Original Article

Amelioration of Hepato-renal Impairment by Natural Chelators in Lead-induced Poisoning in Rats

Quadri Neha Nausheen[1,*], Syed Ayaz Ali[1], Shoaeb Mohammad Syed[2], Tarannum Rashid Patave[3]

[1]Department of Pharmacology, Y.B Chavan College of Pharmacy, Aurangabad, Maharashtra, INDIA.
[2]Department of Pharmaceutics, Dr. Ved Prakash Patil Pharmacy College, Aurangabad, Maharashtra, INDIA.
[3]Department of Pharmacology, Loknete Dr. J. D. Pawar College of Pharmacy, Manur. Nashik. Maharashtra. INDIA.

ABSTRACT

Background: Hepato-Renal impairment refers to renal dysfunction in a liver compromised state concerning lead metal exposure. Natural chelators (marine source) have potent chelating properties claiming to ameliorate hepato-renal dysfunction in heavy metal toxicity **Material and Methods:** A total of 42 male albino Wistar rats weighing between 200 to 250 g were categorised into seven groups ($n=6$). Except for the first group (control), which received sodium-acetate (1,000 mg/L in drinking water), all of the groups received lead acetate 0.4 mg/kg body weight per oral (p.o). Second-group is the negative control group (toxic), the third and fourth received Chitosan and Chitosamine 0.2 g/kg (p.o) respectively. Ethylene diaminetetra acetic acid (EDTA) 495 mg/kg (p.o) was given to the fifth, sixth, and seventh groups, whereas Chitosan and Chitosamine [0.2 g/kg (p.o)] were given to the sixth and seventh groups, respectively. **Results:** There is statistical significant increase in atherogenic indices, serum lipid profile, renal tissue oxidative-stress, renal function biomarkers, kidney weights, and decrease in body weights of experimental animals in the toxic as compared to control whereas these values ameliorated in treatment groups as compared to toxic group. Histopathology of toxic group kidneys revealed histologic and pathological changes in nephrons along with dyslipidemia which healed to normal architecture and analytical values in treatment groups. Thus, the study confirms the nephro protective effect and improvement of dyslipidemia as a consequence of hepato-renal impairment by natural chelators. **Conclusion:** The natural chelators have hepatic and nephro protective effect in lead metal induced poisoning.

Keywords: Lead Toxicity, Nephrotoxicity, Chitosan, Chitosamine, Chelation, Atherogenic indices, Oxidative Stress.

INTRODUCTION

Plumbism, or lead (Pb) toxicity, was recognized as early as 370 BCE when Hippocrates coined the term "lead colic".[1] Human well-being is known to be affected predominantly by lead and its compounds as these are widely distributed in nature and rapidly accumulate in the liver, kidney, and other human organs after intestinal absorption.[2] Lead has an impact on three major organ systems that are the central and peripheral nervous systems, the heme biosynthetic pathway; and the renal system. It is one of the poisonous metals in the environment having a deleterious impact on most organs of the human body viz. physiological, biochemical, neurological, behavioural, impairment of renal system functions and reproductive systems.[3] Lead can translocate through the food chain and cause harmful effects on humans and other living organisms.[4] Lead targets the body mainly through three main routes viz. digestive, respiratory tracts and skin which further causes deleterious effects on several organ systems, but those in the kidney are the most steadily. Acute lead nephropathy is characterized by proximal tubular dysfunction with the distinct syndrome known as Fanconi-type alongside alterations in mitochondrial structure, and the advancement in inclusion bodies (cytosolic and nuclear). Metallothionein, a specific

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Correspondence:
Dr. Quadri Neha Nausheen
Y.B Chavan College of Pharmacy, Pharmacology, Aurangabad-431001, Maharashtra, INDIA.
E-mail: nehakmirza@gmail.com

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protein has a high affinity for lead ions which may further proceed to an unalterable form of nephropathy leading to interstitial fibrosis, both hyperplasia and atrophy of the tubules, glomerulonephritis, and renal failure. The consequences of lead ions on the renal system occur in 3 stages viz. Stage I refers to acute or reversible nephropathy; Stage II refers to chronic nephropathy which is not reversible and Stage III characterized by renal tubular-cell neoplasia or adenocarcinoma. Complications of lead nephropathy include gout and hypertension.[5] Long term low-level acquaintance to lead is associated with a high amount of low molecular weight proteins and lysosomal enzymes via urinary excretion. The link between renal impairment and the risk of future development of chronic renal disease remains indeterminate. Previous Epidemiologic data revealed a relationship between blood lead levels and blood pressure, and hypertension is a cardinal sign of lead nephropathy to identify lead nephropathy. Chronic renal and liver damage may be due to ionized form of lead ions causing acute toxicity or bounded form to metallothionein being equipotent to cause damage. HRS is defined as a definite type of functional Hepato-renal impairment which complicates with advanced liver diseases (acute liver failure, alcoholic hepatitis, etc.). Therefore, this specific type of physiologic renal impairment observed in advanced liver dysfunction needs to be differentiated by a variety of non-functional causes of renal failure in this regard, e.g., causes of pre-renal azotemia or acute tubular necrosis. The discrepancies observed between HRS and other etiological considerations of renal failure occurring in cirrhosis are mainly due to the unavailability of a specific diagnostic test. Hence, the diagnosis of Hepatorenal impairment is currently based on the elimination of other disarrays that may lead to renal failure in cirrhosis counting shock (septic or hypovolemic), ongoing septicemia, dehydration, and recent treatment with nephrotoxic drugs.[6] Treatment regimens include chelation therapy, supportive care, decontamination procedures, and renal replacement therapies out of which the most effective intervention for lead toxicity is early identification and its removal from the source to prevent further exposure.[7] Chelation therapy effectively lowers high blood lead levels using synthetic chelators viz.

