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Original Article

Hyperoxaluria: The role of N-acetyl-L-cysteine and Vitamin E on lithogenic factors and urinary markers in ameliorating calcium oxalate crystallization

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Abstract

Background and Aim: In hyperoxaluria, membrane injury is essential for the binding of oxalate or calcium oxalate (CaOx) crystal to be retained in the renal cell. This oxalate-membrane interaction generates oxidative stress and is considered to be a significant causative factor of membrane damage and stone pathogenesis. The present study uses ammonium oxalate (AmOx) rat model to develop hyperoxaluria and lipid peroxidation, and N-acetyl-L-cysteine (NAC) and NAC + Vitamin E given intraperitoneally to prevent CaOx crystal membrane damage and crystal binding.

Methods: Stone forming risk factors namely, calcium, phosphate, oxalate, and inhibitors of stone formation namely, magnesium, uric acid, citrate, and glycosaminoglycans (GAGs) were studied and for renal function sodium, creatinine, and protein were seen in urine of five groups of animals (6 numbers each). The ratios of Ca/oxalate Mg/oxalate, citrate/calcium, oxalate/creatinine, and others are of clinical importance to assess the recurrence in stone formers. The CaOx supersaturation index and activity product (CaOx) index (rats) were studied as it indicates a high risk for stone formation. Further, the levels of oxalate synthesizing enzymes in liver and kidney were studied for endogenous synthesis of oxalate. Besides, urinary marker enzymes alkaline phosphatase, gamma glutamyl transferase, and lactate dehydrogenase (LDH) were studied indicating tissue membrane damage. Endogenous oxalate synthesizing enzymes of liver and kidney were done and also the tissue risk factors of stone. LDH isoenzyme gel electrophoresis and light microscopy of kidney tissue were done.

Results: Urinary oxalate was increased significantly ($P < 0.05$) urinary calcium too, urinary magnesium was significantly decreased ($P < 0.01$), urinary citrate levels were found to be 1.43 ± 0.15 mg/24 h in control animals but decreased 3-fold in AmOx-treated animals. Urinary marker enzymes showed injured epithelium, a prerequisite for crystal adhesion and decreased GAGs in urine showed damage to the proximal convoluted tubules. LDH 2 isoenzyme as a marker of kidney tissue damage was found increased in hyperoxaluric rats. Levels of urinary GAGs were decreased significantly ($P < 0.001$). NAC and Vitamin E pretreated animals showed a significant decrease in stone forming risk factors in urine and increased inhibitor excretion. Histological sections showed NAC and Vitamin E pretreated hyperoxaluric rats inhibited deposition of CaOx crystals and renal cell damage.

Conclusion: NAC therapy prevents CaOx retention by protecting against membrane injury, thus maintaining a smooth urothelium that does not favor stone formation.

Key word: Antioxidants, hyperoxaluria, oxidative stress, renal stones, risk factors in urine

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INTRODUCTION

Hyperoxaluria is a major risk factor of calcium oxalate (CaOx) stone disease. Urinary stone formation is a

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result of different mechanisms. Microcrystal of CaOx monohydrate, the most common crystal in renal systems, irreversibly bind to cell surface microvilli, are subsequently internalized and proliferation occurs. Calcium phosphate crystals and organic matrix initially are deposited along the basement membranes of the thin loops of Henle and extend further into the interstitial space to urothelium, constituting the so-called Randall plaques [Figure 1].

The progression from hyperoxaluria to nephrolithiasis is multifaceted, and renal tubular injury is a prerequisite. Oxalate ions as well as CaOx crystals promote renal tubular injury (manifested *in vivo*). CaOx stone disease suggests that the generation of reactive oxygen species (ROS) and subsequent lipid peroxidation is an integral part of the process.[1,2]

An increased production of ROS in response to an insult such as hyperoxaluria, triggers the kidney to adapt to this insult by upregulating the antioxidant defense systems such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione. When antioxidant levels are depleted the cells are under "oxidative stress."[3-5]

A balance between stone promoters and inhibitors is necessary to control hyperoxaluria and urolithiasis. Calcium, oxalate phosphorous, and uric acid are the promoters of stones through precipitation of their salts during supersaturation. Inhibitors of crystallization are mainly citrate, magnesium, and Tamm–Horsfall protein and osteopontin.

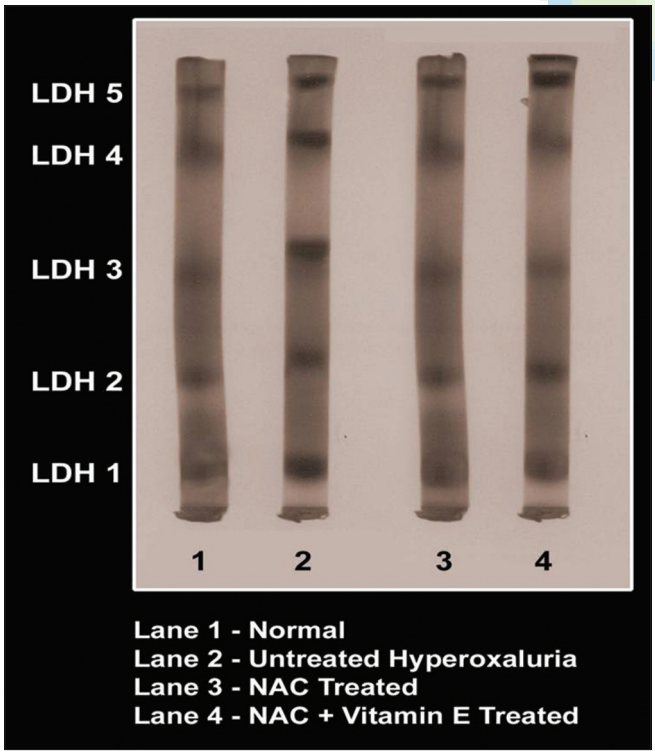

Figure 1: Isoenzyme pattern of serum lactate dehydrogenase. Lane 1: Normal, Lane 2: Untreated hyperoxaluria, Lane: N-acetyl-L-cysteine-treated, Lane 4: N-acetyl-L-cysteine + Vitamin E treated

