with the toxic control group ($P > 0.001$ and $P > 0.01$). The significance level obtained from VI and VII groups reflected that the chelation power of group VI was more pronounced that led to the

Table 1: The experimental design protocol of the study dividing the animals (n = 6) into seven groups

Groups	I	II	III	IV	V	VI	VII
Control (sodium acetate; 1000 mg/L in drinking water)[14]	✓						
Lead acetate (0.4 mg/kg body wt.)[15]		✓	✓	✓	✓	✓	✓
Chitosan (0.2 g/kg body wt.)[16,17]			✓			✓	
Chitosamine (0.2 g/kg body wt.)[16,17]				✓			✓
EDTA (495 mg/kg body wt.)					✓	✓	✓

Group I: vehicle control, group II: toxic group (as received merely the toxic lead acetate), group III: chitosan monotherapy, group IV: chitosamine monotherapy, group V: EDTA monotherapy, group VI: combination therapy of EDTA and chitosan, group VII: combination therapy of EDTA and chitosamine

Table 2: Shifts in hematological indices in lead-induced toxicity in rats after 24 weeks of treatment with chelators

Parameters	Groups						
	Control	Lead	Chitosan	Chitosamine	EDTA	EDTA+ Chitosan	EDTA+ Chitosamine
1. Erythrogram							
Hemoglobin (g/dL)	15.85±0.37	5.58±0.3^{A}	9.53±0.71^{D}	7.87±0.61^{F}	12.15±0.61^{D}	10.78±0.60^{D*}	8.98±0.61$^{F,\#\#}$
RBC (× 10^3/µL)	8.97±0.37	4.8±0.25^{A}	5.38±0.35^{D}	4.45±0.21^{D}	6.87±0.17^{D}	6.08±0.15^{D*}	5.1±0.29$^{D,\#\#}$
MCH (pg)	19.61±0.31	12.18±0.64^{A}	16.38±0.35^{D}	15.91±0.23^{D}	18.56±0.47^{D}	17.7±0.28^{D*}	17.28±0.54$^{D,\#}$
MCHC (%)	36.88±0.25	26.8±0.93^{A}	34.67±0.37^{D}	33.71±0.67^{D}	35.68±0.41^{D}	35.41±0.22^{D}	34.1±0.53^{D}
MCV (FL)	54.65±0.60	47.68±0.62^{A}	50.63±0. 65^{NS}	48.28±0.69^{NS}	53.75±0.74^{D}	51.68±0.77^{E}	50.06±0.98$^{NS,\#}$
PCV (%)	47.73±0.37	29.51±1.26^{A}	45.71±0.47^{D}	45.100±0.44^{D}	45.667±0.84^{D}	46.817±0. 46^{D}	45.87±0.46^{D}
2. Leucogram							
White blood cells (× 10^3/µL)	8.185±0.11	1.235±0.16^{A}	6.168±0.17^{D}	5.0557±0.18^{D}	7.032±0.10^{D}	7.122±0.15^{D,*}	6.665±0.24$^{D,\#}$
Neutrophils (×10^3/µL)	26.283±0.75	4.917±0.71^{A}	20.65±1.4^{D}	21.867±1.4^{D}	21.65±1.69^{D}	19.767±1.70^{D}	20.983±1.76^{D}
Basophils (×10^3/µL)	0.8500±0.06	0.2167±0.04^{NS}	0.5±0.05^{NS}	0.633±0.05^{NS}	0.7±0.05^{NS}	0.6167±0.05^{NS}	2.05±1.39$^{NS,\#\#\#}$
Eosinophils (× 10^3/µL)	3.417±0.21	0.133±0.02^{A}	1.9±0.28^{D}	1.9±0.25^{D}	2.5±0.24^{D}	2.5±0.21^{D,**}	2.65±0.13$^{D,\#\#\#}$
Lymphocytes (× 10^3/µL)	3.89±0.32	1.95±0.32^{A}	3.36±0.25^{D}	2.74±0.27^{D}	3.86±0.42^{D}	3.76±0.13^{D,*}	3.15±0.26$^{D,\#\#}$
Monocytes (× 10^3/µL)	0.87±0.05	0.466±0.08^{C}	0.492±0.06^{NS}	0.59±0.09^{NS}	0.63±0.10^{NS}	0.65±0.07^{NS}	0.66±0.07^{NS}
3. Platelets (× 10^3/µL)	1180±6.24	594.2±21.07^{A}	1010.8±40^{D}	1043.3±58^{D}	1105.5±31^{D}	1052±19^{D*}	1007.2±43.55$^{D\#}$
4. Clotting factors							
Bleeding time (min)	2.35±0.23	9.55±0.42^{A}	6.217±0.81^{F}	6.4±0.94^{F}	5.317±0.99^{E}	6.083±0.84^{F}	5.9±0.94$^{E,\#\#}$
Clotting time (min)	11.68±0.39	19.41±0.50^{A}	15.46±0.95^{E}	16.95±0.96^{E}	12.86±0.45^{D}	13.22±0.95^{D*}	14.46±0.90$^{E,\#\#}$
Prothrombin time (min)	14.73±0.54	18.6±0.25^{A}	15.98±0.49^{E}	15.17±0.64^{E}	11.617±0.55^{A}	12.8±0.58^{E,**}	12.017±0.58$^{A,\#\#}$

RBC = red blood cell count, MCH= mean corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, PCV= packed cell volume. Data are indicated as mean ± SEM. No. of samples (n) =6

[a]P<0.001, [b]P<0.01, [c]P<0.05 as compared to the control group and [d]P<0.001, [e]P<0.01, [f]P<0.05 as compared to the lead (toxic control)-treated group, and ns represents non-significant data

*P<0.5 as compared to the chitosan group

##P<0.01 as compared to the chitosamine group

normalization of results of liver enzymes resembling the control group [Table 3].

