Cholestyramine reduces oxalate mediated renal injury and its manifestations in hyperoxaluric rats

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Abstract- Hyperoxaluria leads to extensive damage to the tubules and causes renal inflammation and fibrosis; and it further advances to nephrolithiasis, nephrocalcinosis as well as renal parenchymal disease. Oxalate synthesis takes place in liver and there is no further metabolism of oxalate in humans. A significant share of oxalate load is caused by metabolism of ingested oxalate precursors. The exposure of renal epithelial cells to oxalate causes ROS generation which is predominantly responsible for renal epithelial cell injury. The injury to the kidney tissue paves the way for crystal nucleation, aggregation and consequently stone deposition in the renal tissue. CST, by virtue of its oxalate binding property, decreased oxalate burden to the renal cells leading to amelioration of renal injury and improvement in renal function. Moreover, the role of CST in minimizing hyperoxaluria induced liver injury was established by a decline in the activities of enzymes SGOT, SGPT and GGT, which were otherwise enhanced by EG and ammonium chloride administration. Our study confirms that in hyperoxaluric animals, CST treatment reduced renal injury and nephrocalcinosis, improved renal function, in renal tissue.

Keywords- Hyperoxaluria; Cholestyramine; Calcium oxalate; Oxidative stress

Abbreviations- Caox Calcium oxalate; EG Ethylene glycol; CST Cholestyramine; ROS Reactive oxygen species; ALP Alkaline phosphatase; LDH Lactate dehydrogenase; SOD Superoxide dismutase; CAT Catalase

1. Introduction

Oxalate is an end product of metabolism that must be removed or sequestered. The kidneys are the primary route of its excretion and the site of its sole function [1]. It is suggested that 20-40% of renal stone formers exhibit hyperoxaluria, which may occur due to increased ingestion of oxalate containing foods, enhanced endogenous production or increased intestinal absorption [2]. Hyperoxaluria causes renal epithelial cell injury and elicits oxidative stress which favors lithogenesis. Oxidative stress at renal cellular level is primarily caused by mitochondria and NADPH oxidase [3,4].The generation of reactive oxygen species (ROS) in response to oxalate

and/or CaOx crystals is also induced by NADPH oxidase and mitochondria [5,6,7]. Despite technical progression, the treatment strategies for hyperoxaluria are limited and are confined to decrease oxalate delivery to intestine by restricting dietary oxalate and administration of drugs like thiazide diuretics or pyridoxine which are more or less efficient in lowering urinary oxalate levels [8]. Cholestyramine (CST) is a bile acid sequestrant which partially removes bile acids from the enterohepatic circulation, thereby directly competing with cholesterol synthesis and resulting in a decrease in serum levels of cholesterol [9]. Bile salts can make the colon more permeable to oxalate and thus facilitate oxalate absorption from the colon. CST supplementation has also been recommended to the hyperoxaluric patients for efficient removal of oxalate from the body [10, 11, 12]. However, till date, no study has shown the potential effect of CST on hyperoxaluria. As stones composed of calcium oxalate comprise the majority of the calculi in kidneys, it is the need of the hour to probe for the potential candidates that may reduce the complications of kidney stone disease. CST is one such drug, which has not been extensively studied with regard to its effect on calcium oxalate kidney stones. The present study was, thus, designed to evaluate the efficacy of CST on hyperoxaluria and its manifestations in an animal model.

2. Materials and methods

2.1. Chemicals

All the chemicals used in present study were of analytical grade and were purchased from Sigma research laboratory, India and the kits were purchased from Reckon diagnostics Private Limited, India. CST used in the study was manufactured by Akums Drugs and pharmaceuticles Ltd., India and marketed by Ajanta pharma limited.

2.2. Experimental design

Healthy male Sprague Dawley rats weighing between 150g and 200g of equivalent age groups were obtained from central animal house of Panjab University, Chandigarh, India. The procedures followed were approved by the institutional Animal Ethics Committee and were in accordance with the Guidelines for Humane Use and Care of Laboratory Animals (PU/IAEC/P/15/03).

