Chapter I
2. CHAPTER I

CLINICAL STUDIES

2.1 INTRODUCTION

Urinary stone disease has afflicted mankind since antiquity and it persists with serious medical consequences, throughout a patient's lifetime. Most calculi in the urinary system arise from components of urine of which calcium oxalate (CaOx) represents up to 80% of the analyzed stones (Daudon et al., 1993). A higher risk has been recorded for stones containing calcium oxalate dihydrate than those for containing calcium oxalate monohydrate (Leusmann et al., 1995). The high incidence of recurrence after an initial stone event underscores the need for an effective medical prophylactic program.

Ferrari et al. (2003) emphasized that importance of medical prevention remains paramount in the management of stone disease. Antioxidant therapy has been found to be effective for treating stone disease (Adhurai and Selvam, 1997; Anbazhagan et al., 1999; Selvam and Ravichandran, 1993). Vitamin E has been increasingly recognized as an essential element in biology and medicine and current research activities are devoted to the possibilities of using these antioxidants for the prevention or treatment of many diseases. α-tocopherol is a promising agent for the prevention of free radical-induced damage (Christiane van den branden et al., 2002). So, the present study was undertaken to find out whether vitamin E therapy could reduce the risk of stone formation.
Although various pharmacological agents are available for urolithiasis and many are effective, such treatment can fail (Rumi et al., 1997). Hence, to identify the suitable agent and to better assess medical therapy, more revolutionized efforts are needed.

Urolithiasis is an art sculpted by the protein architects of the urine, with calcium and oxalate as building blocks and during the process, the architects themselves act as glue and bind the blocks more tightly. Thus, study of these proteins in order to assess the medical management becomes inevitable. So, in the present study, the therapeutic efficacy of vitamin E therapy was assessed using the thiol status and crystallization behaviour of a calcium oxalate crystal binding protein in humans.

2.2 MATERIALS

Normal subjects (age: 25-40 yrs) and stone formers (age: 25-50 yrs) without any complications such as Ischemic heart disease, Renal diseases, Diabetes mellitus were included in the present study. Patients admitted in the Urology ward of Stanley Medical College & hospital and confirmed for the presence of stones by x-rays, KUB, scan and intravenous ureterogram (IVU) and had undergone surgery for the removal stones.

2.2.1 Chemicals and their sources

Fine chemicals, reagents and solvents used during the course of the study were obtained from the following sources.
DEAE cellulose, Coomassie Brilliant Blue (R-250), Thiobarbituric acid, TEMED, β- mercaptoethanol, molecular weight markers and dialysis sacs were obtained from Sigma Chemical Company, St. Louis, U.S.A. 14C- oxalate with specific activity 115mCi/m mole was obtained from Amersham Pharmacia Biotech, UK. Acrylamide, PPO, POPOP and toluene were obtained from SISCO Laboratories, India.

ELISA plates were purchased from Nunc, Denmark. Polyclonal antibodies raised against human urinary fraction I was a gift sample from Dr Asokan, Department of Medical Biochemistry, Taramani, Anti- rabbit IgG conjugated horse radish peroxidase was obtained from National Institute of Immunology, New Delhi.

All other chemicals, reagents and solvents used were of analytical grade from Glaxo laboratories (BDH chemicals), Bombay; SDS fine chemicals, Bombay or Loba-chemie Indo Austramal Company, Bombay, India

2.3 EXPERIMENTAL DESIGN

Group I - Normal healthy individuals (control)

Group II a - Stone patients

Group IIb - Stone Patients supplemented with vitamin E (Evion 400mg) for 3 months

Group IIc - Stone Patients supplemented with vitamin E (Evion 400mg) for 6 months
Group IIId - Stone Patients supplemented with vitamin E (Evion 400mg) for 9 months

2.4 METHODS

24 hrs urine samples were collected from stone formers before supplementation of vitamin E and at three months interval for the duration of nine months after supplementation. Vitamin E supplementation was given along with their regular treatment regime.

2.4.1 Urinary parameters

2.4.1.1 Estimation of oxalate

Oxalate was estimated in 24 hrs urine samples using the method of Hodgkinson and Williams (1972)

Reagents

1. Electrolytic Zinc – approximately 250 mg was washed with 10N nitric acid and then with distilled water before use.

2. Chromotropic acid (4,5-dihydroxy-naphthalene-2, 7 disulphonic acid) – 1 % solution.

3. 2N and 10N H₂SO₄.

4. Standard – 5 mg of potassium oxalate monohydrate in 1.0 ml of distilled water.
**Procedure**

To 2ml of the acidified urine, was added 1.5 ml of distilled water and the pH of the solution was adjusted to 7.0 using dilute sodium hydroxide. 2.0 ml of aqueous saturated calcium sulfate and 14.0 ml of ethanol were added and kept for three hours or preferably overnight. The precipitate was centrifuged at 2000 g. The precipitated calcium oxalate was made up to 2.0 ml using 2 N sulfuric acid. A piece of freshly cleaned zinc was added and kept in boiling water bath unstoppered, till the volume of the solution is reduced to 0.5 ml (avoid drying of the samples) to ensure full colour development.

The zinc piece was removed with a glass rod and 0.5 ml of freshly prepared chromotropic acid was added. The tubes were kept in ice bath and 5.0 ml of chilled concentrated sulfuric acid was added with mixing and heated in a boiling water bath for 30 minutes. The tubes were cooled, diluted to 20 ml with 10 N sulfuric acid and the colour developed was read at 540 nm. Standard curve was prepared using oxalic acid in the concentration range of 10-50 µg and treated similarly.

Urnary oxalate is expressed as mg/24 hours.

**2.4.1.2 Estimation of citrate**

Citrate was estimated using the method of Rajagopal (1984).
**Reagents**

1. TCA 10%
2. Metaphosphoric acid 40%
3. Potassium bromide 2M
4. Potassium permanganate 6.5%
5. Hydrogen peroxide 6%
6. Sulphuric acid 9N
7. Petroleum ether 60 - 80°C
8. Thiourea - Borax solution: 4% thiourea and 2% borax
9. Standard citric acid: 10 mg of anhydrous citric acid dissolved in 100 ml of 1N H₂SO₄. Working standard contains 100 µg / ml.