N-acetyl-L-cysteine (NAC) supplementation provides sulphthiol group of L-cysteine and also exerts direct antioxidant effect by directly scavenging free radicals. NAC pretreatment reduced endothelial dysfunction caused by uremic toxin in kidney disease by reducing ROS and expression of nuclear factor-kappa B.[6] Vitamin E is a lipid soluble alpha-tocopherol scavenge free radicals by incorporating into plasma membranes of cells and halting lipid peroxidation. Dietary oxalate intake in Rajasthan, India, in urban upper-income group is 606 mg/24 h against 200 mg recommended per day. The objective is to reduce membrane injury and crystal growth and aggregation, for a torn membrane (due to oxalate induced Injury) does promote crystal attachment in stone formers. The use of favorable antioxidants and their efficacy singly or in combination is tested NAC has anti-inflammatory, antioxidant, and detoxification function, NAC + Vitamin E combination increases bioavailability of Vitamin E and cysteine.

The aim of the present study was to examine whether oxalate-induced membrane injury occurs as a result of the interaction between oxalate crystals and tubular epithelial cells in this animal model of crystalluria leading to damaging effects of oxidative stress and the use of NAC singly or in combination with Vitamin E (NAC + Vitamin E) in curtailing crystal formation in hyperoxaluric rats.

METHODS

Grouping of animals

Animals were divided into five groups. Weighing approximately 100 ± 15 g.

- Group I: Animals were fed on rat chow with clean drinking water *ad libitum*
- Group II: Animals were fed on rat chow with 3% ammonium oxalate (AmOx) in drinking water for 5 days
- Group III: Animals were given NAC (i.p.) (5 mg/100 g body weight) and Vitamin E (i.p.) (5 mg/100 mg body weight) single dose and fed with rat chow and clean drinking water for the next 5 days
- Group IV: Animals were pretreated with NAC (i.p.) (5 mg/100 g body weight) single dose and fed on rat chow with 3% AmOx in drinking water for the next 5 days
- Group V: Animals were pretreated with NAC (i.p.) (5 mg/100 g body weight) and Vitamin E emulsified in olive oil (5 mg/100 g body weight) (i.p.) single dose and fed on rat chow and were given 3% AmOx in drinking water for the next 5 days.

All the groups of animals were maintained in well-ventilated cages.

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Collection of rat urine and tissues

The present study was conducted with ethical clearance from the institution PGIBMS, India. On the day before sacrifice, rats were housed in metabolic cages for 24 h urine collection free from fecal contamination. The rats were provided with water, but no feed was given. A 50 ml beaker maintained at 0°C in an ice bath was used for collection.

a. A portion of the sample was acidified with concentrated HCl and used for the analysis of oxalate, calcium, magnesium, phosphorus, citric acid, uric acid, and creatinine and after centrifuging for 10 min to remove sediments if any
b. A known aliquot was set aside for the estimation of total glycosaminoglycans (GAGs) as alcian blue precipitable polyanions
c. The remaining portion was dialyzed at 4°C against distilled water for 3 h. Aliquots of the dialyzed urine were then used for the assay of enzymes and determination of protein content.

At the end of experimental period, the animals were killed by cervical decapitation and blood collected. Liver and kidneys were excised immediately and washed with ice-cold saline and their weights recorded.

Preparation of tissue homogenate

A 10% homogenate of the washed tissues (liver and kidney) were prepared in 0.01 M phosphate buffer, pH 7.0 for all the enzymes.

Investigations carried out in urine for urinary lithogenic salts and total protein are protein (Lowry *et al*., 1951), uric acid (Caraway, 1963), creatinine (Owen *et al*., 1954), inorganic phosphorus (Fiske and Subbarow, 1925), total GAGs - (Hwang *et al*., 1988), calcium and magnesium (Emission Spectrophotometry), oxalate - (Hodgkinson and Williams, 1972), citric acid - (Rajagopal, 1984), sodium - flame photometry, and potassium – flame photometry.

Urinary enzymes assayed for tissue injury were alkaline phosphatase - (King, 1965b), lactate dehydrogenase (LDH) - (King, 1965a), alanine transaminase, and pyrophosphatase - (Josse, 1966).

Oxalate synthesizing enzymes are glycolic acid oxidase (GAO) - Richardson and Tolbert (1961), LDH - King (1965a), Xanthine oxidase (XO) - Fried and Fried (1966), and LDH isoenzyme separation - Dietz and Lubrano, 1967, with modifications.

The right kidney was fixed in 10% neutral buffered formalin, processed, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin (H and E) for CaOx crystals, for microscopic examination.

Statistical analysis of data

Statistical analysis was done by Student's *t*-test using SPSS 16.00 (IBM, Chicago, USA). Results are shown and presented as a mean ± standard error of the mean and results were considered statistically significant if $P < 0.05$.

RESULTS

In Table1 urine volume was elevated in Group II animals significantly ($P < 0.001$) when compared to that of control rats. Urinary calcium excretion was increased approximately 2-fold in Group II animals compared to control rats. NAC and NAC and Vitamin E administered AmOx treated rats (Group IV and V) brought down urinary calcium levels compared to controls. Urinary oxalate excretion was found to be 8-fold increased in the lithogenic groups (Group II) compared to controls. It was significantly ($P < 0.05$) lowered in Group IV and Group V animals. The excretion of phosphorous was unaltered after administration of AmOx or therapeutic drugs. Urinary magnesium was found to be decreased in Group II animals compared to controls (Group I) ($P < 0.01$). NAC and NAC and Vitamin E pretreated rats showed increased urinary magnesium [Table 1]. Figure 2 shows decreased calcium/oxalate ratios in Group II rat which was comparatively increased in Group IV and V rats. Magnesium/oxalate ratios were also decreased in hyperoxaluric rats compared to controls.