2.3. Experimental design

All rats were randomly divided into five groups having 5 rats each. Normal rats (NRM) were provided with standard animal feed and water for 9 days. Hyperoxaluric group (HYO) of rats were given 0.4% EG (v/v) with 1% NH_4Cl (w/v) in drinking water for 9 days. Positive control group of rats (KCIT) were given 0.4% EG (v/v) with 1% NH_4Cl (w/v) and potassium citrate in drinking water for 9 days. CST treated rats were administered 3% CST (CST3) and 5% CST (CST5) in diet, respectively for 9 days along with the hyperoxaluric agent i.e. 0.4% EG (v/v) with 1% NH_4Cl (w/v). The standardization of the hyperoxaluric rat model was already done in the lab from previous studies [6].

2.4. Sample collection

At the end of treatment period, rats were placed in metabolic cages and urine was collected for 24 hours period having 20 µl of 20% sodium azide as antibacterial and preservative. Biochemical estimation of creatinine, urea, alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and citrate in the urine were carried out. Rats were anaesthetized with diethyl ether and sacrificed on day 10. After dissection, liver and both kidneys were removed. The transverse sections of kidneys were fixed in formaldehyde for histological analysis.

2.5. Biochemical analysis of urine

The concentrations of creatinine, urea and ALP were estimated by commercially available kit using manufacturer's instructions (Reckon Diagnostic Pvt Ltd). LDH catalyses the reversible reduction of pyruvate to lactate with NADH as the coenzyme; NADH absorbs strongly at 340 nm. Progress of reaction was followed by measuring decrease in extinction at 340nm with pyruvate as substrate. Urinary citrate was estimated by method of White and Davies (1963) with slight modifications [13].

2.6. Studies on Liver injury parameters

Enzyme activity of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), gamma-glutamyl transferase (GGT) estimation were done by using commercially available kits (Reckon diagnostic Pvt Ltd).

2.7. Measurement of oxidant/antioxidant status in renal tissue

Oxidant/antioxidant status in whole renal tissue was determined by assaying catalase (CAT) and superoxide dismutase (SOD) activity. CAT was estimated by the method of Luck, 1971[14]. SOD was estimated by the method of Kono et al., 1978 [15]. NADPH oxidase assay was performed by the method of Kumar et al., 2002 [16]. NADPH has absorbance maxima at 340 nm, and the reduction in absorbance at 340 nm is proportional to the decrease in NADPH through its consumption by the NADPH oxidase.

2.8. Studies on Mitochondria isolated from renal tissue

Mitochondrion from renal tissue was isolated by the method as followed by Chhiber et al., 2016 [6, 7]. The quantitative measurement of Lipid peroxidation (LPO) was performed according to the method of Ohkawa et al., 1979 [17]. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) reduction was used to assess the activity of the mitochondrial dehydrogenases in isolated mitochondria [18]. Activity of Citrate synthase (CS) was assayed by the method of Spinazzi et al.2012 [19]. Mitochondrial swelling as measured by permeability transition pore (PTP) opening was assayed spectrophotometrically as described by Kristian et al., 2000 [20]. Cardiolipin content in mitochondria was measured spectrofluoromertically using the cardiolipin specific dye N-nonyl acridine orange [21, 22].

2.9. Histopathological Studies

The kidneys were removed and the transverse sections were fixed in 10% buffered formalin solution. The tissues were dehydrated and embedded with paraffin wax. The paraffin sections were then cut and finally stained with Delafield's Hemotoxylin and Eosin staining and viewed under polarized light using Leica DM3000 light microscope.

2.10. Statistical analysis

Data were analyzed by one-way ANOVA and the Tukey's test for multiple comparisons using Graph Pad Prism (version 5.0; San Diego, USA). Results were expressed as mean ± SD and were considered significant if $P < 0.05$.