**Procedure**

Urine was pretreated with TCA, centrifuged at 2000 rpm for 10 minutes, supernatant was taken and to this, 1.0 ml of 9N H₂SO₄, 0.25 ml of 40% metaphosphoric acid, 0.5 ml of 2M KBr and 1.0 ml of 6.5% potassium permanganate were added. The tubes were kept in an ice bath for 10 minutes and decolourised by adding 6% H₂O₂. To this, 7.0 ml of petroleum ether was added; the tubes were stoppered and shaken vigorously for 2 minutes. 4.0 ml of the upper organic layer was transferred.
to 15 ml tubes and 6.0 ml of thiourea / borax was added and again shaken well. The tubes were centrifuged after 5 minutes and the top ether layer aspirated and the coloured aqueous layer was read at 455nm.

Values are expressed as mg / 24 hr urine sample.

2.4.1.3 Estimation of total protein

Total protein was estimated using the method of Lowry et al., (1951).

Reagents

1. Lowry reagent
2. Folin – Ciocalteau Reagent:
   1 : 2 diluted with water.
3. Standard protein solution:
   BSA containing 2 mg / 100 ml was prepared.

Procedure

1.0 ml of urine sample was precipitated with 10 % TCA and the precipitated protein was made up to 1.0 ml with distilled water. 4.5 ml of Lowry’s reagent was added, shaken well and allowed to stand for 10 minutes, 0.5 ml of Folin’s – Ciocalteau reagent was added and standard containing 20-100 µg of protein and blank were treated in a similar manner. The colour developed was measured at 620 nm after 20 minutes.

Values are expressed as mg / 24 hr urine sample.
2.4.1.4 Estimation of calcium

Calcium was estimated using the method of Mustafa and Medeiros (1985). 1.0 ml of the urine was digested with 0.5 ml each of conc. Nitric acid and Perchloric acid in a sand bath until it became colorless.

The solution was made up to 5.0 ml using deionized distilled water and the calcium content was measured in atomic emission spectrophotometer. This sequential plasma emission spectrometer (ARL Model 3410 ICP spectrophotometer system) uses argon plasma as the source and utilizes the spectral range of 189 – 800 nm. The temperature of the flame was maintained at 11,000 K. The samples were nebulized in a Meinhardt and MDSN type nebulizer.

Values are expressed as mg /24 hour urine.

2.4.1.5 Estimation of Uric acid

Uric acid was estimated using the method of Caraway (1963).

Reagents

1. Sodium carbonate 10 %.
2. Phosphotungstic acid (PTA) 10 %.
3. Standard: 60 mg of lithium carbonate was dissolved in 15 – 20 ml of water, heated and mixed with 100 mg of uric acid. This mixture was stirred well and warmed if necessary and made up to 100 ml with distilled water. Working standard was diluted such that the concentration is 10μg / ml.
Procedure

0.5 ml of the sample was made upto 3.0 ml with water. To this, 0.6 ml each of Na₂CO₃ and PTA were added. Standards were treated in the same manner. Readings were taken at 660 nm against blank after 20 minutes.

Values are expressed as mg / 24 hr urine sample.

2.4.1.6 Estimation of phosphorus

Phosphorus was estimated using the method of Fiske and Subbarow (1925)

Reagents

1. TCA 10 %.

2. Ammonium molybdate 2.5 % in 5N H₂SO₄.

3. ANSA 0.5g of ANSA was dissolved in 5.0 ml of 20 % Na₂SO₃ and then 195 ml of 15 % sodium meta bisulphate was added, stirred well, filtered and stored in brown bottle.

4. Standard: Working standard concentration of 8μg. 1.0 ml was prepared from the stock KH₂PO₄ (35.1mg / 100 ml).
**Procedure**

To 0.1 ml of sample 4.4 ml of distilled water, 0.5 ml of ammonium molybdate and 0.2 ml of ANSA were added and mixed well. The blue color developed was read at 650nm after 20 minutes.

Values are expressed as mg / 24 hr urine sample.

### 2.4.2 Isolation of COM binding proteins

COM binding proteins were isolated by the method of Kalaiselvi and Selvam (2001).

COM crystals were freshly prepared before use by mixing 1.5M CaCl$_2$ and 0.3M potassium oxalate in the ratio of 1:5 (adjusted to pH 6.5 using Tris – HCl buffer) with constant shaking at room temperature. After 30 minutes of stabilization of the system by agitation, protein (3 mg protein / ing crystal) was added to the system and made up to twice the volume prior to the addition of protein using 0.3 M potassium chloride. The system was allowed to interact with the proteins with constant shaking of the solution for 1 hour. The solution was centrifuged at 4000 g for 10 minutes and precipitated calcium oxalate was washed with water thrice for removal of the extraneously bound protein. 25mM EDTA was used for extraction of the bound protein. EDTA extract was separated by centrifugation at 4°C at 10000 g for 10 minutes and dialyzed against water at 4°C overnight with two changes of water.
2.4.3 DEAE cellulose column chromatography

About 1.2 mg of protein was loaded onto a DEAE cellulose column (10 x 1 cm) pre-equilibrated with 0.05M Tris – HCl buffer. Elution was carried out first with 0.05M Tris – HCl buffer, followed by 0.05M NaCl in the same buffer and 0.3M NaCl in buffer. Twenty 22 ml fractions were collected in each step of elution and the elution of the protein was monitored in a UVIKON 930 spectrophotometer at 220 nm.

Three major proteins were eluted in each buffer, and the protein fractions were designated as fraction I (buffer eluant), fraction II (eluant of 0.05 M NaCl in buffer) and fraction III (0.3 M NaCl in buffer).

2.4.4 Estimation of thiol content

The thiol content of the isolated COM protein fractions was estimated by the method of Ellman (1959).

Reagents

1. Tris – HCl buffer 0.2M, pH 8.2 containing 0.02M EDTA
2. DTNB – 99mg of DTNB was dissolved in 25ml of absolute methanol
3. Std glutathione 100μg GSH / ml

Procedure

30μl containing 100μg of the purified protein was dissolved in 1.5ml buffer, 0.1ml DTNB and 0.1ml methanol vortexed and centrifuged at 3000 x g for 10 minutes. The color developed in the supernatant was read at 412nm.
The values were expressed as \( \mu \text{g} / \text{mg protein} \).

### 2.4.5 Kinetic studies

#### 2.4.5.1 Oxalate binding assay

Oxalate binding assay was carried out using the method of Seethalaksahmi et al. (1986).