Table 1: Effect of NAC, NAC and vitamin E combinatorial therapy for experimental hyperoxaluria on urinary stone forming constituents

Parameters (mg/24 h)	Group I	Group II	Group III	Group IV	Group V
Urine volume (ml/24 hrurine)	5.40±0.32	7.62±0.63^{a***}	5.51±0.48	6.32±0.53^{a*b**}	6.13±0.55^{b**}
Calcium	1.57±0.14	3.75±0.36^{a***}	1.62±0.10	2.40±0.18^{b***}	1.78±0.15^{b***}
Oxalate	0.90±0.09	7.26±0.70^{a***}	0.84±0.08	4.22±0.42^{a**b**}	3.86±0.41^{a**b*}
Phosphorus	9.75±0.95	10.32±1.0	8.42±0.82	9.96±0.94	9.82±0.88
Magnesium	0.91±0.13	0.70±0.05^{a**}	0.90±0.11	0.83±0.08^{b**}	0.92±0.05^{b***}

Treatment of groups: Group I – Control; Group II – 3% Ammonium oxalate in drinking water for 5 days in Group III – Control + N-acetyl-L-cysteine (NAC) (5 mg/100 g body wt (Lp) and vitamin E (5mg/100g g body wt) (i.p); Group IV – Ammonium oxalate + NAC (i.p); Group V – Ammonium oxalate + NAC + Vitamin E. Drugs were administered by interperitonial (i.p) single dose and the vehicle used was saline and olive oil. Comparisons were made as follows : ^{a}With Group I, ^{b}With Group II. The symbols a, b also represent statistical significance at $*P<0.05$; $**P<0.01$; $***P<0.001$. Values are expressed as mean±SD for six animals

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Whereas magnesium/calcium ratios were decreased in hyperoxaluric rats which improved with combinatorial treatment of NAC and Vitamin E.

Table 2 summarizes the effect of NAC and NAC + Vitamin E on other constituents of urine in control and Group II animals. Significant ($P < 0.05$) increase in uric acid excretion was observed in Group II animals. Drug-treated hyperoxaluric animals exhibited lowered uric acid excretion when compared to that of the hyperoxaluric animals. Urinary citrate levels were found to be decreased on AmOx-treated Group II. Partial restoration was observed in drug-treated hyperoxaluric rats. The levels of GAGs were found to be decreased significantly ($P < 0.001$) in Group II animals when compared to controls. NAC and Vitamin E pretreated animals showed improved GAGs in urine ($P < 0.01$).

Proteinuria (1.5-fold increase) and lowered urinary creatinine excretion were seen in AmOx-treated hyperoxaluric rats showing lowered renal function. In NAC and NAC + Vitamin E pretreated groups, the urinary levels of protein and creatinine were well within normal limits. High urinary excretion of sodium and potassium was observed in Group II rats ($P < 0.001$), Group IV showed significant lowering of sodium and potassium ($P < 0.05$), and Group V showed lowered levels ($P < 0.001$) when compared with Group II animals.

Figure 3 shows the changes in ratios of lithogenic substances in urine. Calculogenic rats showed increased oxalate/creatinine ratios compared to controls. NAC (Group IV) and NAC + Vitamin E (Group V) helped in lowering oxalate/creatinine ratios significantly in (Group II) rats. Conversely, creatinine/calcium ratios