3. Results

3.1. Evaluation of renal functioning and renal injury

Creatinine is a waste product produced by muscles from the breakdown of creatine which is found in muscle. It is removed from the body by the kidneys, which filter almost all of it from the blood and release it into the urine. In hyperoxaluric rats levels of creatinine increased indicating renal dysfunction due to nephrocalcinosis. Alkaline phosphatase is a membrane bound zinc containing metalloenzyme. It is activated by Mg^{2+} and other divalent ions. It is a hydrolyze enzyme that dephosphorylates various molecules such as proteins and nucleotides. It is widely distributed in almost every tissue such as bone, liver, placenta, leukocytes and kidneys in the body and is kidney injury biomarker. There was a significant increase in enzymatic activity of alkalíne phosphatase in ethylene glycol treated rats. Administration of 3% cholestyramine led to reduction in its activity and 5% cholestyramine showed significant reduction in its activity comparing to control group. Lactate dehydrogenase (LDH) is an enzyme which is present in liver, kidneys, heart, pancreas, blood cells, skeletal muscles, lymph tissue. It is a marker of tissue damage and injuries. In renal cell injury the enzymatic activity of lactate dehydrogenase significantly increased. Urinary citrate is as an inhibitor of calcium salt crystallization. Citrate is a weak acid which is synthesized inside the Krebs' cycle. It also enters into the body through dietary intake. It reduces supersaturation of calcium salts in urine by forming soluble complexes with calcium ions and inhibits crystal growth and aggregation. The urinary creatinine levels were found to be increased by 72.4% in Hyperoxaluric group (HYO) of rats as compared to the Normal (NRM) group, indicating renal dysfunction due to nephrocalcinosis (Table 1). Supplementation of Cholestyramine (CST) significantly reduced the creatinine levels by 52.44% in 3% Cholestyramine (CST3) rats and by 61.25% in 5% Cholestyramine (CST5) rats, thereby depicting the potential effect of CST in maintaining renal functioning. Renal dysfunction diminishes the ability to filter urea. Urinary excretion of urea in Hyperoxaluric group (HYO) of rats was more by 50.41% as compared to the Normal (NRM) group (Table 1). Treatment with 3% and 5% CST showed significant decrease (28.14% and 28.87% respectively) in urea excretion as compared to Hyperoxaluric group (HYO) of rats. To estimate the level of renal injury due to hyperoxaluria, the enzymatic activities of ALP and LDH were studied in urine. There was a significant increase in activity of ALP

280.60% in Hyperoxaluric group (HYO) of rats as compared to Normal (NRM) group (Table 1). CST treated rats showed a reduction of (67.28% for CST 3 and 73.97% for CST 5) of ALP activity as compared to the Hyperoxaluric group (HYO). Likewise, the enzymatic activity of LDH was increased by 38.93% in Hyperoxaluric group (HYO) as compared to Normal (NRM) group (Table 1). Treatment with CST 3 and CST 5 led to a significant decrease (36.67%, 97.14% respectively) in its activity as compared to the hyperoxaluric group. Urinary citrate level of Hyperoxaluric (HYO) rats was observed to be decreased by 25.83% as compared to Normal (NRM) rats (Table 1). But CST treatment in (CST3) and CST5 rats reversed the change to normal.

3.2. Effect of CST on liver injury

The liver is a major organ responsible for the metabolism of drugs. In liver injury and oxidative stress, cell membrane of liver damage and release enzymes SGPT, SGOT and GGT which are the marker of liver injury. SGPT (serum pyruvate transaminase) is an enzyme found mainly in the liver. SGOT (serum glutamic oxaloacetic transaminase) is an enzyme found in red blood cells and in liver, heart, muscle pancreas and kidney. High levels of liver enzymes SGPT, SGOT and GGT reflect damage to liver cells as a result of several diseases of liver, such as liver tumors or cirrhosis of the liver, chronic viral hepatitis, fatty liver disease, autoimmune hepatitis, hemochromatosis. To study the extent of liver injury in hyperoxaluric rats (HYO) and the potential effect of Cholestyramine (CST), the enzyme activity assays of SGPT, SGOT and GGT were performed. The activities of SGPT, SGOT and GGT were found to be significantly increased in hyperoxaluric (HYO) rats (Table 2). In comparison to hyperoxaluric (HYO) group of rats, 3% Cholestyramine (CST3) and 5% Cholestyramine (CST5) rats showed a significant decline in the activity of SGOT by 70.31% and 73.10%, respectively. While, activity of SGOT was found to be decreased by 40.05% in CST3 rats and 44.35% in CST5 rats. Likewise, the activity of GGT showed a decrease of 20.90% in (CST3) group and 27.89% in CST5 group.