Body and liver weight

Results indicated that the values of body weight of the toxic group decreased significantly ($P < 0.001$) when compared with the control group, whereas the treatment groups showed a significant increase ($P < 0.01$, $P < 0.05$) in body weight when compared with that of the toxic group. The toxic control group showed hepatomegaly with an increase in liver weights ($P < 0.01$), which also shows moderate ulceration when observed grossly when compared with the control group. The treatment groups showed a decrease in liver weights ($P < 0.01$ and $P < 0.05$) when compared with a toxic group with no significant difference as compared to the control group [Table 4].

Oxidative stress parameters

The findings revealed that the oxidative stress in the liver MDA level significantly increased in the toxic group when compared with the control group ($P < 0.001$), whereas antioxidant enzymes SOD, CAT, tissue GSH in the toxic group decreased significantly as compared to the treatment groups III ($P < 0.001$), IV ($P < 0.001$), V ($P < 0.001$), VI ($P < 0.001$), and VII ($P < 0.001$) relatable to the control group [Table 5].

Table 3: Shifts in values of liver function tests in lead-induced toxicity in rats after 24 weeks of treatment with chelators

Parameters	Groups						
	Control	Lead	Chitosan	Chitosamine	EDTA	EDTA+ Chitosan	EDTA+ Chitosamine
AST (U/L)	120.16±3.47	178.83±4.18^{c}	146.33±6.4^{f}	154.17±8.34^{f}	135.5±6.25^{e}	129±6.87^{e,***}	139.83±9$^{f,\#\#}$
ALT (U/L)	44.67±2.1	69.66±4.8^{a}	58.5±3.37^{e}	60±1.317^{e}	51.83±1.4^{d}	46.83±3.506^{d}	55.833±0.8$^{e,\#}$
ALP (U/L)	134±2.98	270.33±6.33^{a}	234.50±6.47^{e}	240.6±7.13^{f}	251.5±5.18^{e}	224.67±6.45^{d}	255±10.4$^{f\#}$
GGT (U/L)	9.66±0.55	20.05±1.78^{a}	10.06±0.8^{d}	10.33±0.75^{d}	10.583±0.95^{d}	13.217±0.75^{d*}	14.15±0.72$^{e,\#\#}$
Total bilirubin (mg/dL)	0.32±0.066	4.017±0.29^{a}	2.1±0.019^{d}	2.9±0.22^{d}	1.1±0.17^{d}	1.4±0.155^{d,**}	1.78±.0245$^{d,\#\#\#}$

AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phosphatase, GGT = gamma-glutaryltransferase
The data are indicated as mean ± SEM. No. of samples (*n*) =6. $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$ when compared with the control group and $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ when compared with the lead (toxic control)-treated group, and ns represents non-significant data
$^{*}P<0.5$ as compared to the chitosan group
$^{\#\#}P<0.01$ as compared to the chitosamine group

Table 4: Shifts in values of body and liver weight in lead-induced toxicity in rats after 24 weeks of treatment with chelators

Parameters	Groups						
	Control	Lead	Chitosan	Chitosamine	EDTA	EDTA+ Chitosan	EDTA+ Chitosamine
Body weight (g)	381±7.34	205±14.66^{a}	290±5.63^{e}	268±4.45^{e}	317±2.68^{f}	344±2.9^{f,*}	277±3.4$^{e,\#}$
Liver weight (g)	8.74±0.64	13±0.45^{a}	9.2±0.36^{d}	9.8±0.38^{d}	9±0.39^{d}	8.1±0.85^{d,*}	9.3±0.84$^{d,\#}$

Data are indicated as mean ± SEM. No. of samples (*n*) =6. $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$ as compared to the control group and $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ as compared to the lead (toxic control)-treated group, and ns represents non-significant data
$^{*}P<0.5$ as compared to the chitosan group
$^{\#\#}P<0.01$ as compared to the chitosamine group

Table 5: Shifts in values of liver tissue oxidative stress in lead-induced toxicity in rats after 24 weeks of treatment with chelators

Parameters	Groups						
	Control	Lead	Chitosan	Chitosamine	EDTA	EDTA+ Chitosan	EDTA+ Chitosamine
SOD (U/mg protein)	26.6±0.63	14.2±1.5^{c}	22.3±0.7^{d}	21.3±0.7^{d}	23.3±0.6^{d}	25.3±0.6^{d,**}	23.5±0.6$^{d,\#\#}$
CAT (nmol of H_2O_2 consumed/min/mg protein)	73±7.01	35.3±4.5^{c}	75.5±5.9^{f}	63.6±6.1^{f}	84.3±8.01^{d}	85.3±6.45^{e,***}	82.7±9.95$^{d,\#\#\#}$
Tissue GSH (µmol/g tissue)	5.65±0.4	1.65±0.24^{a}	7.86±0.33^{d}	6.68±0.34^{d}	8.75±0.28^{d}	8.42±0.32^{d,*}	9.1±0.41$^{d,\#\#\#}$
MDA (µmol/g tissue)	2.4±0.22	11.57±0.67^{a}	3.8±0.36^{d}	4.11±0.33^{d}	2.2±0.93^{d}	3.92±0.76^{d,*}	5.2±0.25$^{d,\#}$