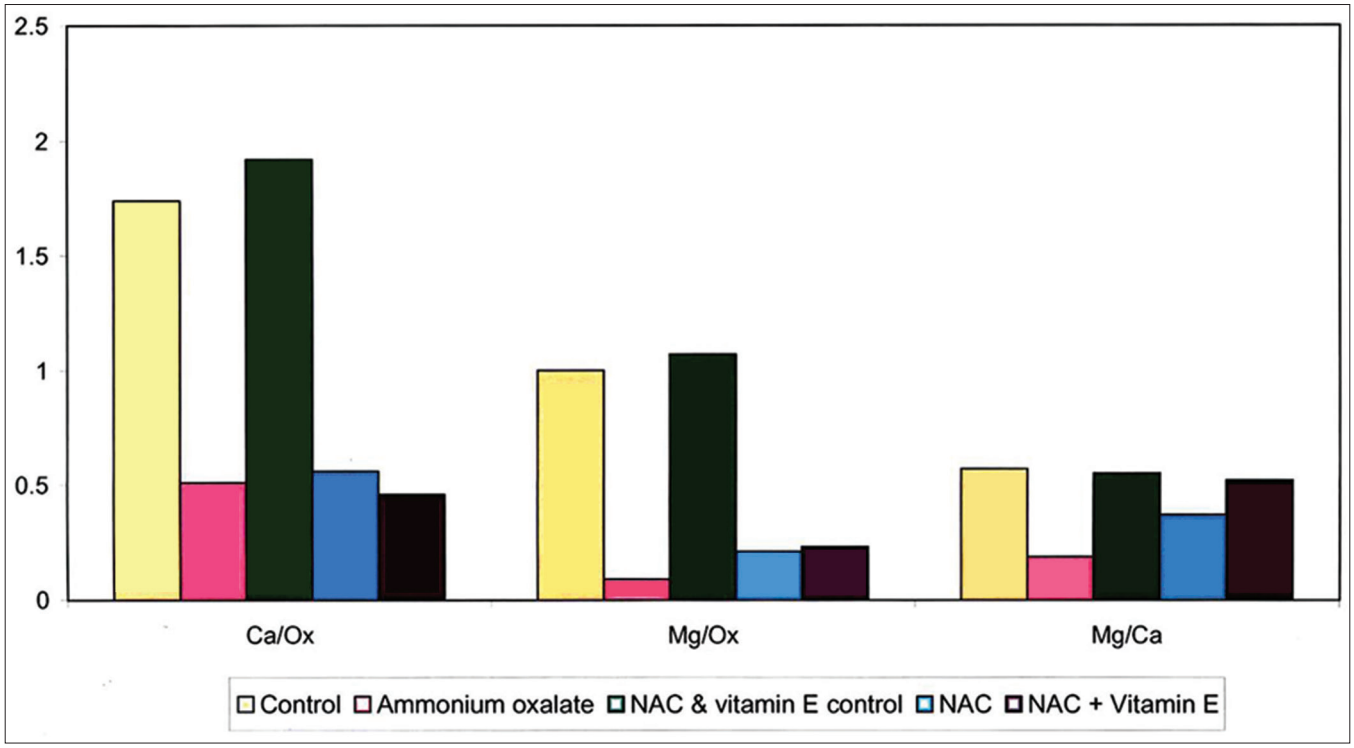

Figure 2: Effect of N-acetyl-L-cysteine and N-acetyl-L-cysteine + Vitamin E therapy on urinary calcium/oxalate ratios

Table 2: Effect of NAC, NAC and vitamin E combination therapy on urinary constituents

Parameters (mg/24 h)	Group I	Group II	Group III	Group IV	Group V
Uric acid	0.36±0.03	0.42±0.03^{a*}	0.35±0.02	0.39±0.03	0.37±0.03^{b*}
Citric acid	1.43±0.15	0.52±0.05^{a***}	1.35±0.14	1.08±0.11^{b***}	1.32±0.09^{b***}
Total GAGs	0.32±0.02	0.24±0.03^{a***}	0.31±0.01	0.27±0.03	0.30±0.03^{b**}
Protein	17.73±1.81	26.0±2.5^{a***}	18.0±1.84	21.21±2.22^{b**}	18.91±2.01^{b***}
Creatinine	13.51±1.33	11.42±1.0^{a*}	13.25±1.26	11.35±1.06	12.22±1.0
Sodium	12.1±1.4	17.6±1.7^{a***}	11.32±1.13	15.2±1.5^{b*}	13.35±1.35^{b***}
Potassium	7.5±0.82	10.8±1.0^{a***}	6.8±0.72	9.2±0.9^{b*}	8.0±1.10^{b**}

Treatment of groups: Group I – Control; Group II– 3% Ammonium oxalate in drinking water for 5 days in Group III – Control + N-acetyl-L-cysteine (NAC) (5 mg/100 g body wt (Lp) and vitamin E (5mg/100g g body wt) (i.p); Group IV – Ammonium oxalate + NAC (i.p); Group V – Ammonium oxalate + NAC + Vitamin E. Drugs were administered by interperitonial (i.p) single dose and the vehicle used was saline and olive oil. Comparisons were made as follows : [a]With Group I, [b]With Group II. The symbols a, b also represent statistical significance at : *P<0.05; **P<0.01; ***P<0.001. Values are expressed as mean±SD for six animals

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showed a 4-fold increase in Group II rats which were found effectively reduced in the drug-treated groups.

Figure 4 Group II rats showed a mild increase in uric acid/GAG ratio compared to other experimental groups. Oxalate/GAGs ratio was also increased, and the increment was thirty-fold in AmOx supplemented hyperoxaluric rats. Oxalate ion causes damage due to oxidative stress. NAC (Group IV) and (Group V) rats showed decreased levels of these ratios showing the effectiveness of combination therapy on hyperoxaluric animals.

Figure 5 shows changes in urinary citrate/creatinine, calcium/citrate, magnesium/citrate, and citrate/calcium in various experimental groups. Citrate/creatinine ratios were found increased in hyperoxaluric rats (Group II). Drug-treated rats showed lowered ratios of the same. Calcium/citrate in Group II animals ratios was increased 7-fold compared to controls. These ratios were lowered in drug-treated animals. Magnesium/citrate ratios were found increased in Group II rats compared to other experimental groups and found to be reversed after drug therapy. Citrate/calcium ratios were severely lowered in hyperoxaluric rats that improved with the double drug combinatorial treatment. Citrate is protective over CaOx precipitation.

Figure 6 shows changes in CaOx supersaturation index that are required for insoluble CaOx to crystallize in various experimental groups. There was a twenty-fold increase in CaOx supersaturation index and activity product (AP) (CaOx) in Group II rats which were normalized significantly in Group IV and V by NAC and NAC + Vitamin E combinatorial treatment.

Table 3 shows activities of oxalate synthesizing enzymes, namely, GAO in liver, XO and LDH in liver and kidney of control and experimental rats. AmOx-treated hyperoxaluric rats (Group II) exhibited a 94% increase in liver GAO activity than that of controls. Therapy resulted in ($P < 0.001$) near normal activities of GAO in Group IV and V hyperoxaluria-induced antioxidant pretreated rats. Liver and kidney LDH activities were elevated by about 24% and 33%, respectively, in Group II rats compared to control. Double drug treatment restored LDH activities to that of control group animals. Liver and kidney XO activities were increased 24% and 28%, respectively, in Group II rats compared to controls. NAC and NAC and Vitamin E pretreated animals restored their activities to that of control ($P < 0.05$). Insoluble uric acid crystals may result from increased XO activity.