An aliquot of the protein (100\(\mu\)g) was incubated in 1.0 ml of potassium phosphate buffer (50 mM, pH 7.4) or acetate buffer (200 mM, pH 4.5) containing 100 nM labeled oxalate (5000 cpm) at room temperature for 20 minutes. Non-specific binding was determined in the presence of 100 \(\mu\)M cold oxalate. The incubation mixture was filtered through 0.45\(\mu\)m membrane filter with aid of constant vacuum and the filter washed twice with the buffer. The filters were placed in mini vials (7.0 ml) to which 2 ml of scintillation fluid [Toluene- 200 ml, Triton- 100 ml, 2,5 diphenyl oxazole (PPO)- 1.5 g, and 1,4- (2-(5-phenyl oxazolyl)- benzene (POPOP)- 0.015g] was added and the radioactivity was measured in the Kontron Betamatic- IV liquid scintillation counter.

The protein was incubated with various group modifiers such as \(\beta\)- mercapto ethanol, dithiothreitol, N- ethyl maleimide, oxidized glutathione and pyridoxal phosphate (1mM concentration for all the modifiers) for 30 minutes prior to \(14C\)- oxalate addition. Oxalate binding was also carried out in the presence of calcium chelators such as EDTA and EGTA (1mM).
Specific binding was calculated by subtracting non-specific binding from total binding and expressed as pmoles of oxalate bound / mg protein.

2.4.5.2 Spectrophotometric crystallization assay

The Spectrophotometric crystallization assay was carried out according to the method of Hess et al. (1995).

To 1 ml of potassium oxalate solution taken in the quartz cuvette, 1 ml of CaCl2 solution was added, so that the final concentration in the incubation mixture was 4.25 mM for calcium and 0.75 mM for oxalate. All the solutions were prepared in deionised water containing 200 mM NaCl and 10 mM sodium acetate (pH 5.7). Automated time course measurements of OD at 620 nm were performed with a UVIKON 930 spectrophotometer (Kontron instruments, Italy). The experiments were carried with the presence of 10 μg of COM binding proteins.

Slope of the nucleation and aggregation phase was calculated using linear regression analysis and the percentage inhibition of the proteins were calculated, using the formula,

\[ \text{Percentage inhibition} = \left(1 - \frac{S_m}{S_c}\right) \times 100, \]

Where \( S_m \) - slope in the presence of the modifier

\( S_c \) - slope of the control

2.4.5.3 In Vitro Calcium Oxalate crystal growth

Crystal growth assay was carried using the modified method of Fellstrom et al. (1986).
Seed crystal preparation

Calcium oxalate seed crystals were prepared by the method of Weissner et al (1987). Equimolar amounts of calcium chloride (20 mM) and sodium oxalate (20 mM) solutions prepared in 10 mM HEPES buffer (pH 7.0, containing 0.9 % NaCl) were mixed in a magnetic stirrer such that the final concentrations of calcium and oxalate varied from 1 mM to 20 mM to form crystals of varying sizes. The crystals were formed within seconds of mixing the solution. The crystals were precipitated and they were washed repeatedly with HEPES buffer (10 mM, pH 7.0) and dried. Stock crystal was prepared by dissolving 25 mg COM crystal in 1 ml deionised water. The aqueous crystalline slurry was stored at room temperature.

Crystal Growth Assay

Metastable calcium oxalate solutions were prepared by combining 10 ml of CaCl2 (3.4 mM) with 1.0 ml of sodium oxalate (0.22 mM) in barbituric acid buffer containing 133 mM NaCl, 0.03 % acetic acid (pH 5.7 prepared in deionised water). 14C- oxalate (5000 cpm) was added and the metastable solution was allowed to equilibrate for 30 minutes. 20 µL of seed crystal slurry (1.5 mg / ml) was added to the incubation medium. Samples were withdrawn from the chamber prior to the addition of seed crystals and at 10 minutes interval for 40 minutes, centrifuged and the aliquot counted in a liquid scintillation counter.

Inhibitory activity was calculated using the formula,

\[
\text{Inhibitory activity} = \frac{I_0 - C_t - C_\omega}{K} \times \frac{C_t - C_\alpha}{C_\omega - C_\alpha}
\]
Where, \( C_1 \) is the initial concentration of \(^{14}\text{C}-\text{oxalate}\)

\( C_t \) is the concentration of \(^{14}\text{C}-\text{oxalate} \) at time \( t \) for inhibitor

\( C_0 \) is the concentration of \(^{14}\text{C}-\text{oxalate} \) at time \( t \) for control

\( C_\infty \) is the concentration of \(^{14}\text{C}-\text{oxalate} \) at infinite time

2.4.6 SDS – PAGE electrophoresis

The isolated proteins were resolved in 10 % (w/v) sodium dodecyl sulphate polyacrylamide gel electrophoresis by the method of Laemmli (1970).

Reagents

1. 30 g Acryl amide and 0.8 g bis acrylamide in 100 ml of distilled Water

2. Running gel buffer: 18.17 g Tris in 100 ml of distilled water, pH 8.8 containing 0.4 % SDS.

3. Stacking gel buffer: 6 g Tris dissolved in 100 ml of water, pH 6.8 containing 0.4 % SDS.

4. Double distilled water.

5. Ammonium persulfate 10 % solution.

6. TEMED

7. Sample solubilising buffer: 63 mM Tris- HCl buffer, pH 6.8, 2.3% SDS, 5 % \( \beta \) - mercapto ethanol and 10 % glycerol.

8. Electrophoresis buffer: 6.05 g Tris, 28.8 g glycine and 1.0 g SDS in 1 L of distilled water.

10. Staining solution: 0.2 % Coomassie Brilliant Blue (R – 250) in fixing solution.


Sample Preparation

20μg of the protein was treated with sample solubilising buffer and heated for 3 minutes in a boiling water bath.