Dimercaprol, edetate calcium disodium (Calcium EDTA) and succimer but with a potential risk of adverse drug events.[8] Penicillamine is less commonly used due to the increased risk of interstitial cells nephritis in adults that hardly appears in children at a dosage of 20 mg/kg/day. Chelation leads to a rapid fall in blood lead levels within the first few days of therapy. It is recommended to recheck blood lead levels one to three weeks after chelation, as they may rebound due to the release of lead from storage sites. Rebound levels may also indicate continued exposure and additional investigation.[9] Therefore, the search for natural chelating compounds with low or minimum adverse effects, to abbreviate lead toxicity, is a pre-requisite for prophylaxis and is warranted.[10] Researchers showed that supplementation of antioxidants along with a chelating agent proves to be a better treatment regimen than monotherapy with chelators concerning oxidative stress.[11] The idea of the usage of Chitin and Chitosan in diet and other specialized applications is a good example of organic solid waste management and their by-products produced by the food industry itself to obtain added-value products.[12] Thus, the chelating agents continue to be the mainstay of treatment for lead poisoning which forms complexes with lead, prevents its binding to cell constituents, and makes it more hydrophilic to eradicate it outside the body harmlessly through the urine.[13] Previous studies revealed that Chitosan has a lipid-lowering effect and has clinical use in obesity management along with lowering liver total and LDL cholesterol in a dose as low as 1.2 g per day. Being from 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. It is also used to treat side effects caused by dialysis in patients with kidney failure, including high cholesterol, "tired blood" (anaemia), loss of strength and appetite, high phosphorous levels (hyperphosphatemia), and trouble sleeping (insomnia) along with antifungal, anti-parasitic and antibacterial effect. Thus, the purpose of the study is to determine the nephrotoxic effects and liver dysfunction as a consequence of Hepato-renal impairment by lead poisoning, ascertain the measures required for their amelioration using natural chelators and analyze their physiologic rebounding occurs if any by use of a combination of synthetic and natural chelating agents as their synergistic effect along with their comparison.

MATERIALS AND METHODS

Animals

In this study male albino Wistar rats weighing between 200-250 g, were used. The animals were maintained at 23 ± 2°C temperature with open access to standard rat feed and water. A (12-12 hr) light cycle was maintained in the animal house. The care and use of experimental animals were performed following the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, New Delhi, India,

and were permitted by the Institutional Animal Ethical Committee of Y.B. Chavan College of Pharmacy, Aurangabad having the approval number (CPCSEA/ IAEC/ P'col-58/2017-18/139).

Chemicals and Reagents

Chitosan and Chitosamine were purchased from Thomas bakers chemicals Pvt. limited, Mumbai, India, and S.D Fine–Chem Limited, Mumbai, India, respectively. The Blood urea nitrogen (BUN), Creatinine, uric acid, serum urea, and serum albumin detection kits were purchased from Biosystems, Mumbai, India. Serum lipid profile (Triglycerides, HDL, LDL, VLDL) detection kits were obtained from Sigma Aldrich, Pune. The solvents and chemicals employed were of analytical grade and chemicals necessary for sensitive biochemical assays were purchased from Merck. All drug solutions were freshly prepared in distilled water for daily dosing.

Experimental design

The animals were divided into seven groups (*n*=6) at random. The treatment was extended for an additional 24 weeks (6 Months).

Experimental Design Protocol

Table 1 shows experimental design protocol, where the animals were divided as per the study requirements. At the end of the experimental period, animals were fasted overnight, anesthetized using a carbon dioxide chamber and their blood samples were collected in test tubes. All serum specimens were separated and stored at 8°C until they were analyzed.

Biochemical Analysis

Preparation of serum: blood was collected from the retro-orbital plexus from the inner canthus of the eye (under light CO_2 anaesthesia) using glass capillary tubes. Serum was separated using an R-24 research centrifuge (Remi Instruments Ltd., Mumbai) at 3000 rpm for 15 min.[14] The Blood urea nitrogen (BUN),[15] Serum Creatinine,[16] Serum uric acid, Serum Urea, and Serum Albumin were estimated using respective detection kits on a biochemical autoanalyzer (Preitest, Robonik) following the manufacturer's instructions. The serum lipid profile parameters were assessed using Sigma Aldrich detection kits following the user manual by the Coulometric Method of Analysis. The atherogenic indices were calculated using the following formulae: Cardiac risk ratio (CRR) = Total cholesterol / HDL Cholesterol, Atherogenic coefficient (AC) = Total cholesterol- HDL Cholesterol / HDL Cholesterol, Atherogenic index of plasma = Triglycerides / HDL Cholesterol.[17]

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; 1000mg/L drinking water).	✓						
Lead Acetate (0.4 mg/kg body wt.)		✓	✓	✓	✓	✓	✓
Chitosan (0.2 g/ kg body wt.)			✓			✓	
Chitosamine (0.2 g/kg body wt.)				✓			✓
EDTA (495 mg /kg body wt.)					✓	✓	✓

Preparation of Tissue Homogenate

The animals were sacrificed using a CO_2 anaesthetic chamber to render them unconscious (Euthanasia) followed by cutting of the carotid artery. The kidneys were quickly removed, rinsed in ice-cold saline, dried on a filter paper, and weighed. A 10% homogenate was prepared in 0.15 M Potassium Chloride (KCl) for the estimation of tissue malondialdehyde and the homogenate for the tissue glutathione was prepared in 0.02 M EDTA (Figure 1).