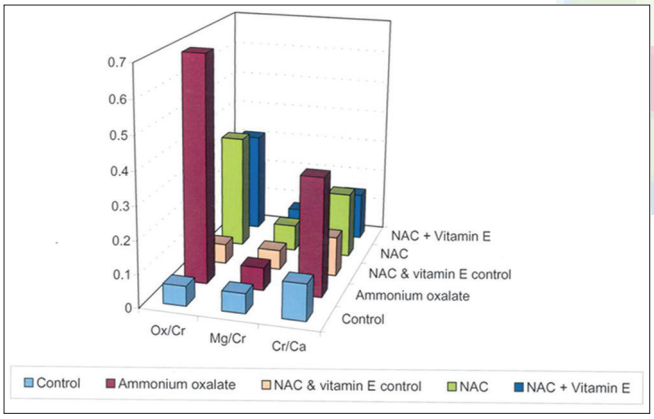

Figure 3: Effect of N-acetyl-L-cysteine and N-acetyl-L-cysteine + Vitamin E on urinary oxalate/creatinine ratios in experimental groups

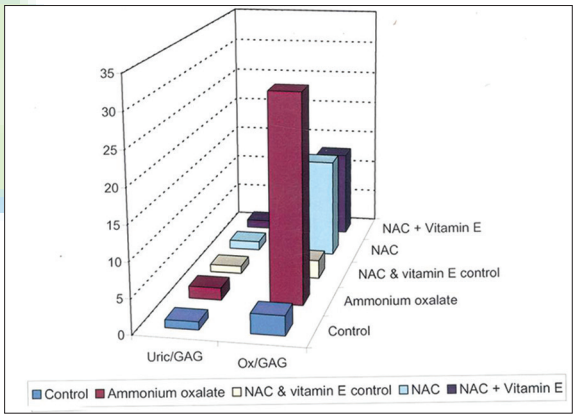

Figure 4: Effect of N-acetyl-L-cysteine and N-acetyl-L-cysteine + Vitamin E on urinary uric acid/Gags and Oxalate/Gags ratio

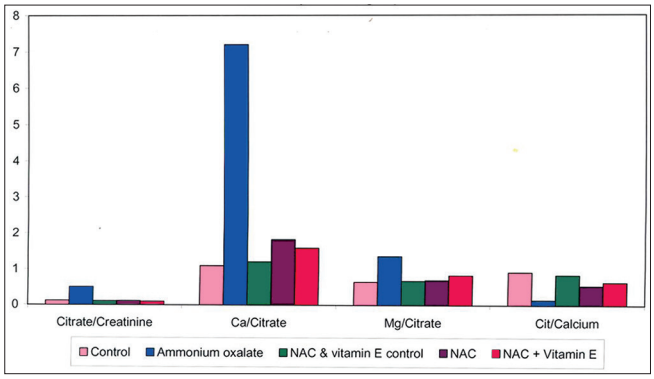

Figure 5: Effect of N-acetyl-L-cysteine and N-acetyl-L-cysteine + Vitamin E on urinary citrate to creatinine Ratios in various experimental groups

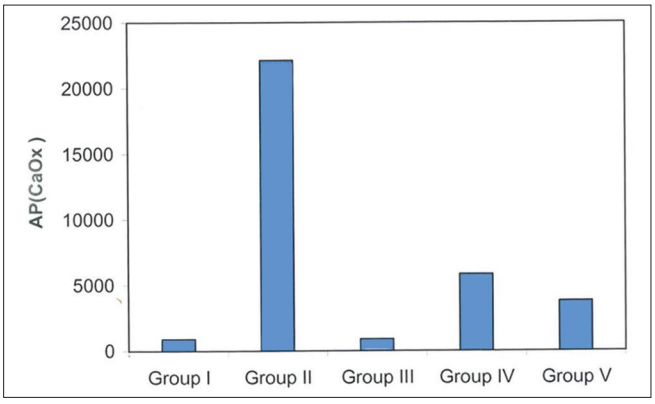

Figure 6: Calcium oxalate supersaturation index in various experimental groups

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Table 4 shows the effects of NAC and NAC + Vitamin E on tissue risk factors of stone formation. In kidney and liver, the levels of calcium and oxalate were found to be elevated ($P < 0.001$) in Group II animals which were controlled by NAC (Group IV) and NAC and Vitamin E (Group V) pretreated animals. Increased magnesium levels ($P < 0.001$) were found in liver and kidney of hyperoxaluric rats, and partial restoration of normal levels of magnesium was observed in renal tissue of hyperoxaluric rats following single and combinatorial treatment of NAC and Vitamin E. Tissue levels of oxalate were significantly increased in the liver and kidney tissue in Group II animals and efficiently brought under control by single drug (Group IV) and combinatorial therapy (Group V) pretreated animals. Phosphorous in liver and kidney tissue shows no changes in all experimental groups. CaOx stones are thus predominant in kidney.

Tables 5 and 6 illustrates the effect of NAC and NAC and Vitamin E pretreatment on activities of renal enzymes in Group II rats. The activities of both alkaline phosphatase and acid phosphatase were decreased in Group II animals both being cellular, and

Table 3: Effect of NAC, NAC and vitamin E combinatorial therapy on the activities of urinary marker enzymes

Parameters (mg/24 h)	Group I	Group II	Group III	Group IV	Group V
Alkaline phosphatase	0.96±0.08	2.8±0.25^{a***}	1.06±0.09	1.82±0.17^{b***}	1.32±0.18^{b***}
Lactate dehydrogenase	0.55±0.05	1.7±0.15^{a***}	0.58±0.06	1.05±0.09^{b***}	0.65±0.05^{b***}
γ-Glutamyl transferase	1.05±0.10	3.02±0.31^{a***}	1.03±0.13	2.05±0.15^{b***}	1.43±0.14^{b***}
Pyrophosphatase	1.40±0.14	1.25±0.09	1.42±0.12	1.30±0.12	1.38±0.13