Procedure

A 10% gel was prepared and the samples were loaded onto the wells. Electrophoresis was carried out at 100 V at 4 ° C. The gel was immersed in 10 % TCA for 1 hour. After washing with distilled water, the gel was soaked in staining solution for 1 hour and destained in destaining solution. Molecular weight of the proteins were calculated using the formula,

\[ R_f = \frac{\text{Distance traveled by the protein}}{\text{Distance traveled by the dye front}} \]

2.4.7 Statistical evaluation

Data are presented as mean ± S.D. statistical analysis was carried out using ANOVA SPSS for windows, Release 9.05.
2.5 RESULTS AND DISCUSSION

2.5.1 Urinary profile of stone forming risk factors

2.5.1.1 Oxalate

The 24 hr urinary excretion of oxalate was 2.22 fold higher in the stone formers group when compared with that of the control [Table 1]. However, this increase in oxalate excretion was brought to near normal during treatment with vitamin E. It decreased by 33 %, 49 % and 52 % when compared to stone formers [p<0.001] progressively during 3rd, 6th, 9th month of treatment with vitamin E. An incidence of 5 – 50 % increased oxalate excretion in stone formers have been already reported by Lamunski et al. (1991).

The cause of hyperoxaluria might be due to increased absorption of dietary oxalate and renal clearance or endogenous production of oxalate (Hatch, 1993). Mikam et al. (2003) found that absence of oxalate degrading bacteria in the gut could promote the absorption of oxalate, there by increasing the level of urinary oxalate excretion and appear to be a risk factor for the presence of absorptive hyperoxaluria.

Hyperoxaluria is a far more significant risk factor in the pathogenesis of renal stones than hypercalciuria (Tiselius, 1996). Oxalate in urine can cause tubular damage by the production of free radicals leading to cell death (Chen et al., 2001). Jonassen et al. (2003) proposed that high concentrations of oxalate promote stone formation in two ways 1. By providing urinary conditions favorable to the formation of calcium oxalate crystals and 2. By inducing renal injury that generates cellular debris and promotes crystal nucleation and attachment.
In our present study, the urinary oxalate excretion is controlled upon supplementation of vitamin E to stone formers.

2.5.1.2 Citrate

Citrate levels were found to be significantly lowered in stone formers when compared to control subjects (p<0.001). Supplementation of vitamin E to these patients leads to a gradual increase in the excretion of citrate. By nine months of treatment, their excretory levels were corrected to the normal limits.

Low urinary citrate excretion is found in stone formers. The incidence of hypocitraturia is found to be 87% for calcium oxalate stone formers by Ratan et al. (2002). Earlier reports also demonstrate the lower incidence of stones in children with increased excretion of citrate (Malnick et al., 2000).

Citrate is an important inhibitor of urolithiasis by forming soluble complexes with calcium and inhibits precipitation of calcium oxalate and phosphate and growth of these crystals (Francois et al., 1986). Hypocitraturia is recognized as one of the important risk factors for stone pathogenesis (Perido et al., 1996; Kaminska et al., 2000).

Both hypocitraturia and hyperoxaluria are conducive to stone formation through several mechanisms including super saturation, oxidative stress on tubular cells, and interference with some natural inhibitors (Marangella et al., 2000).
Table 1: Urinary constituents of control, stone patients and vitamin E treated stone patients

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Oxalate</th>
<th>Total protein</th>
<th>Calcium</th>
<th>Citrate</th>
<th>Phosphorus</th>
<th>Uric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 50)</td>
<td>23.9 ± 5.2  b***</td>
<td>58.5 ± 8.7 b***</td>
<td>189.4 ± 20.7 b***</td>
<td>428.1 ± 63.7 b***</td>
<td>343.0 ± 28.2 b***</td>
<td>268.0 ± 31.5 b***</td>
</tr>
<tr>
<td>(GP I)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>KSP (n = 42)</td>
<td>53.2 ± 8.4  a***</td>
<td>85.9 ±11.6 a***</td>
<td>302.4 ± 40.5 a***</td>
<td>161.4 ± 24.4 a***</td>
<td>503.4 ± 40.3 a***</td>
<td>394.0 ± 33.6 a***</td>
</tr>
<tr>
<td>(GP IIa)</td>
<td></td>
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**Vitamin E Treated**

<table>
<thead>
<tr>
<th></th>
<th>Oxalate</th>
<th>Total protein</th>
<th>Calcium</th>
<th>Citrate</th>
<th>Phosphorus</th>
<th>Uric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd Month (n = 30) (GP IIb)</td>
<td>35.7 ± 3.8  a<em><strong>b</strong></em></td>
<td>66.4 ± 7.9 a<em><strong>b</strong></em></td>
<td>236.6 ± 19.6 a<em><strong>b</strong></em></td>
<td>216.5 ± 23.9 a<em><strong>b</strong></em></td>
<td>422.2 ± 38.9 a<em><strong>b</strong></em></td>
<td>344.2 ± 26.1 a<em><strong>b</strong></em></td>
</tr>
<tr>
<td>6th Month (n = 26) (GP IIc)</td>
<td>27.1 ± 4.6  a<em><strong>b</strong></em></td>
<td>63.8 ± 8.7 a<em><strong>b</strong></em></td>
<td>208.3 ± 23.2 a<em><strong>b</strong></em></td>
<td>375.1 ± 43.3 a<em><strong>b</strong></em></td>
<td>384.6 ± 50.9 a<em><strong>b</strong></em></td>
<td>291.3 ± 26.9 a<em><strong>b</strong></em></td>
</tr>
<tr>
<td>9th Month (n = 24) (GP IIId)</td>
<td>25.2 ± 2.6  aNSb***</td>
<td>61.2 ± 8.1 aNSb***</td>
<td>198.4 ± 18.9 aNSb***</td>
<td>399.0 ± 32.1 aNSb***</td>
<td>359.9 ± 50.1 aNSb***</td>
<td>279.7 ± 22.7 aNSb***</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., expressed as mg/24hr. Comparisons made with a = control Vs various groups, b = KSP Vs various groups, NS = not significant.

*** p< 0.001, ** p< 0.01, * p< 0.05, NS Non - Significant
On vitamin E treatment, there was a progressive increase in the urinary citrate levels. Changes in urinary citrate manifest as altered calcium oxalate crystallization by inhibiting the stage of nucleation via the action of free citrate and the formation of calcium citrate complex (Fan et al., 2001).

2.5.1.3 Calcium

The urinary excretion of calcium was observed to be approximately 1.5 fold increased in the stone forming patients. Vitamin E treatment to these patients normalized the excretion of calcium. The levels of calcium, phosphorus and uric acid were found to be significantly lowered when compared to stone patients by 6 months after vitamin E administration, while they were well within the normal limits by 9 months of treatment.