SOD = superoxide dismutase, CAT = catalase, GSH= glutathione, MDA= malondialdehyde
Data are indicated as mean ± SEM. No. of samples (*n*) =6. $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$ as compared to the control group and $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ as compared to the lead (toxic control)-treated group, and ns represents non-significant data
$^{*}P<0.5$ as compared to the chitosan group
$^{\#\#}P<0.01$ as compared to the chitosamine group

Lead toxicity marker

The lead toxicity marker, viz., several porphobilinogen molecules formed/h/mL, CK-MB, and LDH, was estimated which showed a remarkable decrease in the number of molecules of porphobilinogen and increase in CK-MB and LDH in groups receiving only lead acetate when compared with that of control group. These numbers reverted back to normal limits when treated with chelators of groups III ($P < 0.001$), IV ($P < 0.001$), V ($P < 0.001$), VI ($P < 0.001$), and VII ($P < 0.001$) in range values of the control group. The synthetic and natural chelators showed the nearly same level of significance value ($P < 0.001$) in chelation when compared with the toxic group [Table 6].

Histopathological interpretations

The histopathological findings revealed that the normal hepatocyte's parenchymal architecture observed in the control group changed due to heavy metal poisoning which can be perceived in the following slides [Figure 1].

Table 6: Shifts in values of the number of porphobilinogen molecules formed, CK-MB, and LDH (lead toxicity marker) in lead-induced toxicity in rats after 24 weeks of treatment with chelators

Parameters	Groups						
	Control	Lead	Chitosan	Chitosamine	EDTA	EDTA+ Chitosan	EDTA+ Chitosamine
No. of PPB	0.372±0.024	0.19±0.021[a]	0.35±0.0189[d]	0.39±0.018[d]	0.51±0.0145[d]	0.47±0.032[d,**]	0.45±0.018[d,#]
CK-MB (U/L)	922±81.6	2049±203.2[a]	1431±57.2[d]	1477±72.8[d]	1345±45.6[d]	1353±28.5[d,*]	1357±75.6[d,#]
LDH (U/L)	840±244	1811±411[a]	1298±210[d]	1309±258[d]	1190±169[d]	1119±165 [d,*]	1212±250[d,#]

PPB= porphobilinogen molecules, CK-MB= creatinine kinase-muscle brain, LDH= lactate dehydrogenase
Data are indicated as mean ± SEM. No. of samples (*n*) =6. [a]*P*<0.001, [b]*P*<0.01, [c]*P*<0.05 as compared to the control group and [d]*P*<0.001, [e]*P*<0.01, [f]*P*<0.05 as compared to lead (toxic control)-treated group, and ns represents non-significant data
**P*<0.5 as compared to the chitosan group
[##]*P*<0.01 as compared to the chitosamine group