3.3. Studies on oxidant/antioxidant status in renal tissue

Excessive oxidative stress in the Hyperoxaluric (HYO) rats disturbed the redox environment of renal cells which manifested as a decline in the activities of antioxidant enzyme CAT and SOD by 86.89% and 27.10%, respectively (Figure 1A, 1B). Interestingly, 3% Cholestyramine (CST3) and 5% Cholestyramine (CST5) rats showed restored activities of CAT and SOD. Activity of NADPH oxidase (a major source of ROS in renal tissue exposed to oxalate) was found to be significantly higher in Hyperoxaluric group (HYO), CST3 and CST5 groups showed a significant reduction in its activity by 22.85% and 40.66% respectively (Figure 1C), (Table 3).

3.4. Histological analysis

On staining with Haematoxylin and Eosin, the kidney sections from Normal (NRM) rats showed normal architecture and did not display any crystal deposition (Figure 2A). Positive control group of rats (KCIT) displayed crystal deposits at a few sites and an otherwise normal histology (Figure 2B). Hyperoxaluric (HYO) rats revealed shrunken glomeruli and deposition of crystals at various sites (Figure 2C, 2D). 3% Cholestyramine

(CST3) and 5% Cholestyramine (CST5) rats depicted an improved histological architecture and better glomeruli with no crystal deposits (Figure 2E, 2F).

3.5. Effect of CST on renal mitochondria

To further evaluate the effect of Cholestyramine (CST) on oxidative stress induced by oxalate, studies on mitochondria were carried out. Mitochondrial lipid peroxidation was studied by measuring malondialdehyde level. As expected, lipid peroxidation was significantly elevated (11.83%) in Hyperoxaluric (HYO) rats as compared to Normal (NRM) rats (Figure 3A). Treatment with 3% Cholestyramine (CST 3) and 5% Cholestyramine (CST 5) significantly reduced the level of lipid peroxidation by 44.92% and 49.57%, respectively. Cardiolipin, a mitochondrial damage marker, was studied to examine the extent of damage to the mitochondrial inner membrane. The level of Cardiolipin was decreased by 31.06% in Hyperoxaluric (HYO) rats (Figure 3B). Supplementation with 3% Cholestyramine (CST 3) and CST 5 refurbished the Cardiolipin level by 19.28% and 26.92% in CST3 rats and CST5 rats, respectively. The mitochondrial enzyme, citrate synthase, showed dwindling activity in Hyperoxaluric (HYO) rats as compared to the Normal rats (NRM) rats (Figure 3C). The enzyme activity was restored by treatment with CST at both the doses in comparison to the Hyperoxaluric (HYO) group. The perturbed activities of ETC enzymes caused increased mitochondria swelling in Hyperoxaluric (HYO) rats (Figure 4A). On the contrary, CST3 and CST5 rats exhibited a decline in mitochondrial swelling by 62.57% and 66.25% respectively. MTT reduction assay was performed to evaluate cell viability. There was a significant decline in the extent of MTT reduction by the mitochondria isolated from the Hyperoxaluric (HYO) group of rats, when compared with Normal (NRM) group (Figure 4B). However, administration of 3% Cholestyramine (CST 3) and 5% Cholestyramine (CST 5) showed a significant increase in the MTT reduction.

4. Discussion

Hyperoxaluria leads to extensive damage to the tubules and causes renal inflammation and fibrosis; and it further advances to nephrolithiasis, nephrocalcinosis as well as renal parenchymal disease. Oxalate synthesis takes place in liver and there is no further metabolism of oxalate in humans. A significant share of oxalate load is caused by metabolism of ingested oxalate precursors [2]. The exposure of renal epithelial cells to oxalate causes ROS generation which is predominantly responsible for renal epithelial cell injury. The injury to the kidney tissue paves the way for crystal nucleation, aggregation and consequently stone deposition in the renal tissue [3]. In agreement with our previous studies (6), we also encountered extensive renal injury as well as renal dysfunction after the rats were administered with EG (a precursor of oxalate) and ammonium chloride (prompts crystallisation by urinary acidification). CST, by virtue of its oxalate binding property, decreased oxalate burden to the renal cells leading to amelioration of renal injury and improvement in renal function. The enhanced urinary excretion of citrate, an inhibitor of crystallisation, in CST supplemented rats, further strengthened the potential of CST to improve the ugly manifestations of hyperoxaluria. Moreover, the role of CST in minimizing hyperoxaluria induced liver injury

was established by a decline in the activities of enzymes SGOT, SGPT and GGT, which were otherwise enhanced by EG and ammonium chloride administration.