Estimation of Tissue Glutathione

A known weight of tissue ranging from (100-150 mg) was homogenized in 5 ml of EDTA (0.02 M) and then, 4 ml of cold distilled water was added to it. After mixing 1ml of TCA (50%) was added and shaken intermittently for 10 min using vortex mixer. After 10 min the content was transferred to centrifuge tubes (rinsed in EDTA) and centrifuged at 6,000 r.p.m for15 min at 4°C. After centrifugation, 2 ml of supernatant was mixed with 4 ml of tris buffer (0.4 M, pH 8.9). After mixing the entire solution, 0.1ml of DTNB (0.01 M) was added. The absorbance was read within 5 min of addition of DTNB at 412 nm against the appropriate blank.[18]

Figure 1: Kidney Tissue Oxidative Stress Parameters study procedure.

Measurement of tissue MDA

Measurement of lipid peroxidation is carried out by determination of kidney malondialdehyde content by the thiobarbituric acid (TBA) method. 10% kidney homogenate was prepared in buffered 0.9% KCl pH 7.4 for the estimation of tissue MDA. To 1 ml of homogenate, 0.5 ml of trichloroacetic acid (30%) and 0.5 ml of thiobarbituric acid (0.8%) were added and shaken for 5 min. The tubes were then subjected to heating on the water bath at 80°C for 30 min followed by cooling in ice-cold water for 10 min and centrifugation at 5,000 r.p.m for 15 min. The clear supernatant was separated and absorbance was measured at 540 nm using an appropriate blank.[19]

Preparation of Post Mitochondrial Supernatant (PMS)

Using a Remi homogenizer, the tissues were homogenised in cold potassium phosphate buffer (50mM, pH 7.4). The homogenate was centrifuged in a refrigerated centrifuge at (10,500 rpm) for 20 min at 4°C to obtain the PMS, which was used for various biochemical analyses. The post mitochondrial supernatant (PMS) was used for the estimation of antioxidant enzymes such as Catalase and Superoxide Dismutase.

Assessment of Catalase (CAT)

The cytosolic supernatant, (50µl) 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 change in absorbance was read at 240 nm on the Shimadzu spectrophotometer at 1 min interval for 3 min.[20]

Estimation of Superoxide Dismutase (SOD) Assay

Following the suppression of pyrogallol auto-oxidation, the supernatant was tested for superoxide dismutase (SOD) activity. 100 ml of cytosolic supernatant was added to Tris-HCl buffer, pH 8.5. The final volume of 3 ml was adjusted with the same buffer. At last 25 ml of pyrogallol was added and changes in absorbance at 420 nm were recorded at the 1-min interval for 3 min. The increase in absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD.[21]

Estimation of Body and Organs Weight

In each group, the body weight of rats was taken before and after treatment. Isolated kidneys were weighed after keeping them in ice-cold saline and squeezing out the blood.

Histopathological Studies

The kidney was fixed in 10% formalin. The specimens were then processed for the standard procedure and were embedded in paraffin wax. The blocks were then sectioned according to the hematoxylin and eosin methods. The sections were examined under the light microscope and photographs were taken under 10X.[22]

Statistics Analysis

The mean ± SEM values were calculated for each group. One-way ANOVA followed by Tukey's tests was used for statistical analysis. Values of $p<0.05$ were considered statistically significant. The entire statistical analysis was performed using the statistical package, Graph Pad Instat Version 8.0 (Graph Pad Software Inc., USA) software at a level of significance of $p < 0.01$, 0.05, and 0.1.

RESULTS

Biochemical Parameters (Kidney Function Test)

The effect of natural chelators from marine sources (Chitosan and Chitosamine), the synthetic chelator, and their combinations on lead exposure were studied concerning kidney function tests presented in Table 2. The results revealed a highly significant increase in BUN ($p<0.001$), Serum Creatinine ($p<0.001$), Serum Urea ($p<0.001$), Serum uric acid ($p<0.001$), and Serum Albumin ($p<0.001$) in a toxic group as compared to the control group whereas these parameters found to be decreased statistically in treatment groups as compared to the toxic group.

Kidney Function Test parameters

The data is indicated as Mean ± SEM. No. of samples (n) =6. a $p<0.001$, b $p<0.01$, c $p<0.05$ as compared to control group and d $p<0.001$, e $p<0.01$, f $p<0.05$ as compared to lead (toxic control) treated group while ns represents non-significant data* $p<0.5$ as compared to Chitosan group, ## $p<0.01$ as compared to Chitosamine group.

Serum Lipid Profile

Table 3 shows the serum lipid profile data viz. Triglycerides, Total Cholesterol, HDL, LDL and VLDL. The findings revealed that the toxic group showed increased values of triglycerides $p<0.001$, LDL $p<0.001$, VLDL $p<0.001$, Total Cholesterol $p<0.001$, and decrease in HDL $p<0.001$ values as compared to the control group whereas these values found to decrease significantly in treatment groups as compared to the toxic group.

Table 2: The effect of Chitosan, Chitosamine, and EDTA on Biochemical Parameters (Kidney Function Test) in lead-induced toxicity in rats after 24 weeks of treatment.