Enzyme units are expressed as : ALP – μ moles of phenol liberated; LDH – μ moles of pyruvate released; γ-GT – μ moles of p.nitroaniline; PPase – μ moles of inorganic phosphorus formed. Treatment of groups: Group I – Control; Group II – 3% Ammonium oxalate in drinking water for 5 days in Group III – Control + N-acetyl-L-cysteine (NAC) (5 mg/100 g body wt (Lp) and vitamin E (5mg/100g g body wt) (i.p);Group IV – Ammonium oxalate + NAC (i.p); Group V – Ammonium oxalate + NAC + Vitamin E. Drugs were administered by interperitonial (i.p) single dose and the vehicle used was saline and olive oil. Comparisons were made as follows : ^{a}With Group I; ^{b}With Group II. The symbols a, b also represent statistical significance at : *P<0.05; **P<0.01; ***P<0.001. Values are expressed as mean±SD for six animals

Table 4: Effect of NAC, NAC and vitamin E combination on experimental hyperoxaluria on oxalate synthesizing enzymes in liver and kidneys

Parameters	Group I	Group II	Group III	Group IV	Group V
Liver					
Xanthine oxidase	1.78±0.17	2.22±0.21^{a***}	1.79±0.14	2.03±0.20	1.87±0.25^{b*}
Glycollic acid oxidase	1.43±0.14	2.78±0.26^{a***}	1.49±0.12	1.97±0.11^{a*b***}	1.55±0.16^{b***}
Lactate dehydrogenase	1.12±0.09	1.39±0.12^{a**}	1.10±0.11	1.26±0.11	1.14±0.12^{b**}
Kidney					
Xanthine oxidase	1.75±0.15	2.25±0.22^{a***}	1.72±0.16	1.95±0.19^{b*}	1.80±0.19^{b*}
Lactate dehydrogenase	1.09±0.10	1.45±0.21^{a**}	1.08±0.16	1.26±0.12	1.05±0.10^{b**}

Enzyme units are expressed as (Units/min/mg protein): Xanthine oxidase – units/mg protein (1 unit=amount of enzyme that brings about a change in O.D of 0.01/min) GAO – n moles of glyoxalate released; LDH – μ moles of pyruvate released. Treatment of groups: Group I – Control; Group II – 3% Ammonium oxalate in drinking water for 5 days in Group III – Control + N-acetyl-L-cysteine (NAC) (5 mg/100 g body wt (Lp) and vitamin E (5 mg/100g g body wt) (i.p);Group IV – Ammonium oxalate + NAC (i.p); Group V – Ammonium oxalate + NAC + Vitamin E. Drugs were administered by interperitonial (i.p) single dose and the vehicle used was saline and olive oil. Comparisons were made as follows: ^{a}With Group I; ^{b}With Group II. The symbols a, b also represent statistical significance at : *P<0.05; **P<0.01; ***P<0.001. Values are expressed as mean±SD for six animals

Table 5: Assessment of tissue risk factors of stone formation in various experimental groups

Constituents (mg/g wet tissue)	Group I	Group II	Group III	Group IV	Group V
Liver					
Calcium	0.16±0.01	0.22±0.02^{a***}	0.17±0.01	0.19±0.02^{b*}	0.17±0.02^{b**}
Magnesium	0.09±0.01	0.18±0.01^{a***}	0.10±0.01	0.14±0.01^{b***}	0.11±0.01^{b***}
Oxalate	1.05±0.10	1.97±0.19^{a***}	1.13±0.11	1.40±0.16^{a***b***}	1.16±0.15^{b***}
Phosphorus	3.0±0.28	3.25±0.32	3.01±0.30	3.18±0.32	3.10±0.28
Kidney					
Calcium	0.20±0.02	0.26±0.03^{a***}	0.19±0.02	0.24±0.02^{b*}	0.20±0.02^{b***}
Magnesium	0.25±0.02	0.56±0.05^{a***}	0.23±0.02	0.28±0.03	0.27±0.03^{b*}
Oxalate	0.50±0.05	7.29±0.72^{a***}	0.48±0.05	4.52±0.45^{a***b***}	1.49±0.14^{b***}
Phosphorus	3.25±0.32	3.57±0.35	3.23±0.30	3.21±0.31	3.20±0.30

Treatment of groups: Group I – Control; Group II – 3% Ammonium oxalate in drinking water for 5 days in Group III – Control + N-acetyl-L-cysteine (NAC) (5 mg/100 g body wt (Lp) and vitamin E (5mg/100g g body wt) (i.p); Group IV – Ammonium oxalate + NAC (i.p); Group V – Ammonium oxalate + NAC + Vitamin E. Drugs were administered by interperitonial (i.p) single dose and the vehicle used was saline and olive oil. Comparisons are made as follows: ^{a}With Group I; ^{b}With Group II. The symbols a, b also represent statistical significance at: *P<0.05; **P<0.01; ***P<0.001. Values are expressed as mean±SD for six animals

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Table 6: Effect of NAC, NAC and vitamin E combination on hyperoxaluria on the activities of renal enzymes

Enzymes (units/min/mg protein)	Group I	Group II	Group III	Group IV	Group V
Alkaline phosphatase	2.77±0.27	2.32±0.20[a]*	2.62±0.26	2.63±0.20[b]*	2.72±0.32[b]*
Acid phosphatase	0.29±0.04	0.24±0.05	0.28±0.04	0.25±0.01	0.27±0.02
Alanine transaminase	0.18±0.01	0.16±0.01[a]**	0.19±0.02	0.17±0.02	0.18±0.02
Pyrophosphatase	4.42±0.42	3.39±0.33[a]***	4.28±0.42	4.22±0.45[b]**	4.38±0.44[b]**