As stated earlier, and as recognized by a number of reports, the exposure of renal tissue to oxalate crystals leads to dwindling of redox balance and a further decline in the activities of enzymes (CAT and SOD) responsible for maintaining the antioxidant environment in the renal milieu[3,6,7]. NADPH oxidase is a major contributor of superoxide radicals in both renal cortex and medulla [5]. As observed in the present study, an increased activity of NADPH oxidase is associated with tremendous oxidant damage to the renal tissue. CST effectively maintained the redox equilibrium and conferred protection against deleterious oxidant damage by chelating oxalate.

Another offender responsible for oxalate mediated renal injury and renal damage, as claimed by recent studies, is the mitochondrion [6, 7]. Mitochondria are a source of ROS, and as well as are affected by the ROS in a hyperoxaluric scenario. As a result of oxalate exposure, there is malfunctioning of enzymes of electron transport chain and the leakage of electrons. The mitochondrial oxidative damage due to oxalate was manifested as lipid peroxidation, the decline in the cardiolpin content, diminished activity of citrate synthase, mitochondrial swelling and a decline in MTT reduction. CST offered significant protection against these damaging effects of increased oxalate content.

A number of reports mentioned the use of CST in treatment of hyperoxaluria as it is an oxalate chelator [23, 24, 25, 26]. But our study for the first time, confirms that in hyperoxaluric animals, CST treatment reduced renal injury and nephrocalcinosis, improved renal function, and counter-acted the oxidative imbalance caused by increased oxalate load in renal tissue as well as renal mitochondria.

Acknowledgment

Conflict of interest

The authors state no conflict of interest.