Increased excretion of calcium has been already reported in human as well as rat (Bek-Jensen and Tiselius, 1997; Rengaraju and Selvam, 1987; Jayanthi and Varalakshmi 1993). Hypercalciuria remains the most prevalent risk factor both in male and female renal calcium stone formers (Hess et al., 1997). Idiopathic hypercalciuria is common in nephrolithiasis occurring in more than 50 % of patients and raises the urinary supersaturation with respect to calcium oxalate and brushite (Monk and Bushinsky, 1996).

Marangella et al. (2000) explained by molecular biology that some types of hypercalciuria are due to genetic mutations altering tubular function. Hypercalciuria may be due to defective renal tubular reabsorption of dietary calcium or renal calcium leak (David et al., 1988).
Supplementation of vitamin E to stone formers lowered the calcium excretion.

2.5.1.4 Phosphorus

Phosphorus excretion was found to be 1.4 fold increased in stone formers when compared to control. The excretion was brought back to normal limits upon supplementation with vitamin E by nine months.

Controversial reports are available stating increased (Ettinger et al., 1986) as well as decreased (Selvam and Varalakshmi, 1990; Selvam and Bilkurien, 1991 and Ali Tekin et al., 2000) excretion of phosphate. Increased urinary phosphorus excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces calcium oxalate deposition (Roger et al., 1997).

2.5.1.5 Uric acid

Uric acid excretion was elevated by 47% in stone formers when compared to non-stone formers. There was a gradual decrease in the excretion of uric acid by subjecting stone forming subjects to vitamin E therapy. The levels were normalized by nine months of vitamin E treatment.

Increased urinary uric acid levels have been reported in clinical studies (Rajpurohit et al., 1999). Uric acid plays a major role in stone formation (Grover et al., 1993). Uric acid is implicated in the etiology of calcium oxalate kidney stones. The predominance of uric acid crystals in
Calcium oxalate stones suggests that it plays a major role in calcium oxalate crystallization. It not only interferes with the solubility of calcium oxalate, but also reduces the inhibitory activity of glycosaminoglycans by binding with them (Grover and Ryall, 1994).

Uric acid binding proteins are capable of binding to calcium oxalate and modulate its crystallization and are suggested to play a primary role in stone formation (Kalaiselvi et al., 1999).

2.5.1.6 Proteins

Urinary protein excretion for normal subjects was found to be 58.5 ± 8.7 mg, while in stone formers, it was found to be 85.9 ± 11.6 mg, which is nearly 1.5 fold higher. On treatment with vitamin E, it gradually decreased and by nine months, the protein excretion was non-significant from the control.

Proteinuria is an important manifestation of renal disease. Increased excretion of proteins has already been reported in stone formers (Grover and Kesnick, 1995). Seven unique proteins with molecular weights ranging from 18.5 kD to 43 kD with over expression of two other proteins have been identified in stone formers urine (Morse and Resnick et al., 1995).

Proteins are the predominant macromolecules in the urine and are the major components of urinary stone matrices (Khan, 1997a). Urinary proteins modulate the crystal nucleation, growth, aggregation and retention within the kidneys. Many proteins like Tamm-Horsfall protein (Bachman et al., 1990), osteopontin (Brown et al., 1992; Kleimann et al., 1995), Bikunin...
Increased protein excretion observed in the present study may be due to membrane damage caused by hyperoxaluria or overexpression of proteins. Damage to urothelium and loss of membrane integrity in urolithic condition has been well established in our laboratory (Selvam, 2002). Membrane fragments are known to induce calcium oxalate nucleation in in-vitro studies (Khan et al., 1996). Recently, brush border membrane vesicles are found to be involved in the promotion of calcium oxalate crystallization in in-vitro model (Fasano and Khan, 2001).

Vitamin E supplementation to humans reduce the urinary excretion of proteins and thereby showing that vitamin E protected the membrane from damage. Vidya et al. (2002) observed that treatment with lupeol and lupeol linoleate to pyridoxine deficient hyperoxaluric rats reduced the extent of tubular damage and minimized proteinuria.

2.5.2 COM binding proteins

2.5.2.1 Excretion of Total COM binding protein

Control subjects excreted nearly 0.875 mg of COM binding proteins / 24 hrs. It accounted for only 1.5% of the total protein excretion. Stone formers exhibited 1.46 fold-increased excretion of total protein, while the increase was nearly 2.4 fold for COM binding protein alone. The COM binding protein excretion was normalized by 6 months of vitamin E treatment (Table 2).
Table 2: Effect of vitamin E treatment on the urinary excretion of total COM binding protein

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Total Protein</th>
<th>Total COM</th>
<th>% Excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (GP I)</td>
<td>58.5± 4.7</td>
<td>0.875 ± 0.079</td>
<td>1.495</td>
</tr>
<tr>
<td>KSP (GP IIa)</td>
<td>85.9± 7.6</td>
<td>2.104 ± 0.288</td>
<td>2.444</td>
</tr>
</tbody>
</table>

Treated

<table>
<thead>
<tr>
<th>Period</th>
<th>Total Protein</th>
<th>Total COM</th>
<th>% Excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd month (GP IIb)</td>
<td>66.4± 5.9</td>
<td>1.535 ± 0.124</td>
<td>2.304</td>
</tr>
<tr>
<td>6th month (GP IIc)</td>
<td>63.8 ±5.7</td>
<td>0.956 ± 0.116</td>
<td>1.498</td>
</tr>
<tr>
<td>9th month (GP IId)</td>
<td>61.2± 5.1</td>
<td>0.915 ± 0.098</td>
<td>1.495</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. for 21 patients. Values are expressed as mg of COM binding protein / 24 hrs.
COM adsorbing protein excretion is increased in kidney stone patients. Over-expression of proteins involved in lithogenesis has been already reported in humans as well as in urolithic rats (Selvam and Kalaiselvi, 2003). Increased expression / excretion of histones and mitochondrial oxalate binding proteins during experimental hyperoxaluria (Selvam and Kannabiran, 1996; Selvam and Sridevi, 1997), nuclear pore complex protein gp210 in cultured cells on exposure to oxalate (Vijaya et al., 1999) and various proteins involved in stone formation like uropontin (Min et al., 1998), 23 kDa COM binding protein (Asokan et al., 2004a) and calcium oxalate binding proteins (Adhirai and Selvam, 1998a) in stone forming kidneys have already been reported.