Enzyme units are expressed as : ALP and ACP – μ moles x 10^{-1} of phenol liberated; ALT – μ moles of pyruvate released; PPase – μ moles x 10^{-1} of inorganic phosphorus (Pi) released. Treatment of groups: Group I – Control; Group II – 3% Ammonium oxalate in drinking water for 5 days in Group III – Control + N-acetyl-L-cysteine (NAC) (5 mg/100 g body wt (Lp) and vitamin E (5mg/100g g body wt) (i.p);Group IV – Ammonium oxalate + NAC (i.p); Group V – Ammonium oxalate + NAC + Vitamin E. Drugs were administered by interperitonial (i.p) single dose and the vehicle used was saline and olive oil. Comparisons were made as follows : [a]With Group I; [b]With Group II. The symbols a, b also represent statistical significance at: *P<0.05; **P<0.01; ***P<0.001. Values are expressed as mean±SD for six animals

membrane-bound enzymes of the kidney. The activities of pyrophosphatase were decreased in Group II rats. It improved in Group IV and Group V animals showing decreased crystal formation.

Staining viewed by light microscopy

Results were correlated with histopathological sections and H and E staining. Plates showed normal glomerulus and tubules in control kidney section. Lumen with cellular debris and swollen dilated tubules with increased lysosomal activities were seen in Group II rats. Group IV and V rats showed normal tubules and glomerulus with fewer lysosomal activities [Plates 1a-d].

DISCUSSION

Hyperoxaluria is regarded as the major risk factor for CaOx urolithiasis.[7] Oxalate is eliminated by the kidneys. It is 100% ultrafilterable and filtered in glomeruli and excreted in proximal tubule and along with calcium phosphate and oxalate crystals causing tubular damage. Obesity is a risk factor for uric acid urolithiasis. Gastric lipase inhibitors induce fat malabsorption associated with hyperoxaluria.[8] Urinary volume was increased in hyperoxaluric drug-treated rat to reduce CaOx supersaturation. Increase in the concentration of urinary stone forming constituents results in precipitation and growth of crystals within the urinary tract.[9] Hypercalciuria was observed in hyperoxaluric rats. Similar increase has been observed in ethylene glycol supplemented rats.[10] It may be due to defective renal tubular reabsorption of dietary calcium or renal calcium leak.[11] Hypercalciuria can be caused by systemic acidosis and protein overload. On NAC treatment, a considerable decrease in urinary calcium levels was found, and on combination therapy, it was still lowered. Phyllanthus niruri is a plant alkaloid normalizes elevated urinary calcium levels in calcium stone formers.[12] NAC and Vitamin E therapy prevented loss from membrane leak and affords protection to membrane.

Urinary oxalate was significantly increased in hyperoxaluric rats. Similar results were earlier reported.[13] A mild increase in urinary oxalate is an important determinant of CaOx supersaturation[14] facilitating the risk for CaOx crystal formation in earlier sites of nephron.[15] Urinary marker enzymes alkaline phosphatase, acid phosphatase, and alanine transaminase indicate renal cell injury. Jonassen *et al*.[16] proposed that high oxalate promotes stone formation and induces renal injury that generates cellular debris and promotes crystal nucleation and attachment. NAC and NAC and Vitamin E decreased urinary oxalate in hyperoxaluric rats. Similar results have been reported.[17]

AmOx-induced hyperoxaluric rats showed an increase in phosphorous excretion compared to controls in lithiatic condition.[18] This increased excretion is of clinical significance in the formation of calcium phosphate crystals as it is linked to higher rate of recurrence in stones with calcium phosphate supersaturation. Supersaturation may occur first in the loop of Henle[19,20] or in the distal part of the distal tubule.[21] These small crystals can be expelled from the nephron, but these minute calcium phosphate crystals may act as a nidus for CaOx deposition in intermedullary collecting ducts with associated interstitial scarring.[22] However, in this study, phosphorous excretion was unaltered. It may be presumed that phosphorous excretion is less because of decreased calcium phosphate supersaturation and shows less recurrence of stones. NAC and NAC + Vitamin E administration is likely to prove beneficial in preventing CaOx crystallization and thus subsequent stone formation by reducing stone forming constituents.

Urine magnesium levels have been shown to be decreased in hyperoxaluric rats. Hypomagnesuria is commonly found in calcium stone formers.[23] The protective effect of magnesium citrate enhances urinary citrate excretion.[24] Furthermore, magnesium binds with oxalate to form magnesium oxalate. Urinary magnesium deficiency accelerates renal tubular CaOx deposition in hyperoxaluric rats. Thus, lowered Mg/Ox and Mg/Ca ratios were found in stone forming rats.[25]

Interference with crystal growth and aggregation therefore seems a possible therapeutic strategy for the prevention of recurrent stone disease. NAC interacts with urinary