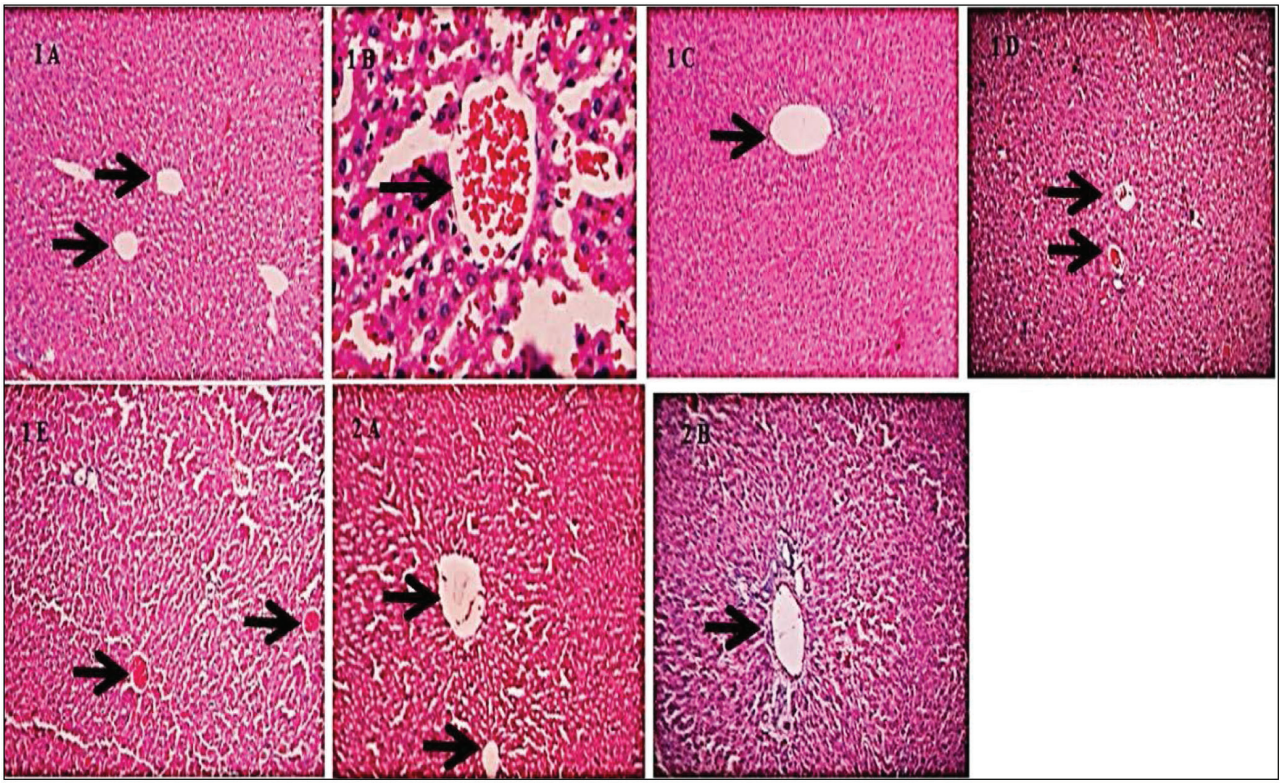

Figure 1: H & E-stained transverse sections of hepatocytes of the groups in the study

H&E-stained liver organ section I A represents male rats (control group) showing normal hepatic architecture with central vein and centrilobular hepatocytes (marked by arrows). I B showed a toxic group receiving lead acetate (0.4 mg/kg p.o.) reflecting sharp demarcated centrilobular necrosis with loss of hepatocyte cytological details (demonstrated by arrows) with sinusoidal bleeding. Section I C showed the transverse section of hepatocytes of groups that received EDTA (500 mg/kg p.o.) along with lead acetate (0.4 mg/kg p.o.) showing reversal of necrotic insults gaining normal architecture with typical central vein perfusion. Section I D showed histopathology of groups receiving chitosan (400 mg/kg p.o.) along with lead acetate (0.4 mg/kg p.o.) showing terminal hepatic vein with the organized microstructure of hepatocytes. Section I E showed a section of groups receiving chitosamine (400 mg/kg p.o.) along with lead acetate (0.4 mg/kg p.o.) showing central vein with radiating hepatocytes surrounding it along with the reversal of cellular details of hepatocytes. Section 2D showed the section of groups receiving chitosan (0.4 mg/kg p.o.) + EDTA (500 mg/kg p.o.) along with lead acetate (0.4 mg/kg p.o.), which showed portal tracts (arrow) surrounding the classical lobules. The normal architecture reverts to normal along with the interlobular portal vein (arrow).

Section 2E represents the group receiving chitosamine (0.4 mg/kg p.o.) + EDTA (500 mg/kg p.o.) along with lead acetate (0.4 mg/kg p.o.), which showed liver architecture consisting of a massive inter anastomosing network of

hepatocytes arranged in solitary-cell dense plates separated by vascular sinusoids. The hepatocytes along with vascular channels reflect structured micro-constructions, central vein (arrow) surrounded by radiating hepatocytic covers resembling typical liver histology reflecting their protective effect against heavy metal toxicity.

Discussion

Rapid urbanization in the modern world gave rise to several harms such as pollution, adulteration, population explosion, health hazards, and so on.[34] One of the inevitable issues is the high incidence of heavy metal exposure in the environment. The matter of concern here is that they are non-biodegradable, non-eco-friendly, bioaccumulated in the body, toxic to nature, and most importantly have the potential to detriment normal anatomy and physiology of the body.[35] Chelation therapy is a remedy for heavy metal intoxication. Our study made an effort to put a light on the chelation process which eliminates the same, rendering it harmless. Chelation takes place by chelating agents which tend to claw heavy metals and mark them water-soluble which eases them to get eradicated from the body.[36] Chelators are synthetic and natural. Synthetic chelators are effective but have serious side effects on the body.[37,38] Chelators from natural origin, viz., marine sources such as chitosan and its monomer chitosamine, have several advantages such as cholesterol-lowering properties, as a styptic in obstetrics hemorrhages, biomedical nanoparticles, anticoagulant, antioxidant, pharmaceutical aid, and so on.[39-42] Heavy metal outburst in the environment since the early nineteenth century marked an alarming issue concerning several hazardous effects on the organ system. Due to prolonged exposure of lead for years, a much slower clearance takes place. The experimental protocol of our study is illustrated in Table 1 which showed the grouping of animals for better interpretation of results in lead toxicity with respect to natural and synthetic chelation aspects. Our study made an effort to find the impact of lead exposure on hemogram indices, which revealed a significant decrease in their levels due to lead poisoning. Lead is responsible to disrupt the cellular constitution and cell membrane making RBCs more fragile leading to its clumping which ends up in anemia, our point recorded in concordance with other studies.[43]