The urinary excretion of bikunin is elevated in stone formers when compared to control subjects (Suzuki et al., 2001). Stapleton et al. (1993b) reported that the urinary excretion of crystal matrix protein is abundant in stone formers and its excretion is associated with hyperoxaluria. Osteopontin excretion is high in stone forming condition, but its excretion is further elevated when urinary calcium level is increased. Excretion of COM binding protein is associated with injurious hyperoxaluria, and is within normal limits in non-injurious hyperoxaluria (Asokan, 2003; Asokan et al., 2004b).

Vitamin E supplementation to kidney stone forming patients is found to normalize the COM protein excretion. Vitamin E alleviates the oxalate mediated tissue injury, thereby reducing shedding of proteins from tubular cells. Earlier report shows reduced protein excretion by antilithic
agents like *Aeroa lanata* and Vediuppu Chunnam (Selvam *et al.*, 2001). However, this is the first report of its kind to demonstrate the normalization of excretion of proteins involved in lithogenesis by vitamin E therapy.

Urinary proteins adsorb COM crystals and the crystal adsorbed proteins are extracted with EDTA. Studies by Vivek Kumar *et al.* (2003) and Wiessner (2003) stated that macromolecules present in normal urine could effectively coat crystals and block their adhesion to renal tubular cells. Macromolecules present in whole urine acted to decrease binding to cells by coating crystals and four proteins were detected on coated crystals. They are bikunin, osteopontin, prothrombin 1+2 and THP. The coating of urinary crystals with macromolecules during episodes of crystalluria could prevent growth, and aggregation of crystals, as well as prevent the attachment of crystals to healthy urinary epithelia. Coating of calcium oxalate crystals with urine proteins, as would occur *in vivo*, inhibits attachment of the crystals to normal healthy epithelia. These crystal binding proteins isolated in the present study might either prevent adhesion by coating it or aggravate adhesion by bridging between the crystal and renal epithelium. Hence, increased adsorption observed in hyperoxaluric individuals might lead to series of serious consequences that lead to stone formation.

### 2.5.2.2 Elution profile and % distribution of human urinary COM adsorbing proteins

COM binding proteins isolated from human urine were subjected to DEAE cellulose column chromatography and eluted with a stepwise gradient of NaCl; three major protein peaks were obtained [Fig 1a-d]. The
peak obtained in the Tris-HCl buffer [0.01M; pH 7.0] was designated as fraction I and the peaks obtained in 0.05M and 0.3M sodium chloride in buffer were designated as Fraction II and Fraction III respectively. All the three fractions were present in all the groups. Among the various COM binding proteins, the proportion of FII was maximum in control and it was found to be 60.2 %, while in stone formers it was only 37.5 %, which accounts for only half of that excreted in non-stone formers. FI was increased by 43 % in KSP and FIII by 68 %[Fig 2].

Upon supplementation of vitamin E for a period of nine months identical patterns of COM adsorbing protein elution was observed for both control and stone formers. It might be attributed to the beneficial role of vitamin E in minimizing the stone forming risk factors.

2.5.2.3 Thiol content of human urinary COM binding protein fractions

Among the COM binding proteins, control fraction I had the maximum -SH content, which is followed by fraction II. The -SH content of all the three COM binding protein fractions were significantly decreased in KSP [Table 3], when compared to that of control [p<0.001]. Vitamin E supplementation restored the -SH content in all three fractions by nine months.

Depletion in -SH contents have been reported in peroxidised tissue fractions by Selvam and Kalaiselvi (2001a). Upon supplementation of vitamin E, the depletion of -SH content is minimized and there by suggesting that vitamin E prevented the peroxidation.
Fig 1: EFFECT OF VITAMIN E THERAPY ON THE DEAE CELLULOSE ELUTION PROFILE OF COM BINDING PROTEINS

a. Control and KSP

b: KSP and 3 months VKSP

c: KSP and 6 months VKSP

d: KSP and 9 months VKSP
Effect of Vitamin E Treatment on the % Distribution of COM Binding Proteins

CONTROL

KSP

KSP + VIT E 9TH MONTH
Table 3: Effect of vitamin E treatment on the total COM thiol content of COM binding proteins

<table>
<thead>
<tr>
<th>Particulars</th>
<th>F I</th>
<th>F II</th>
<th>F III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (GP I)</td>
<td>4.8 ± 0.05 b***</td>
<td>4.0 ± 0.04 b***</td>
<td>3.6 ± 0.03 b***</td>
</tr>
<tr>
<td>KSP (GP IIa)</td>
<td>3.6 ± 0.04 a***</td>
<td>2.9 ± 0.03 a***</td>
<td>2.9 ± 0.02 a***</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd month (GP IIb)</td>
<td>3.9 ± 0.03 a***b NS</td>
<td>3.2 ± 0.03 a*<strong>b</strong></td>
<td>3.2 ± 0.03 a*<strong>b</strong></td>
</tr>
<tr>
<td>6th month (GP IIc)</td>
<td>4.5 ± 0.05 aNSb***</td>
<td>3.5 ± 0.03 a<em><strong>b</strong></em></td>
<td>3.4 ± 0.03 aNSb***</td>
</tr>
<tr>
<td>9th month (GP IIId)</td>
<td>4.7 ± 0.04 aNSb***</td>
<td>3.7 ± 0.03 aNSb***</td>
<td>3.5 ± 0.03 aNSb***</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. for 10 experiments expressed as µ g / mg protein.

Values are statistically different compared to a – control, b – EG when *** p < 0.001, ** p < 0.01, NS – Non - Significant.
2.5.2.4 Oxalate binding activity

The oxalate binding activities of the COM binding proteins were assayed (F I and F II - pH 7.4; F III - pH 4.5). All the three fractions had oxalate binding activity and the results are shown in Table 4. Maximal oxalate binding activity was associated with F II. Fraction I isolated from kidney stone formers exhibited 78.35% increase in oxalate binding activity \[ p < 0.001 \] when compared to control. Vitamin E supplementation to these renal stone formers for a follow up period of 9 months normalized the oxalate binding activities of all the three fractions.