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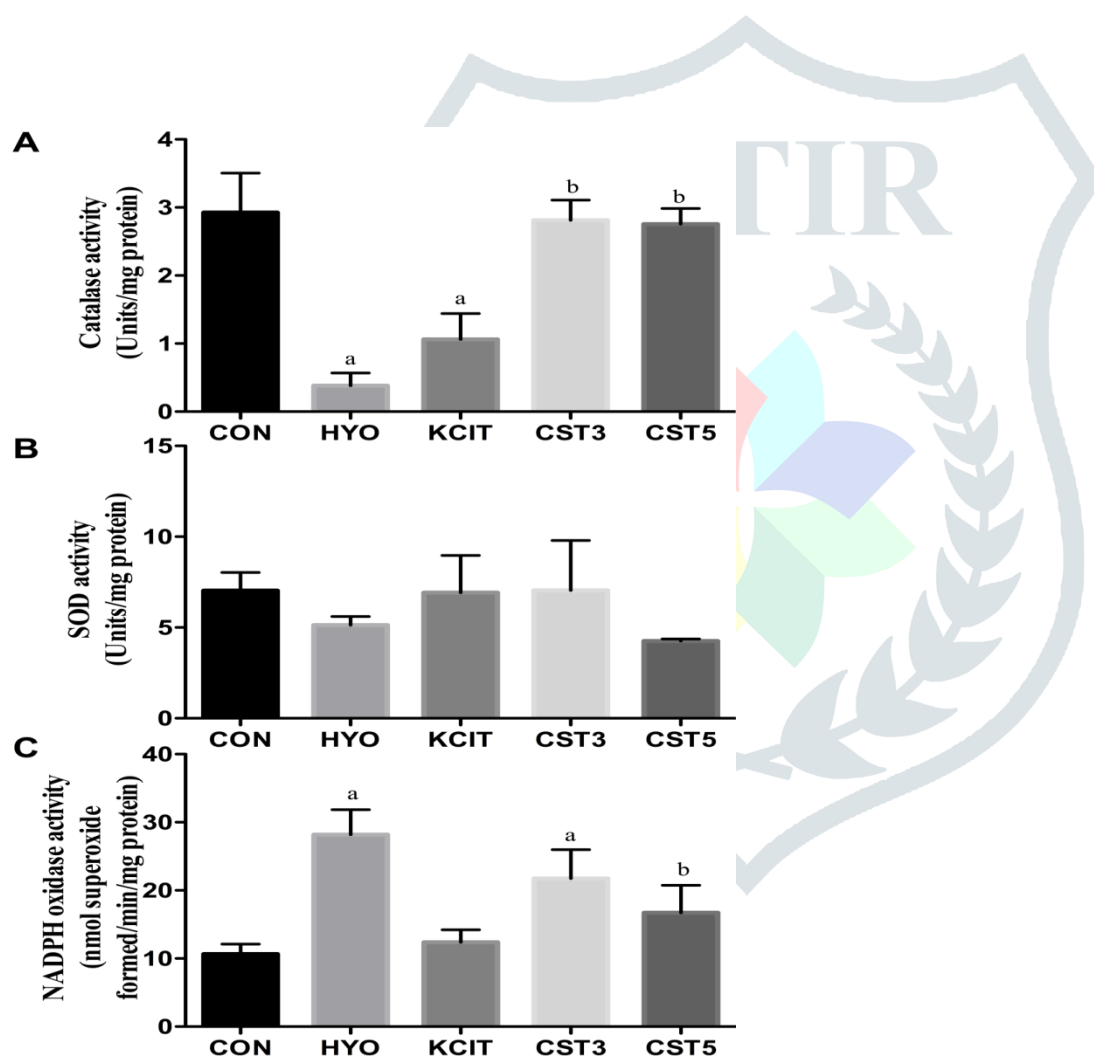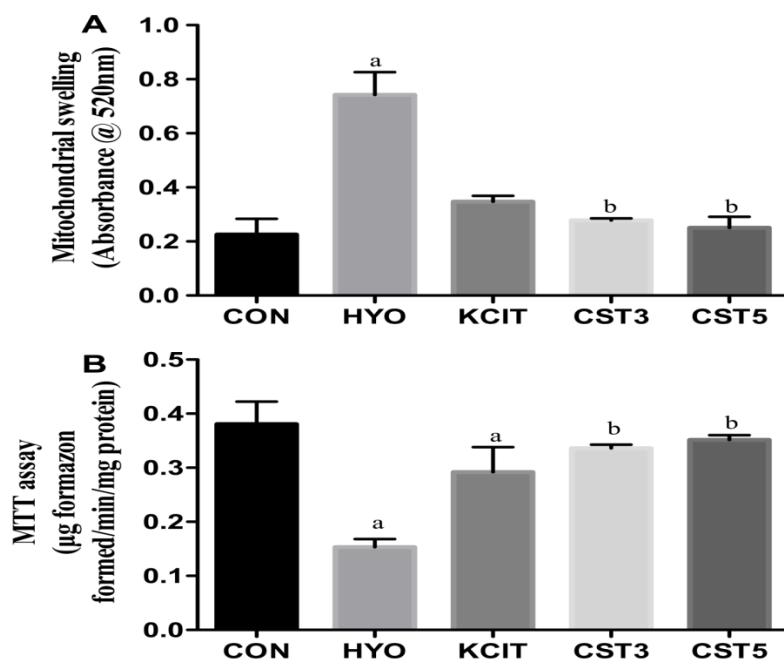
Fig 1. Effect of Cholestyramine on (a) Catalase; (b) Superoxide dismutase; (c) NADPH oxidase activity in renal tissue of hyperoxaluric rats. CON: normal rats, HYO: hyperoxaluric rats, KCIT: hyperoxaluric rats treated with potassium citrate, CST3: Hyperoxaluric treated with 3% cholestyramine, CST5: hyperoxaluric rats treated with 5% cholestyramine. Values are expressed as Mean± SD of 5 rats per group. Results of all the groups are compared with CON group. The result of CST3 and CST5 group are also compared with HYO group. The results are compared by ANOVA with Tukey's multiple comparison post hoc test, a: $P < 0.05$ significantly different from CON group, b: $P < 0.05$ significantly different from HYO group.
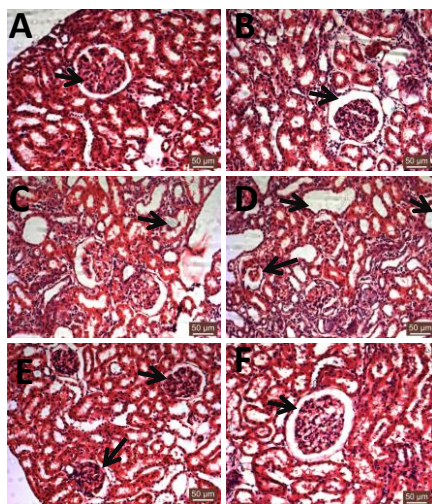
Fig 2. Renal histology stained with Haematoxylin and Eosin (original magnification 200X) (a) CON: normal rats showed no crystal deposition(arrows), (b) KCIT: hyperoxaluric rats treated with potassium