Lead intoxication showed characteristic changes in RBCs such as hypochromic and microcytic anemia along with basophilic stippling, which was found to be normalized in treatment groups. Moreover, lead is also responsible to alter the permeability of blood vessels and collagen synthesis.[44] The group treated merely with lead acetate showed a fall in the levels of erythrocytes showing severe anemia along with a decrease in the number of porphobilinogen molecules ($P < 0.01$), as explained in Figure 2. This can be elucidated by the interference of lead with essential enzymes, namely, Delta-ALAD is responsible for the biosynthesis of heme, a cofactor in hemoglobin.[45] This number improved to normal value in chelation groups which indicated the decrease in lead molecules, where ALAD is again available for heme synthesis. The lead acetate led to inhibition of the conversion of coproporphyrinogen III to protoporphyrin IX causes decreased erythrocyte life span and thus results in low Hb.[46] The results showed nearly the same chelation outcomes of natural and synthetic origin, which indicates that natural chelators can opt instead of synthetic ones. On application of Tukey Kramer's test of statistical significance, it is also revealed that the chitosan-treated group showed higher value of significance of chelation than chitosamine with respect to the toxic group, which may be due to more exposure of groups that bind with ligands.[47] A considerable decrease in the leucogram and concentration of platelets in the intoxicated animals was observed when compared with the treatment groups. This may be because of thrombocytopenia after lead intoxication, followed by thrombocytosis[48,49] [Table 2]. This further results in decreased efficiency of clotting factors leading to an increase in bleeding ($P < 0.001$), clotting time ($P < 0.001$), and prothrombin time ($P < 0.001$) in groups exposed to lead acetate.[50] This may be due to injury of hepatocytes responsible for Prothrombin synthesis which in turn decreases thrombin synthesis in blood plasma which delays blood clotting as reflected in our study as explained in Figure 3.[51] The groups treated with EDTA, chitosan, and chitosamine showed an increase in the levels of clotting proteins due to healing of liver cells as a result of chelation. These results illustrate that the combination of natural and synthetic ones showed better protection and is helpful against lead-induced stress which may be due to the synergistic action together enhancing the chelation potency.

The outcomes obtained from the liver function test revealed that the groups receiving lead acetate have elevated levels of serum enzymes and total bilirubin which were reduced to normal limits in EDTA-, chitosan-, and chitosamine-treated groups. This suggested that the lead destroyed hepatocytes which elevated the levels of serum enzyme.[52] In liver injury, the hepatocyte's transport mechanism gets disrupted, resulting in the plasma membrane leakage, hence causing an amplified enzyme level in serum.[53] These enzymes can be discharged into the blood flow due to the incidence of necrosis or cell membrane damage.[54]

It was also discovered in our results that the chitosan-treated group showed better tolerance against lead toxicity than chitosamine when all the above parameters were examined. The chitosan (polymer) is trivalent, whereas the chitosamine (monomer) is pentavalent but their spatial configuration allows maximum exposure and thus more bonding with chitosan than chitosamine due to the least steric hindrance for bond formation with ligand (lead) as confirmed by the chemistry of both,[55] which may be the reason of difference of their chelating potency.

The findings of the study also reflected that the results obtained from combination of natural and synthetic chelators were more statistically significant than respective