Parameters (mg/dL)	Ctrl	Pb	Cx	Cn	EDTA	EDTA+ Cx	EDTA+ Cn
BUN	16.8±1.2	38.8±4.2^{a}	19.2±1.0^{d}	20.5±0.99^{d}	18.8±2.1^{d}	17.3±1.2^{d,*}	18.8±1.08$^{d,\#}$
Serum Creatinine	0.5±0.13	6.52±0.48^{a}	1.3±0.25^{d}	1.41±0.26^{d}	0.92±0.2^{d}	0.39±0.3^{d,**}	0.48±0.25$^{d,\#\#}$
Serum Uric Acid	2.12±0.39	6.12±0.46^{a}	2.86±0.22^{d}	2.98±1.13^{d}	2.39±0.11^{d}	2.46±0.66^{d,*}	2.77±0.32$^{d,\#}$
Serum Urea	41.42±1.4	92.1±1.12^{a}	63.2±0.54^{d}	66.6±0.28^{d}	73.8±0.16^{d}	57.7±1.08^{d,*}	52.2±2.11$^{e,\#\#}$
Serum Albumin (g/dL)	3.82 ± 0.25	0.23±0.56^{a}	4.39±0.21^{d}	4.47±0.4^{d}	4.16±0.22^{d}	4.66±0.6^{d,*}	4.72±0.71$^{d,\#}$

Table 3: The effects of Chitosan, Chitosamine and EDTA on serum lipid profile data in lead induced toxicity in rats after 24 weeks of treatment.

Parameters (mg/dL)	Ctrl	Pb	Cx	Cn	EDTA	EDTA+Cx	EDTA+ Cn
TG	130.2 ± 3.1	198.7 ±3.3^{a}	142.2±7.02^{d}	156±4.38^{e}	98.13±2.38^{d}	123.21± 1.82^{d,**}	131.7±0.48$^{d,\#\#}$
TC	118.8±4.2	199.8±2.6^{a}	129.4±7.1^{d}	134±4.2^{d}	113.99± 2.81^{d}	118.4± 6.3^{d,**}	121.3±3.5$^{d,\#\#}$
HDL	36.82±3.09	8.38±5.4^{a}	41.38±2.1^{d}	32.3±2.1^{d}	49.14±1.82^{d}	49.9±4.6^{d,**}	38.6±3.8$^{d,\#\#}$
LDL	46.84±2.9	112.2±4.4^{a}	66.2±.06^{d}	72.4±9.3^{d}	51.26±1.88^{d}	54.21±5.8^{d,**}	63.4±3.9$^{d,\#\#}$
VLDL	18.46±2.5	55.66±1.06a	21.09± 3.2^{d}	24.8±8.7^{d}	18.3±3.9^{d}	16.8±0.6^{d,**}	19.36±2.1$^{d,\#\#}$

Table 4: The effects of Chitosan, Chitosamine, and EDTA on Atherogenic indices in lead-induced toxicity in rats after 24 weeks of treatment.

Parameters	Ctrl	Pb	Cx	Cn	EDTA	EDTA+Cx	EDTA+Cn
Cardiac Risk Ratio	3.23±0.97	18.84±3.91^{a}	3.13±0.84^{d}	4.15 ± 1.1^{d}	2.32±1.6^{d}	2.97± 1.1^{d*}	3.51± 1.6$^{d\,\#}$
Atherogenic coefficient	2.23±0.6	19.84±4.2^{a}	2.13±0.4^{d}	3.15±0.7^{d}	1.32±0.4^{d}	1.97±0.33^{d*}	2.51±1.1$^{d\,\#}$
Atherogenic index	0.55±0.22	1.30±0.56^{a}	0.54±0.39^{d}	0.68±0.54^{e}	0.30±0.12^{d}	0.49±0.29^{d*}	0.58±0.9$^{e\,\#}$

Serum Lipid Profile Data

TC=Total Cholesterol, HDL=High-density lipoproteins, LDL= Low-density lipoproteins, VLDL=Very low-density lipoproteins. The data is indicated as Mean ± SEM. No. of samples (n) =6. a $p<0.001$, b $p<0.01$, c $p<0.05$ as compared to control group and d $p<0.001$, e $p<0.01$, f $p<0.05$ as compared to lead (toxic control) treated group while ns represents non-significant data* $p<0.5$ as compared to Chitosan group, # # $p<0.01$ as compared to Chitosamine group.

Atherogenic Indices

Table 4 shows the data of results obtained from the Atherogenic indices (Cardiacriskratio, Atherogenic coefficient, Atherogenic index of plasma) of experimental animals of each group. The findings revealed that the cardiac risk ratio was higher in the toxic group as compared to the control ($p<0.01$) group. The Atherogenic coefficient and Atherogenic index of plasma were found to be on the higher side in the toxic group indicating dyslipidemic behaviour of lipid metabolism in comparison with the control ($p<0.01$) group whereas these numbers were found to be decreased in treatment ($p<0.01$) groups as compared to the toxic group.

Atherogenic İndices

The data is indicated as Mean ± SEM. No. of samples (n) =6. a $p<0.001$, b $p<0.01$, c $p<0.05$ as compared to control group and d $p<0.001$, e $p<0.01$, f $p<0.05$ as compared to lead (toxic control) treated group while ns represents non-significant data* $p<0.5$ as compared to Chitosan group, # # $p<0.01$ as compared to Chitosamine group.

Relative Weight of Kidney

Table 5 shows the data of results obtained from changes in body weight of experimental animals of each group and kidney (left) weight. The findings revealed that the bodyweight of the toxic group is in lower units as compared to the control ($p<0.01$) whereas, there was no significant reduction in body weight of the treatment group concerning the toxic group. The kidney weights of the toxic group showed nephromegaly-like characteristics due to lead toxicity as compared to the control group ($p<0.01$) and treatment groups ($p<0.01$).