Fraction I and II exhibit oxalate binding activity at pH 7.4, which is nearer to physiological urinary pH, suggesting that they can bind urinary oxalate leading to biological consequences that are involved in lithogenesis. Fraction III has oxalate binding activity only at pH 4.5. Oxalate binding histones (H1) exhibits binding activity at pH 4.2 (Selvam and Prassannalakshmi, 1996). In our laboratory, we have isolated three major calcium oxalate binding protein from human kidneys using the same methodology (Selvam and Kalaiselvi, 2000; Kalaiselvi et al., 1999) and the corresponding molecular weights of the DEAE cellulose fractions are found to be 45 kDa, 20 kDa and 23 kDa. Fraction III derived from human kidney is found to be basic in nature and has oxalate binding activity at pH 4.5 (Kalaiselvi and Selvam, 2001).

A significant high oxalate binding activity is observed for hyperoxaluric fractions. The total urinary calcium binding protein activity is significantly greater in the active calcium oxalate stone formers when
Table 4: Oxalate binding activity of urinary COM binding proteins of control and various groups

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Fraction I (7.4)</th>
<th>Fraction II (7.4)</th>
<th>Fraction III (4.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (GP I)</td>
<td>252.6 ± 20.7 b***</td>
<td>285.6 ± 22.7 b***</td>
<td>255.1 ± 20.3 b***</td>
</tr>
<tr>
<td>KSP (GP IIa)</td>
<td>334.2 ± 33.1 a***</td>
<td>230.4 ± 16.1 a***</td>
<td>197.7 ± 13.7 a***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treated</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd month (GP IIb)</td>
<td>282.7 ± 17.4 a*b**</td>
<td>262.0 ± 13.8 a<em><strong>b</strong></em></td>
<td>225.1 ± 12.8 a<em><strong>b</strong></em></td>
</tr>
<tr>
<td>6th month (GP IIc)</td>
<td>279.0 ± 18.6 a*b**</td>
<td>270.4 ± 16.4 a**<em>b</em> ***</td>
<td>235.3 ± 17.4 a*b**</td>
</tr>
<tr>
<td>9th month (GP IIId)</td>
<td>258.5 ± 21.0 aNSb***</td>
<td>273.8 ± 19.2 a<em>b</em>**</td>
<td>247.5 ± 20.1 aNSb***</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. for six experiments. Values are expressed as pmoles/mg protein, values were significantly different from a=control, b=KSP when compared with the treated groups. Symbols represent statistical significance *** p < 0.001, ** p < 0.01, * p < 0.05, NS - Non - Significant.
compared with either inactive calcium oxalate stone formers or non-stone forming controls. Cyclosporin A administration or ammonium oxalate treatment increased rat renal calcium oxalate binding, which correlate with lipid peroxidation and concomitant decrease in total thiol in human kidney (Adhirai et al., 2002).

There are reports suggesting the link between lipid peroxidation induced protein modification and the resultant modulatory activity of them (Govindaraj and Selvam, 2001; Kalaiselvi and Selvam, 2001) considered to be vital for oxalate binding activity. Treatment of proteins with -SH group oxidizing agents have either increased or decreased their binding activities (Selvam and Sridevi, 1991).

Vitamin E supplementation normalized the oxalate binding activities of all the three fractions, which may be due to restoration of the thiol status of the proteins.

2.5.2.4.1 Group modifiers

Table 5 presents the oxalate binding activities of the various control and hyperoxaluric fractions after treatment with group modifiers. Calcium chelator EGTA abolished the oxalate binding activity of FII, while, the oxalate binding activity of FI and FIII were unaltered by calcium chelators -SH group modifiers affected the oxalate binding activity of all the three fractions. GSSG increased the oxalate binding activity of all the three fractions, whereas NEM abolished the oxalate binding activity of FIII. The binding activities of fraction I and II were decreased. -SH group reducing
Table 5: Oxalate binding activity of COM binding proteins in presence of group modifiers

<table>
<thead>
<tr>
<th>Particulars</th>
<th>None</th>
<th>β-mercaptoethanol</th>
<th>DTT</th>
<th>NEM</th>
<th>GSSG</th>
<th>Pyridoxal phosphate</th>
<th>EDTA</th>
<th>EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (GP I)</td>
<td>252.6 ± 20.7</td>
<td>NB</td>
<td>NB</td>
<td>106.8 ± 7.6***</td>
<td>293.1 ± 18.5</td>
<td>166.0 ± 17.2**</td>
<td>223.6 ± 15.2</td>
<td>210.6 ± 19.8'</td>
</tr>
<tr>
<td>KSP (GP II)</td>
<td>334.2 ± 33.1</td>
<td>NB</td>
<td>NB</td>
<td>73.0 ± 5.7***</td>
<td>412.4 ± 29.1***</td>
<td>199.6 ± 16.6***</td>
<td>315.7 ± 21.3</td>
<td>300.00 ± 21.6'</td>
</tr>
<tr>
<td>Fraction II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>285.6 ± 22.7</td>
<td>NB</td>
<td>NB</td>
<td>170.1 ± 10.5**</td>
<td>307.4 ± 20.6</td>
<td>265.9 ± 17.1</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>KSP</td>
<td>230.4 ± 16.1</td>
<td>NB</td>
<td>NB</td>
<td>142.4 ± 8.7**</td>
<td>327.8 ± 19.6**</td>
<td>192.2 ± 14.7**</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>Fraction III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>255.1 ± 20.3</td>
<td>216.2 ± 10.8'</td>
<td>199.6 ± 9.6'</td>
<td>NB</td>
<td>300.0 ± 13.7</td>
<td>NB</td>
<td>185.8 ± 15.5</td>
<td>200.5 ± 11.5'</td>
</tr>
<tr>
<td>KSP</td>
<td>197.7 ± 13.7</td>
<td>173.4 ± 9.0</td>
<td>149.4 ± 9.8'</td>
<td>NB</td>
<td>373.9 ± 11.8***</td>
<td>NB</td>
<td>138.1 ± 16.2</td>
<td>158.8 ± 15.5'</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. for six experiments expressed as pmoles / mg protein

Values were significantly different when compared to the respective controls (None). ***p<0.001, **p<0.01, *p<0.05
agents such as β-mercaptoethanol and DTT completely abolished the oxalate binding activities of FI and FII. Fraction III exhibited reduced binding activity. Lysine ε - amino group modifier such as pyridoxal phosphate abolished the binding activity of FIII. The binding activity was considerably decreased in FI and FII.