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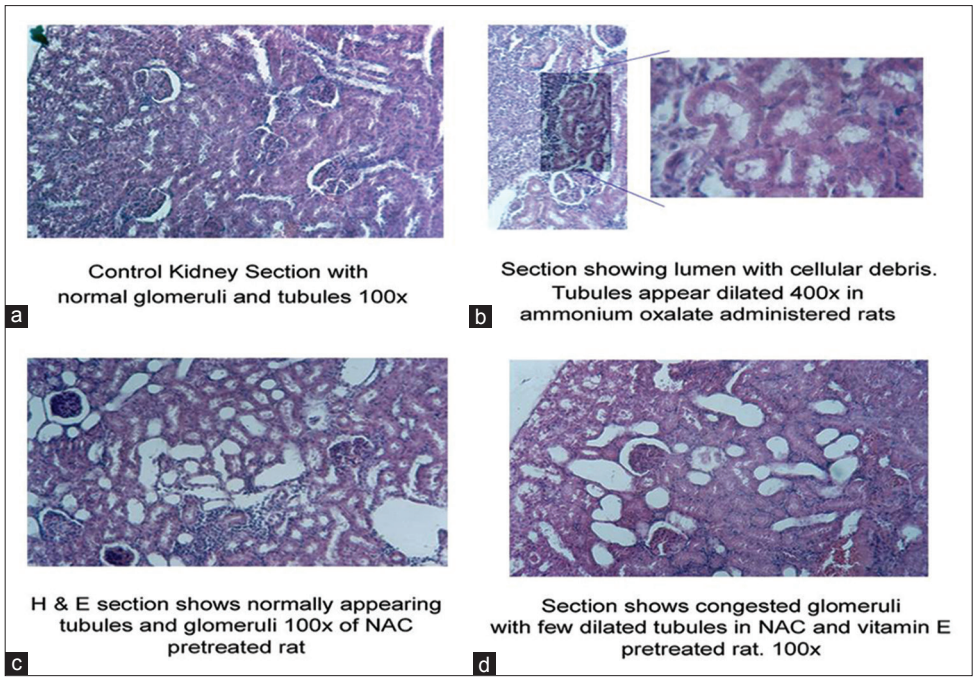

Plate 1: (a-d) Hematoxylin and eosin staining of kidney sections viewed under the light microscope from control, untreated, N-acetyl-L-cysteine, and N-acetyl-L-cysteine + Vitamin E pretreated hyperoxaluric rats. (a) Control, (b) untreated hyperoxaluria, (c) N-acetyl-L-cysteine pretreated, (d) N-acetyl-L-cysteine + Vitamin E pretreated

macromolecules and reduces CaOx nucleation[26] that inhibit crystal aggregations, thus keeping CaOx particles dispersed in solution, so they can be easily eliminated.[27] Furthermore, increased uric acid crystals in urine of stone formers[28] enhance CaOx precipitation. Increased uric acid/GAGs ratio has been reported in stone patients.[29]

Citrate is found to be significantly decreased in this study which decreases the CaOx AP. It also prevents stone formation by lowering osteopontin mRNA expression[30] also modifies shape and inhibits the growth of CaOx crystals. An association between insulin resistance and calcium stone formation related to lowered citrate excretion was seen in calcium stone formers. NAC and Vitamin E improved citrate/calcium ratio suggesting that this combination can act as a better therapeutic agent and are useful biomarkers in assessing stone recurrence in patients.

Urinary GAGs was found lowered in this study. Nesse *et al*.[31] have observed lowered concentration and excretion of urinary GAGs in stone forming patients as compared to normal patients, oxalate/GAGS ratio improved with NAC and Vitamin E. Urinary GAGs restore anticrystal adhering properties to the injured epithelium.[32] Increased proteinuria was corrected by NAC and Vitamin E pretreatment, and this could improve renal function.

Creatinine excretion was significantly decreased in lithogenic rats derived from systemic metabolism of ammoniumoxalat and decreased secretion. Decreased magnesium/creatinine and increased oxalate/creatinine ratios were observed in calculogenic rats. A similar trend was observed by Hussain *et al*.[33] in stone patients. Oxalate/creatinine ratios represent true oxalate levels in hyperoxaluria. Urinary sodium excretion was shown to be significantly higher in patients with hypercalciuria.[34] Similar findings in this study showed treatment with dual drugs decreased urinary sodium and potassium level causing increased reabsorption of calcium thereby leading to lowered urinary CaOx supersaturation.

Urinary ion AP of CaOx is an index of urinary CaOx supersaturation. A marked increase in urinary oxalate and a slight reduction in urinary magnesium results in increased AP (CaOx) index elevating the risk of stone formation.[35] NAC and Vitamin E lowered this index in rats indicating the lowered risk of crystallization.

Urinary marker enzyme studies are indicative of cell injury.[7] NAC and Vitamin E mitigate the toxic effects of oxalate on proximal tubular epithelium. Vitamin E acts as a membrane stabilizer by circumventing the oxyradical-dependent cascade destruction of membrane.[36]

Oxalate synthesizing enzymes were increased in liver and kidney tissues of hyperoxaluria-induced rats. NAC and Vitamin E lowered the activities of these enzymes. LDH is also a marker for membrane integrity and is a regulator for many biochemical reactions. Less intense bands for LDH2 were found in drug-treated hyperoxaluric rats, and they also suppressed GAO activity in liver thereby decreasing kidney oxalate load in these animals. Increased release

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of cellular enzymes into spent media has been reported by Hackett *et al*.[37] which establishes the interaction of CaOx crystals with cells.

Light microscopic studies showed tubular atrophy, inflammatory infiltrates, crystal deposits, and interstitial fibrosis indicating tubulointerstitial damage in hyperoxaluric rats. There was pronounced cystic dilation of tubule with mild congestion of glomeruli, also focal cystic dilations of tubules and little tubular dilation with complete occlusion. However, in NAC and NAC and Vitamin E pretreated animal fields were normal except for focal inflammation and cystic dilations of tubules in kidney. CaOx crystals in the tubular fluid lead to denudation and shedding of membrane particles which opened up sites of injured tissue for further crystal attachment as seen in light microscopy.

Limitations of the study

We have not assessed the NAC therapy on long term basis in different type of renal stone formers.

CONCLUSION

Thus, reduction of excretion of stone-forming constituents in urine, CaOx supersaturation, protective effects of increased magnesium, citrate, and GAGs on NAC and NAC and Vitamin E pretreatment to rats proves to be beneficial in preventing CaOx nucleation, aggregation, and subsequent crystal growth in stone formation. NAC alone can be effective in controlling these events intracellularly. However, in combination with Vitamin E, the toxic effects of kidney oxalate on cell membrane were subdued.

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Conflicts of interest

There are no conflicts of interest.

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