Table 5: The effect of Chitosan, Chitosamine, and EDTA on Body and kidney (left) weight in lead-induced toxicity in rats after 24 weeks of treatment.

Parameters	Control	Lead	Chitosan	Chitosamine	EDTA	EDTA+ Chitosan	EDTA+ Chitosamine
Kidney/Body weight (X10-3)	3.4±0.8	9.36±2.3a	4.8±1.9d	5.7±1.5d	4.2±0.7d	4.1±1.1d*	5.4±1.8d#

Table 6: The Effect of Chitosan, Chitosamine, and EDTA on Kidney Tissue Oxidative Stress Parameters in Lead Induced Toxicity in rats after 24 weeks of treatment.

Parameters	Ctrl	Pb	Cx	Cn	EDTA	EDTA+Cx	EDTA+Cn
SOD	32.3±1.17	12.83±2.3a	45.71±3.46d	41.5±3.13d	48.55±3.5d	38.52±3.4d,***	33.17± 2.8d,###
CAT	73.33±7.1	30.3±3.24 a	77.33±6.96 d	73.16±7.19 d	82.67±7.08 d	85.0±5.73 d, **	78.0±7.26 d,#
Tissue GSH	5.77±0.43	1.88±0.27a	5.98±0.35d	5.48±0.24d	6.5±0.36d	6.57±0.29d,***	6.45±0.3d,###
MDA	2.6±0.12	9.88±0.62a	2.9 ± 0.12d	3.62±0.13d	2.1 ±0.88 d	3.86± 0.46d,***	4.92 ± 0.12d,###

Relative weight of Kidney

The data is indicated as Mean ± SEM. No. of samples (n) =6. a $p<0.001$, b $p<0.01$, c $p<0.05$ as compared to control group and d $p<0.001$, e $p<0.01$, f $p<0.05$ as compared to lead (toxic control) treated group while ns represents non-significant data* $p<0.5$ as compared to Chitosan group, ## $p<0.01$ as compared to Chitosamine group.

Oxidative stress

The data revealed in Table 6 showed results of oxidative stress in the toxic group and its comparison with control and treatment groups. The SOD, CAT, and tissue GSH levels were found to be decreased significantly in the toxic group as compared to the control group ($p<0.001$), the levels of which ameliorated in treatment groups in their comparison. The levels of MDA were increased as a result of the high lead blood burden in the toxic group as compared to the control group. The data obtained from the oxidative stress of the toxic control group ameliorated due to the chelation effect of chelators in treatment groups.

Kidney Tissue Oxidative Stress Parameter

SOD = Superoxide Dismutase, CAT = Catalase, GSH= Glutathione, MDA= Malondialdehyde. The data is indicated as Mean ± SEM. No. of samples (n) =6. a $p<0.001$, b $p<0.01$, c $p<0.05$ as compared to control group and d $p<0.001$, e $p<0.01$, f $p<0.05$ as compared to lead (toxic control) treated group while ns represents non-significant data* $p<0.5$ as compared to Chitosan group, ## $p<0.01$ as compared to Chitosamine group.

Serum electrolytes

Figure 2 represents the data of the serum electrolyte of the experimental animals treated for the period of 24 weeks.

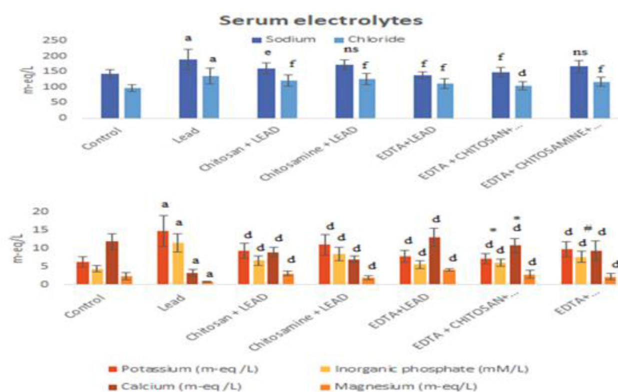

Figure 2: Serum electrolyte concentration of experimental animals.

The data is indicated as Mean ± SEM. No. of samples (n) =6. a $p<0.001$, b $p<0.01$, c $p<0.05$ as compared to control group and d $p<0.001$, e $p<0.01$, f $p<0.05$ as compared to lead (toxic control) treated group while ns represents non-significant data* $p<0.5$ as compared to Chitosan group, # # $p<0.01$ as compared to Chitosamine group.

Histopathological Interpretations

The histopathological findings of the experimental animals were highlighted in Figure 3, where the results reflect the normal parenchymal architecture of the kidney observed in the control group with its respective changes owing to heavy metal (lead) intoxication as perceived in the following slides can be studied.