citrate showed no crystal deposition, (c) HYO: hyperoxaluric rat kidney section showing crystal deposition (arrows), (d) HYO: hyperoxaluric rat kidney section showing shrunken glomeruli (arrows), (e) CST3: Hyperoxaluric treated with 3% cholestyramine, (F) CST5: hyperoxaluric rats treated with 5% cholestyramine (arrows).
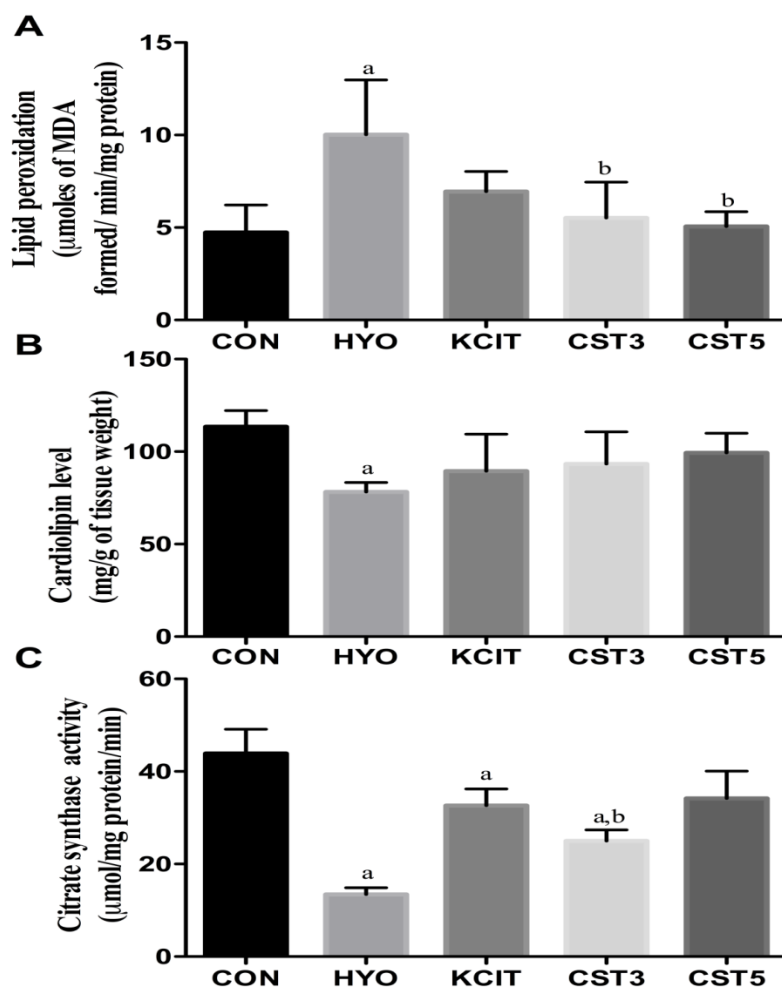
Fig 3. Effect of Cholestyramine on (a) Lipid peroxidation; (b) Cardiolipin level; (c) Citrate synthase activity in renal mitochondria of hyperoxaluric rats. CON: normal rats, HYO: hyperoxaluric rats, KCIT: hyperoxaluric rats treated with potassium citrate, CST3: Hyperoxaluric treated with 3% cholestyramine, CST5: hyperoxaluric rats treated with 5% cholestyramine. Values are expressed as Mean± SD of 5 rats per group. Results of all the groups are compared with CON group. The result of CST3 and CST5 group are also compared with HYO group. The results are compared by ANOVA with Tukey's multiple comparison post hoc test, a: $P<0.05$ significantly different from CON group, b: $P<0.05$ significantly different from HYO group.