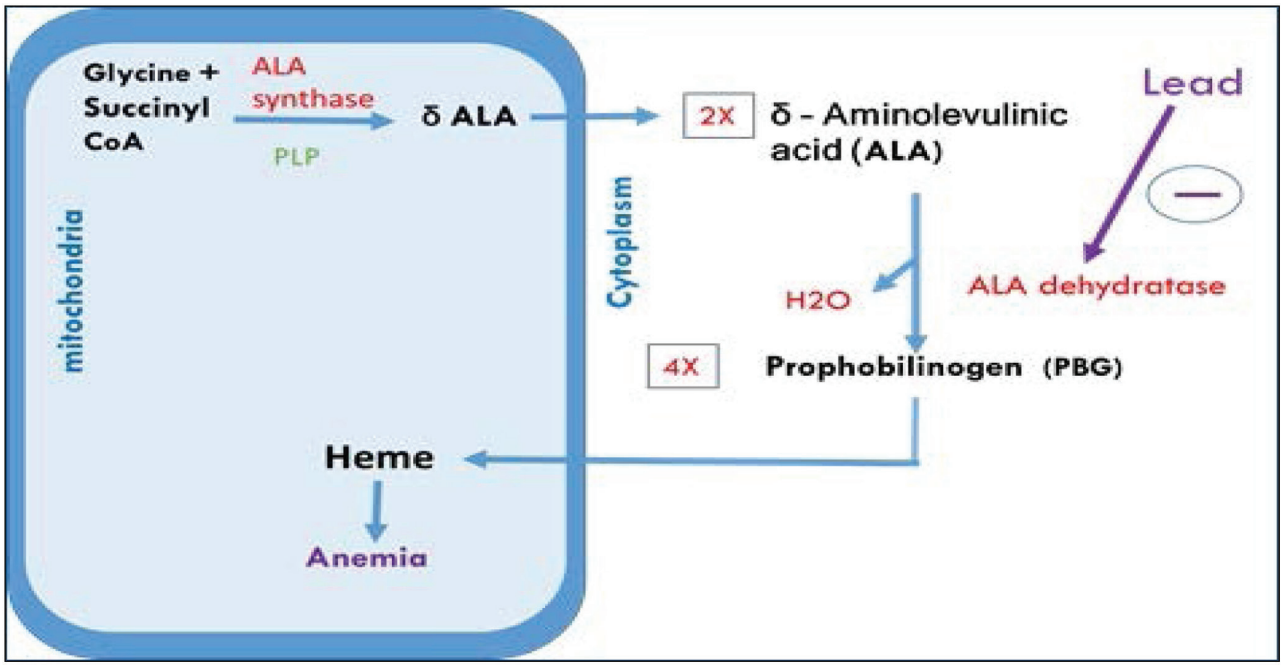

Figure 2: The effect of lead on heme synthesis causing anemia

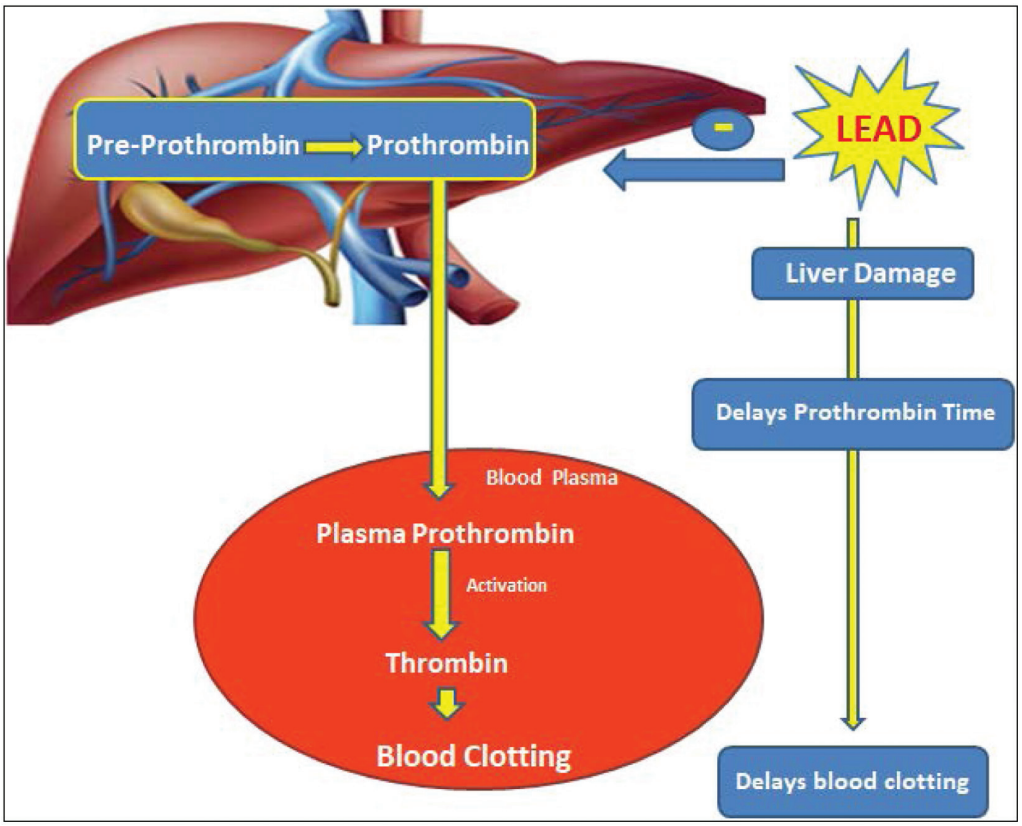

Figure 3: Effect of lead metal exposure on the liver causing delayed clotting of blood

monotherapy with respect to hemogram parameters, liver function tests, and hepatocyte oxidative stress studies.[56] Tukey Kramer's multiple comparison tests highlighted the comparative results of combination of EDTA+Chitosan to be more pronounced than EDTA+Chitosamine with respect to chitosan and chitosamine alone. Our results are concordant with previous studies reflecting that the combination of chelators along with antioxidants enhanced

the detoxification process.[57] The significance of combination of synthetic with natural chelators is an attempt to decrease the therapeutic dose of synthetic chelators which in turn will decrease its adverse effects and this grouping will also synergize the mechanism of detoxification as confirmed by previous researchers.[58]

Conclusion

The chelation properties of natural chelators, viz., chitosan and chitosamine, are mainly due to their ability to claw and complex with lead ions to eradicate them out of the body harmlessly. This process of detoxification not only cleanses the body but also enhances the overall immune system to fight various ailments. The study thus accentuates the prophylactic use of these chelators over synthetic ones as these are completely safe and of natural origin and has enormous different advantages as its being from marine source. Our study confirmed the chelating potency of natural as well as synthetic ones to be comparable and thus requests to encourage the practice of natural chelators in day-to-day life as a measure of detoxification. The findings also revealed that the combination of natural and synthetic chelators showed more significant results when compared with their respective monotherapy, as a function of their potentiation effect and also may be helpful in reduction of the dose and thus the adverse effects of synthetic chelators when used in combination with the natural chelators.

Acknowledgement

The authors are grateful to Y.B. Chavan College of Pharmacy, Dr. Rafiq Zakaria Campus, Aurangabad for providing their provision and support as a research center.

Financial support and sponsorship

The study is self-funded and is not associated with any funding sources.

Conflicts of interest

There are no conflicts of interest.

Ethical approval

CPCSEA/lAEC Approval-"CPCSEA/lAEC/P" Cology-58/2017-l8/l39.