1A – normal histology of kidney of the control group, 1B- disruption in normal parenchyma of medulla region of kidney of the toxic group receiving merely lead acetate (0.4 mg/kg), 1C- showed protective changes in glomeruli cells and tubules in groups receiving Chitosan (0.2mg/kg), 1D- showed restoration of morphology in

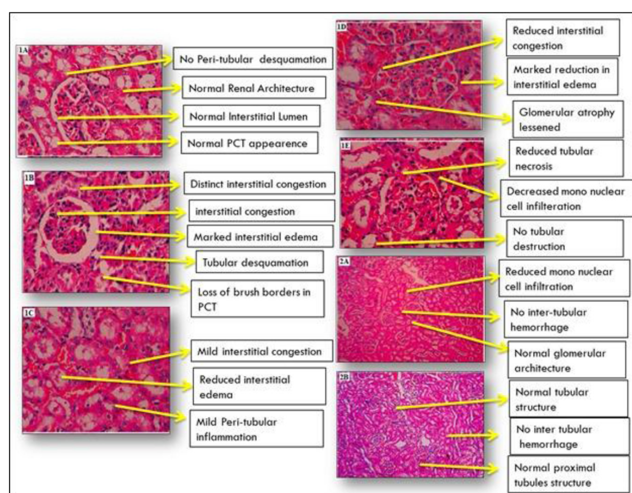

Figure 3: H&E Stained transverse sections of renal tissues (left-kidney) of the groups in the study.

groups receiving Chitosamine (0.2mg/kg), 1E – showed histology of kidney cells of groups receiving EDTA (495 mg/kg) comparable to the parenchyma of control groups, 2A- showed cortex area of kidney cells of the group receiving combination doses EDTA +Chitosan, 2B showed the parenchymal structure of renal cortex of groups receiving combination doses EDTA+ Chitosamine.

DISCUSSION

Biochemical Parameters

Renal system plays a major role in the clearance of xenobiotics while the Hepatic system is mainly concerned with the biotransformation of metal-containing agents and hence, is the primary target organ for metal-induced toxicity. Urea is the primary and Creatinine in the advanced stages of renal damage are considered biomarkers responsible for renal damage as reflected in our study on chronic exposure of lead acetate, structurally characterized by tubular necrosis, interstitial oedema, tubular desquamation, glomerular atrophy, mononuclear cell infiltration, intertubular haemorrhage, and peri-tubular congestion, etc.[23] Lead ions were found to directly inhibit renal tubular reabsorption of sodium ions probably by its action on Na+/K+-ATPase to alter intracellular concentrations of sodium and calcium ions. A change in cellular volume may elevate plasma renin activity and thus causing hypertension. Lead may also affect cytosolic free calcium ions in juxtaglomerular cells which finally alters renal vascular reactivity to adrenergic agents. The data presented in Table 2 showed an increased level of Creatinine, urea, serum uric acid, and decreased levels of serum albumin in animals of the toxic group as compared to the control and treatment groups. An increase in blood burden of lead ions could increase blood urea by more than one mechanism, including enhancement of proteins catabolism, conversion of ammonia to urea by induction of arginase-enzyme synthesis, and inhibition of amino acids incorporation in proteins an indicative of parenchyma tissue injury after tubular necrosis.[24] The hyperuricemia induced in the toxic group might result from over-production and/or reduced renal excretion of uric acid, and elevation of endogenous oxygen species levels. Chelation treatment ameliorated lead-induced nephrotoxicity, as indicated by significant restoration of serum creatinine, urea, and uric acid levels to normal limits relatable to control groups in the treatment groups. These findings are concordant with previous studies of lead-induced toxicity in animals. Moreover, there was a significant decrease in the levels of serum albumin in the groups receiving merely lead acetate as a result of increased urinary excretion of high molecular weight protein viz. albumin indicating glomerular toxicity which was rectified in other treatment groups.[25] This can be explained by two mechanisms i.e. due to loss of charge or size restriction of the glomerular capillary wall there is transglomerular passage of albumin leading to albuminuria or impairment of reabsorption of proximal tubules due to toxic injury by lead ions.[26] Chelation rectified this issue leaving a decreased amount of lead ions to cause toxic effects resulting in increased levels of serum albumin in treatment groups as compared to toxic ones.

Figure 2 represents the serum electrolyte concentration of rats, where the data found to increase in serum potassium, inorganic phosphate, sodium and chloride ions in the toxic group compared to the control group whereas, these levels ameliorated back to normal in treatment groups. The levels of calcium and magnesium were found to be decreased in the toxic group whereas, these levels were rectified in treatment groups. The total sodium ions concentration is the key element for fluid homeostasis and hence is mainly concerned with blood volume depletion and overload. The agents such as heavy metals having an impact on potassium excretion via influence on glomerular filtration loss are the main reason for hyperkalemia as revealed in our study. The disturbance in balanced serum normal electrolyte concentration by heavy metals caused hyponatremia, hypomagnesemia, hypochloremia and hyperkalemia with hyperphosphatemia and hypocalcemia. Our studies are in contrast with other studies stating that mercury heavy metal has an influence on tubular reabsorption which further have an impact on Anti Diuretic Hormone, suppressing it to cause hypervolemia and loss of essential normal electrolytes in urine.[27]

The histopathological examinations confirmed the glomerular necrosis, shrinkage and congestion with the absence of glomerular tuft, dilation of tubular and congestion of peritubular capillaries observed in the toxic group which might be the reason for electrolyte imbalance. These amelioration aspects observed in the treatment group automatically rectified serum electrolyte imbalance confirming chelators possess potent chelating properties as revealed in our findings.