Fig 4. Effect of Cholestyramine on (a) Mitochondrial swelling and (b) MTT reduction in renal mitochondria of hyperoxaluric rats. CON: normal rats, HYO: hyperoxaluric rats, KCIT: hyperoxaluric rats treated with potassium citrate, CST3: Hyperoxaluric treated with 3% cholestyramine, CST5: hyperoxaluric rats treated with 5% cholestyramine. Values are expressed as Mean± SD of 5 rats per group. Results of all the groups are compared with CON group. The result of CST3 and CST5 group are also compared with HYO group. The results are compared by ANOVA with Tukey's multiple comparison post hoc test, a: $P<0.05$ significantly different from CON group, b: $P<0.05$ significantly different from HYO group.

Table 1. Effect of Cholestyramine on renal injury and renal functioning in hyperoxaluric rats

Urinary parameters	CON	HYO	KCIT	CST3	CST5
Creatinine (mg/dl)	0.1022±0.02156	0.1762±0.006967	0.1021±0.01014	0.08379±0.005203	0.06827±0.01358
Urea (mg/dl)	73.00±4.894	109.8±0.5164	77.30±13.70	78.90±7.779	78.10±10.34
ALP (IU/L)	12.89± 5.188	49.06±6.411	19.26±6.594	16.05±5.435	12.77±5.239
LDH (Units/mg protein)	0.2445±0.02371	0.3397±0.05469	0.04913±0.05756	0.2151±0.08436	0.09790±0.07139
Citrate (mg/dl)	0.9704±0.08431	0.7197±0.05023	1.215±0.04230	0.9068±0.01116	0.9277±0.02348

CON: normal rats, HYO: hyperoxaluric rats, KCIT: hyperoxaluric rats treated with potassium citrate, CST3: Hyperoxaluric treated with 3% cholestyramine, CST5: hyperoxaluric rats treated with 5% cholestyramine. Values are expressed as Mean± SD of 5 rats per group. Results of all the groups are compared with CON group. The result of CST3 and CST5 group are also compared with HYO group. The results are compared by ANOVA with Tukey's

multiple comparison post hoc test, a: P<0.05 significantly different from CON group, b: P<0.05 significantly different from HYO group.

Table 2. Effect of Cholestyramine on liver injury in hyperoxaluric rats

Liver injury parameters (IU/L)	CON	HYO	KCIT	CST3	CST5
SGPT	17.40±5.256	95.74±21.30	28.44±4.795	28.42±4.336	25.75±1.935
SGOT	45.15±2.907	118.4±12.88	78.34±6.118	70.97±9.808	65.88±15.10
GGT	72.51±11.73	112.7±11.68	89.03±10.93	89.14±7.078	79.04±16.09

CON: normal rats, HYO: hyperoxaluric rats, KCIT: hyperoxaluric rats treated with potassium citrate, CST3: Hyperoxaluric treated with 3% cholestyramine, CST5: hyperoxaluric rats treated with 5% cholestyramine. Values are expressed as Mean± SD of 5 rats per group. Results of all the groups are compared with CON group. The result of CST3 and CST5 group are also compared with HYO group. The results are compared by ANOVA with Tukey's multiple comparison post hoc test, a: P<0.05 significantly different from CON group, b: P<0.05 significantly different from HYO group.

Table 3. Effect of Cholestyramine on Renal tissue of hyperoxaluric rats.

Renal tissues parameters	CON	HYO	KCIT	CST3	CST5
CAT	2.924±0.5805	0.3834±0.1862	1.062±0.3801	2.812±0.2948	2.754±0.2324
SOD	7.036±0.9974	5.129±0.4746	6.913±2.058	7.039±2.760	4.257±0.113
NOX	0.521±0.2561	0.871±0.9232	0.362±0.3754	0.651±0.7651	0.421±0.5232

CON: normal rats, HYO: hyperoxaluric rats, KCIT: hyperoxaluric rats treated with potassium citrate, CST3: Hyperoxaluric treated with 3% cholestyramine, CST5: hyperoxaluric rats treated with 5% cholestyramine. Values are expressed as Mean± SD of 5 rats per group. Results of all the groups are compared with CON group. The result of CST3 and CST5 group are also compared with HYO group. The results are compared by ANOVA with Tukey's multiple comparison post hoc test, a: P<0.05 significantly different from CON group, b: P<0.05 significantly different from HYO group.