References

1. Dapul H, Laraque D. Lead poisoning in children. Adv Pediatr 2014;61:313-33.
2. Mansouri MT, Cauli O. Motor alterations induced by chronic lead exposure. Environ Toxicol Pharmacol 2009;27:307-13.
3. Abdul LW, Anjum A, Jawed AU. Lead toxicity: A review. Interdiscip Toxicol 2015;8:55-64.
4. Stanley JS. Pediatric Clinical Advisor (Second Edition). Instant Diagnosis and Treatment. Mosby Publishers; 2007. p. 332.
5. Gagan F, Deepesh G, Archana T. Toxicity of lead: A review with recent updates. Interdiscip Toxicol 2012;5:47-58.
6. Ahamed M, Siddiqui MK. Environmental lead toxicity and nutritional factors. Clin Nutr 2007;26:400-8.
7. Vaibhav S, Priyanka S, Avanish T. Lead poisoning. Indian J Med Specialities 2018;9:146-9.
8. Flora SJ, Mittal M, Mehta A. Heavy metal induced oxidative stress and its possible reversal by chelation therapy. Indian J Med Res 2008;128:501-23.
9. Goycoolea FM, Argüelles-Monal W. Chitin and chitosan. Dev Food Sci 2000;41:265-308.
10. El-Sharif AA, Hussain MH. Chitosan-EDTA new combination is a promising candidate for the treatment of bacterial and fungal infections. Curr Microbiol 2011;62:739-45.
11. Andrew BJ, Cliona NM, Derrick AB, Christel AE, Dunshea M, Anthony R. Chitosan for overweight or obesity. Cochrane Systematic Review—Intervention version published: 16 July 2008.
12. Gallaher DD, Gallaher CM, Mahrt GJ, Carr TP, Hollingshead CH, Hesslink R, *et al*. Glucomannan and chitosan fibre supplement decreases plasma cholesterol and increases cholesterol excretion in overweight normocholesterolemic humans. J Am Coll Nutr 2002;21:428-33.
13. Jennings CD, Boleyn K, Bridges SR, Wood PJ, Anderson JW. A comparison of the lipid-lowering and intestinal morphological effects of cholestyramine, chitosan, and oat gum in rats. Proc Soc Exp Biol Med 1988;189:13-20.
14. Velaga MK, Yallapragada PR, Williams D, Rajanna S, Bettaiya R. Hydroalcoholic seed extract of *Coriandrum sativum* (coriander) alleviates lead-induced oxidative stress in different regions of rat brain. Biol Trace Elem Res 2014;159:351-63.
15. Alwaleedi SA. Hematobiochemical changes induced by lead intoxication in male and female albino mice. Natl J Physiol Pharm Pharmacol 2016;6:46-51.
16. Toz H, Deger Y. The effect of chitosan on erythrocyte antioxidant potential of lead toxicity-induced rats. Biol Trace Elements Res 2018;184:114-8.
17. Ibrahim HM, Ali EHA, Sabry HA. Haloperidol-loaded chitosan nanocomposites improve liver and kidney functions and lipid profile of male rats. Res J Pharmaceut Biol Chem Sci 2017;8:1135.
18. Quadri NN, Syed AA, Subur K. Metal chelating activity of glycine max seed extract on ferrous + doxorubicin-induced cardiotoxicity in rats. Ann Exp Biol 2014;2:43-8.
19. Fritsma GA. In: Rodak B, editor. Evaluation of Hemostasis. Hematology: Clinical Principles and Applications. Philadelphia: W.B. Saunders Company; 2002. p. 719-53.
20. Houdijk WP, Van Den Besselaar AM. International Multicenter International Sensitivity Index (Isi) calibration of a new human tissue factor thromboplastin reagent derived from cultured human cells. J Thromb Haemost 2004;2:266-70.
21. Lehman CM, Blaylock RC, Alexander DP, Rodgers GM. Discontinuation of the bleeding time test without detectable adverse clinical impact. Clin Chem 2001;47:1204-11.
22. Peterson P, Hayes TE, Arkin CF, Bovill EG, Fairweather RB, Rock Jr, *et al*. The preoperative bleeding time test lacks clinical benefit: College of American Pathologists' and American Society of Clinical Pathologists' position article. Arch Surg 1998;133:134-9.
23. Dayyal DG. Bleeding time (BT) and clotting time (CT). BioScience Pakistan 2016; ISSN 2521-5760.
24. Lindsay RH, Sedlak J. Estimation of total, protein bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968;25:192-205.

25. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70-7.
26. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
27. Clairborne A. Catalase activity. In: Greenwald RA, editor. Handbook of Methods for Oxygen Radical Research. Boca Raton: CRC Press; 1985. p. 283-84.
28. Marklund SL. Pyrogallol autooxidation. In: Greenwald RA, editor. Handbook of Methods for Oxygen Radical Research. Boca Raton: CRC Press; 1985. p. 243-47.
29. Berlin A, Schaller KH. European standardized method for the determination of delta amino levulinic acid dehydratase activity in blood. Zeitschrift für Klinische Chemie Klinische Biochemie 1974;12:389-90.
30. Kruijswijk H. Clinical Chemistry, Principle and Techics. 2nd ed. Hagerstown, MD: Harper and Row; 1974. p. 819-31.
31. Lin LM, Chen YK. Creatine kinase isoenzymes activity in serum and buccal pouch tissue of hamsters during DMBA induced squamous cell carcinogenesis. J Oral Pathol Med 1991;20: 479-85.
32. Belur B, Kandaswamy N. Laboratory techniques in histopathology. In: Mukherjee K, editor. Medical Laboratory Technology: A Procedure for Routine Diagnostic Tests. Delhi. Tata McGraw Hill Publishing Company Ltd; 1990. p. 1124-90.
33. Smith A, Burton J. A color atlas of histological staining techniques. In: Smith A, Burton J, editors. London: Wolfe Medical Publications; 1977. p. 177-8. Available from: https://lib.ugent.be/catalog/rug01:000186281. [Last accessed on 2018 Oct 18].
34. Naghmeh S, Behnam K, Moore F, Tavakol T, Lahijanzadeh AR, Jaafarzadeh N. Ecological and human health hazards of heavy metals and polycyclic aromatic hydrocarbons (PAHs) in road dust of Isfahan metropolis, Iran. Sci Total Environ 2015;505:712-23.
35. Muataz AA, Yun J, Viktor K. Metals in the environment: Toxic metals removal. Bioinorg Chem Appl 2017; 2017:2.
36. Rebeca CG, Wayne RS. Lead toxicity and chelation therapy. Am J Health Syst Pharm 2007;64:45-53.
37. Thuppil V, Tannir S. Treating lead toxicity: Possibilities beyond synthetic chelation. JKIMSU 2013;2:4-31.
38. Swaran JS. Flora and VidhuPachauri. Chelation in metal intoxication. Int J Environ Res Public Health 2010;7:2745-88.
39. Carles G, Dabiri C, Mchirgui A, Saoudi EO, Hcini N, Pouget K, *et al*. Uses of chitosan for treating different forms of serious obstetrics hemorrhages. J Gynecol Obstet Hum Reprod 2017;46:693-5.
40. Elieh-Ali-Komi D, Hamblin MR. Chitin and chitosan: Production and application of versatile biomedical nanomaterials. Int J Adv Res (Indore) 2016;4:411-27.
41. Subhapradha N, Suman S, Ramasamy P, Ramachandran S, Shanmugam V, Srinivasan A, Shanmugam A. Anticoagulant and antioxidant activity of sulfated chitosan from the shell of donacid clam *Donax scortum* (Linnaeus, 1758). J Nutr Pharmacol Neurol Dis 2013;3:39-45.
42. Koide SS. Chitin-chitosan: Properties, benefits and risks. Nutr Res 1998;18:1091-101.
43. White LD, Cory-Slechta DA, Gilbert ME, Tiffany-Castiglioni E, Zawia NH, Virgolini M, *et al*. New and evolving concepts in the neurotoxicology of lead. Toxicol Appl Pharmacol 2007;225:1-27.
44. Needleman H. Lead poisoning. Annu Rev Med 2004;55:209-22.
45. Patrick L. Lead toxicity, a review of the literature. Part 1: Exposure, evaluation, and treatment. Altern Med Rev 2006;11:2-22.
46. Klaassen CD. Casarett and Doull's Toxicology: The Basic Science of Poisons, 6th ed. New York: McGraw-Hill Medical Publishing Division; 2001. p. 812-41.
47. Mourya VK, Inamdar NN. Chitosan-modifications and applications: Opportunities galore. React Funct Polym 2008;68:1013-51.
48. Sudakova AI, Shevchenko ZhT, Nosova LI. [Peripheral blood and bone marrow cell status of white rats with long-term lead exposure]. Tsitol Genet 1983;17:3-7.
49. Yagminas AP, Franklin CA, Villeneuve DC, Gilman AP, Little PB, Valli VE. Subchronic oral toxicity of triethyl lead in the male weanling rat. Clin Biochem Hematol Histopathol Effects Fundam Appl Toxicol 1990;15:580-96.
50. Robert A, Chazouillères O. Prothrombin time in liver failure: Time, ratio, activity percentage, or international normalized ratio? Hepatology 1996;24:1392-4.
51. Tripodi A, Caldwell SH, Hoffman M, Trotter JF, Sanyal AJ. Review article: The prothrombin time test as a measure of bleeding risk and prognosis in liver disease. Aliment Pharmacol Ther 2007;26:141-8.
52. Alshawsh MA, Abdulla MA, Ismail S, Amin ZA. Hepatoprotective effects of *Orthosiphon stamineus* extract on thioacetamide-induced liver cirrhosis in rats. Evid Based Complement Alternat Med 2011;2011:103-39.
53. Cichoż-Lach H, Michalak A. Oxidative stress as a crucial factor in liver diseases. World J Gastroenterol 2014;20:8082-91.
54. Salahshoor MR, Mohammadi MM, Roshankhah S, Najari N, Jalili C. Effect of *Falcaria vulgaris* on oxidative damage of liver in diabetic rats. J Diabetes Metab Disord 2019;18:15-23.
55. Kumar MNV, Muzzarelli RAA, Muzzarelli C, Sashiwa H, Domb AJ. Chitosan chemistry and pharmaceutical perspectives. Chem Rev 2004;104:6017-84.
56. Hatcher HC, Singh RN, Torti FM, Torti SV. Synthetic and natural iron chelators: Therapeutic potential and clinical use. Future Med Chem 2009;1:1643-70.
57. Tsang MM, Trombetta LD. The protective role of chelators and antioxidants on mancozeb-induced toxicity in rat hippocampal astrocytes. Toxicol Ind Health 2007;23:459-70.
58. Govarthanan M, Kamala-Kannan S, Kim SA, Seo YS, Park JH, Oh BT. Synergistic effect of chelators and *Herbaspirillum* sp. GW103 on lead phytoextraction and its induced oxidative stress in Zea mays. Arch Microbiol 2016;198:737-42.