Serum Lipid Profile

Table 3 refers to the data of serum lipid profile of experimental animals where the levels of total cholesterol, triglycerides, LDL, and VLDL were found to be increased significantly in the toxic group as compared to control and other treated groups. The dyslipidemic behaviour in lipid metabolism as a result of hepatotoxicity possibly is owing to either increase in the synthesis or a decrease in the removal of lipoproteins causing the incidence of hepatic hypercholesterolemia involving the activation of biosynthetic cholesterol enzymes viz. squalene synthase, lanosterol 14-demethylase, 3-hydroxy- 3-methyglutaryl-CoA reductase, farnesyl diphosphate synthase, etc. with simultaneous suppression of cholesterol-catabolic enzymes viz. hydroxylase caused by lead poisoning as reflected in our studies.[28] The uneven levels of LDL and VLDL in the toxic control group indicate the accumulation of bad cholesterol as a consequence of liver dysfunction resulting in hypercholesterolemia. The HDL levels on the other side were found to be at lower levels in the negative control group which may increase the chances of ischemic heart diseases. These values were found to resemble the numbers obtained from the control group indicating the reduced blood lead burden in treatment groups, thus ameliorating dyslipidemia[29] Tukey's test allowed us to signify the results showing results from chitosan and chitosamine to be equipotent with synthetic chelators to be used as a corrector in lipoproteins fluctuation disorders.

Atherogenic Indices

It refers to the logarithmically transmuted proportion of molar concentrations of triglycerides to high-density lipoproteins in plasma with HDL values. Dyslipidemia is a precursor mechanism that escalates hyperlipidemia and atherosclerosis triggering cardiovascular diseases viz. coronary and ischemic heart diseases, hypertension, shock and stroke. The results highlighted the increase in total cholesterol along with triglycerides, LDL, and VLDL in the toxic control group which led to the increase in atherogenic indices viz. cardiac risk ratio, atherogenic index and atherogenic co-efficient. The HDL levels in the toxic control group were found to be decreased as compared to the control and treated group due to which there was a decrease in atherogenic indices. This shows that total cholesterol, triglycerides, and uneven levels of lipoproteins were positively co-related with atherogenic indices whereas HDL was negatively correlated with the same.[30] Our findings revealed that chelation rectified the lead ions burden in serum leading to correction in dyslipidemia thus reducing atherogenic indices and cardiac risk ratio in groups treated with chitosan and chitosamine as compared to the toxic group. The findings of our study suggest equal potency of chelation of EDTA and natural chelators to be used in heavy metal poisoning as a detoxifier.

Relative weight of the Kidney

Table 5 shows the bodyweight of toxic group animals which was found to be decreased as compared to the control group, which might be due to a reduction in absorption of nutrients through the Gastrointestinal tract as a result of increased lead ion exposure as discussed earlier.[31] The treatment group animals showed a mild to moderate increase in body weight as compared to toxic animals as a result of the chelation of lead ions. On the other hand, the animals exposed to only lead acetate showed an increase in kidney weight as compared to control and treatment groups. Accumulation of lipids in kidney cells of lead intoxicated rats has been reported previously.[32] which could be an indicator for the increased weight of different organs. An increase in the dry weight of the kidney and liver relative to the body weight was observed, which might be because of nutritional disturbances caused by pair feedings which further showed amelioration in the treatment groups indicating a decrease in lead burden due to the chelation mechanism.[33]

Oxidative Stress Parameters

The oxidative stress parameters viz. SOD, CAT, and tissue GSH were estimated (Table 6) in experimental animals which revealed their significant reduction in the toxic group as compared to control and treatment groups, whereas there were increased levels of MDA observed in the merely lead treated group as compared to control and treatment groups. Lead exposure causes liver damage as confirmed by previous studies, which results in hypoxia causing oxidative stress leading to the formation of ROS (O_2, H_2O_2, and OH-). These migrate to the whole of the system in the body causing their oxidation and ageing. The body's antioxidant defence mechanisms attack this reactive oxygen species to protect from the cytotoxicity of vital organs of the body, which

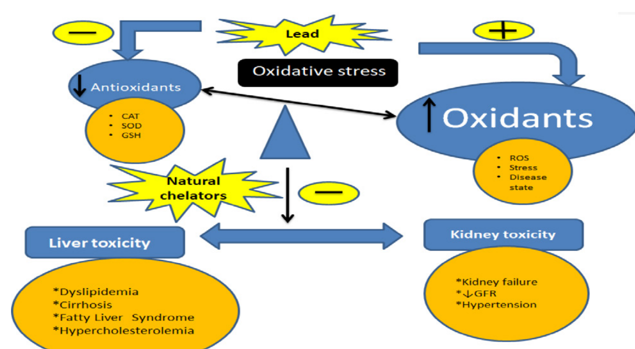

Figure 4: Represents the mechanism of oxidative stress by lead ions and their chelation by natural chelators.

in turn causes their depletion and acts as a prognostic indicator of increased cytotoxicity in renal cells, the level of SOD, CAT, and GSH thus founds to be decreased in toxic group indicating lead poisoning (Figure 4). The amelioration effect can be observed in treatment groups where these levels reverted to normal limits indicating chelation of lead ions detoxifies the lead burden in renal cells which further confirms that the polyphenols viz. Chitosan and Chitosamine are potent chelating agents to overcome renal lead poisoning. The present study also revealed increased MDA levels in a toxic group which may be due to the toxic effect of lead on membrane structure and functions. The uptake of lead ions via the renal brush border does not require any specific carriers. The mechanism of absorption of lead ions may be due to the conjugation of lead ions to a specific surface site present on membranes of brush borders causing its internalization by endocytosis.[34] Hence, altered lipid and protein composition of membranes due to the increased lead burden is accompanied by an increase in the concentration of MDA which results in altered membrane integrity, permeability, and function increasing the susceptibility to lipid peroxidation and generation of free radicals resulting in nephrotoxicity. Scavenging of free radicals and decreasing hydroxyl radical generation can be achieved by the arrest of lead ions by chelating agents reverting the oxidative stress parameters to normal relatable to control groups.

Histopathological Findings

To further confirm the renal injury at the cellular morphological level, histopathological estimations were carried out for each group by staining kidney tissues with H&E stains and studying detailed cellular changes of treatment groups in comparison with the toxic group to ratify the nephron-protective effects of chelators. As shown in Figure 3, the control group showed normal renal parenchymal architecture of the medulla region with no signs of morphological changes concerning any sort of reversible or irreversible cell injury causing damage. The toxic group showed morphological alterations viz. noticeable tubular necrosis, edematous swelling, and destruction of proximal tubular cells with loss of brush borders. Many studies showed a high relation between lead exposure and nephrotoxicity where high lead blood burden causes proximal tubular impairment causing Fanconi syndrome (aminoaciduria, glycosuria, and hyperphosphaturia). In our study, it was found that administration of lead in experimental animals produced changes in the proximal tubular cells indicating its nephrotoxic effects on renal cortical tissue as concordant with previous studies. The study further shows that continuous lead administration with increasing periods leads to a more pronounced and progressive increase in toxic effects on proximal tubular cells, indicated by changes seen in micrometric observations.[35] These changes were prominent in toxic groups which were found to be reversed in treatment groups as a result of chelation. This therapy not only restored normal architecture in treatment groups but also reestablished the altered biochemical data to normal limits confirming natural chelators to be effective in lead poisoning. Moreover, the histopathological changes observed in the natural chelators treated group were more relatable with the control group along with synthetic chelators which confirms the equipotency of natural chelators with synthetic ones.

Natural chelation confirmed a protective effect against respective toxicities in organ systems as results are comparable with the control group. Tukey's multiple comparison test allowed us to study the comparative effect of Natural and Synthetic chelators, Natural chelators (Chitosan and Chitosamine), and Monotherapy (Chitosan and Chitosamine alone) Vs Combination Therapy (EDTA+ Chitosan and EDTA+ Chitosamine), Natural chelators in monotherapy and combination Vs control group.

Findings revealed the results obtained from synthetic and natural chelators showed ameliorative effects compared to the toxic group with minor differences in their chelation potency as compared to each other claiming both to be equipotent chelators. Moreover, the results obtained from Chitosan and Chitosamine monotherapy showed pronounced protection in chitosan compared to Chitosamine treated groups. This may be due to the spatial configuration of Chitosamine with an attached bulkier group creating steric hindrance for binding with heavy metal.

The combination therapy of synthetic and natural chelators is the preferred mode of choice to overcome

the adverse effects of synthetic chelators. The results obtained from EDTA+ Chitosan treated group were found to exhibit more protection against lead toxicity than in EDTA+ Chitosamine treated groups. The reason could be the same justification for spatial configuration of bulkier groups of Chitosamine creating steric hindrance in bond formation with heavy metal as compared to the spatial arrangement of chitosan (polymer) groups. On the other hand, the results obtained from the combination of EDTA+ Chitosan are more protective in comparison with monotherapy of Chitosan alone. This may be due to the synergistic effect of chelation giving added results with more pronounced amelioration. The same can be observed in the combination of EDTA +Chitosamine group results which are more attenuating than Chitosamine monotherapy alone. Thus, the purpose of a combination of synthetic and natural chelators to reduce the deleterious effects of synthetic chelators alone and to boost up detoxification mechanism by the natural source is a good approach in chronic heavy metal poisoning.

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 boosts up the overall immune system to fight various ailments alongside rectifying hepato-renal impairment due to lead toxicity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

Pb: Lead; **EDTA:** Ethylenediamine Tetraacetic Acid; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **GSH:** Glutathione; **MDA:** Malondialdehyde; **H&E:** Hematoxylin And Eosin; **b.w:** Body Weight; **p.o:** Per Oral; **CPCSEA:** Committee For The Purpose Of Control And Supervision Of Experiments On Animals; **ANOVA:** Analysis Of Variance; **ROS:** Reactive Oxygen Species.

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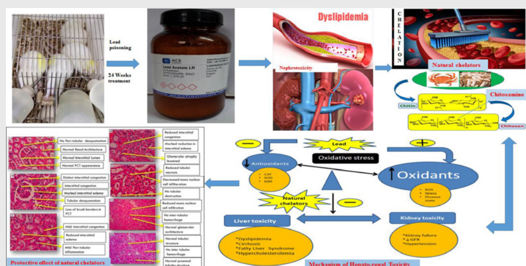
PICTORIAL ABSTRACT



SUMMARY

- Lead has toxic effects on blood, liver, kidney, heart, brain, lungs, gastrointestinal system, spleen, pancreas and Male reproductive system as confirmed by our study.
- Natural chelation confirmed a protective effect against respective toxicities in organ systems as results are comparable with the control group. Tukeys test allowed the study of Natural chelators in monotherapy and combination with synthetic chelators. The results obtained from a combination of EDTA+ Chitosan are more protective in comparison with monotherapy of Chitosan alone. This may be due to the synergistic effect of chelation giving added results with more pronounced amelioration.
- The same can be observed in the combination of EDTA +Chitosamine group results which are more attenuating than Chitosamine monotherapy alone.
- Thus, the purpose of a combination of synthetic and natural chelators to reduce the deleterious effects of synthetic chelators alone and to boost up detoxification mechanism by natural sources is a good approach in chronic heavy metal poisoning.
- Thus, the study displays that the natural chelators are as effective as synthetic ones with no side effects as these are non-Xenobiotic and can be used prophylactically as a detoxifier.

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