The calcium oxalate binding proteins seems to be highly specific for oxalate and is only partially inhibited by substrate analogs. Calcium chelators EGTA and EDTA abolished the oxalate binding activity of Fraction II, while for fraction I and III, it is only slightly decreased, inferring that Fraction II might be calcium / calcium oxalate binding protein

- SH group modifiers like GSSG increased the oxalate binding activity of all the three fractions suggesting that oxidation of the protein favors more oxalate binding. Similar observations have been reported for rat kidney mitochondrial oxalate binding protein (Selvam and Sridevi, 1996). It has been postulated that if the – SH group, which is involved in oxalate binding, is altered by GSSG more oxalate binds to the protein (Selvam, 2002). This may be true for this protein as well. Increase in oxalate binding activity in the presence of GSSG will be significant in vivo as GSSG has been found to increase during urolithic condition. Increased oxalate binding activity of these proteins may have an impact on calcium oxalate retention.

Lysine ε - amino group modifiers such as pyridoxal phosphate also abolish the oxalate binding activity of fraction III indicating the involvement of lysine group in the binding site.
2.5.2.5 Spectrophotometric crystallization

Table 6 shows the effect of COM binding protein fractions on nucleation and aggregation of COM crystals. The stone formers protein had an increased promoting effect. The promoting effect was nearly 2.2 fold increased in stone formers when compared to control. Fraction I promoted calcium oxalate nucleation by about 21.9 % and aggregation 8.32 % respectively, when the concentration of the protein was 10 mg / L in the medium. This promoting effect was gradually restored to near normalcy upon treatment with vitamin E. Fraction II and III inhibited nucleation and aggregation in both the control subjects and patients. There was significant loss in the inhibitory activity in these fractions among the stone formers. Supplementation of vitamin E for a period of 9 months to these patients normalized the percentage inhibitory activity of calcium oxalate nucleation and aggregation in the stone formers.

Fraction I promotes nucleation and aggregation of calcium oxalate crystals and fraction II and III inhibits them. Osteopontin rich in aspartic acid residues is another urinary protein present in calcium oxalate crystals and stones (Shiraga et al., 1992). Removal of these acidic residues from the protein, Van de Loo et al., (1987) showed that the inhibitory activity of the protein on calcium oxalate precipitation was lost. Hence, amino acid composition of these proteins might be responsible for their differential behaviour in the crystal nucleation and aggregation system.

Macromolecules that inhibit the nucleation, growth, aggregation and retention of crystals in the kidneys serve as a defense against
Table 6: % Inhibition of urinary FI, FII, FIII of control, stone formers, vitamin E treated groups in *in vitro* calcium oxalate crystal nucleation and aggregation

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NUCLEATION</td>
<td>AGGREGATION</td>
<td>NUCLEATION</td>
</tr>
<tr>
<td>Control (GP I)</td>
<td>-21.9 ± 2.8 b***</td>
<td>-8.3 ± 0.98 b***</td>
<td>36.5 ± 2.9 b***</td>
</tr>
<tr>
<td>KSP (GP IIa)</td>
<td>-48.5 ± 4.6 a***</td>
<td>-11.6 ± 1.01 a***</td>
<td>23.2 ± 2.6 a***</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd month (GP IIb)</td>
<td>-43.8 ± 5.6 a***</td>
<td>-10.7 ± 0.87 a***NS</td>
<td>26.0 ± 3.3 a**<em>b</em></td>
</tr>
<tr>
<td>6th month (GP IIc)</td>
<td>-35.7 ± 2.7 a******</td>
<td>-10.1 ± 0.82 a******</td>
<td>29.7 ± 3.2 a******</td>
</tr>
<tr>
<td>9th month (GP IIId)</td>
<td>-28.4 ± 2.1 a******</td>
<td>-9.3 ± 0.84 a***NS</td>
<td>30.9 ± 2.5 a******</td>
</tr>
</tbody>
</table>

Values are expressed as % inhibition and are mean ± S D for six experiments. Statistical significance was calculated using student's t test.  
***P<0.001, **P<0.01, *P<0.05, NS Non-Significant, comparison made with a-control, b-KSP
nephrolithiasis. Most of the proteins like crystal matrix protein, nephrocalcin, uronic acid rich protein (Khan, 1997a) and osteopontin (Wesson et al., 2003) pertaining to urolithiasis act as inhibitors, but only very rare evidences are there to show the promoting activity of the proteins (Khan, 1997a). Cellular degradation products induced heterogeneous nucleation of crystals at lower and physiologic levels of oxalate and they also promoted aggregation (Khan and Thamilselvan, 2000; Khan et al., 2002). THP purified from healthy subjects it promotes the nucleation and inhibits the growth and aggregation of COM crystals invitro (Chen et al., 2001). Fibronectin, (230 kDa), a multifunctional alpha 2 glycoprotein, inhibits the calcium oxalate crystal aggregation (Tsujita et al., 2000).

Carvalho et al. (2002) studied the effect of two proteins Tamm – Horsfall and uromodulin in calcium oxalate crystallization invitro. THP affects nucleation and inhibits crystal aggregation and UM promotes aggregation of highly polymorphic calcium oxalate crystals. Both THP and UM have the same protein framework but UM is more heavily glycosylated than THP and contains unprocessed mannose – rich chains (Kumar and Muchmore, 1990). Lieske and Coe (1996) revealed that differences in glycosylation of these two molecules exhibited different functional properties. Similarly, the differences between hyperoxaluric proteins and control proteins might be due to certain modifications induced by oxalate or post – translational modifications. Hess et al. (2000) demonstrated that citrate as a main determinant of CaOx nucleation and aggregation as well as crystal morphology. The effect of citrate appears to predominate over those of THP. Citrate potentates the effects of THP, since promotory stone former THP is
turned into a crystallization inhibitor in presence of citrate, leading to formation of smaller and less aggregated crystals. Hence, the role of this protein in *in vivo* crystallization events cannot be predicted as it is exposed to a complex urinary milieu.

Proteins such as dentin, albumin and polyaspartic acid promote crystallization only when they are immobilized, otherwise they have inhibitory activity (Campbell et al., 1989). Addadi and Weiner (1992) suggested that immobilized proteins could act as excellent nucleation sites for calcium salt by exposing an array of carboxylate groups locked in an ordered, rigid conformation. During periods of high calcium oxalate supersaturation, a new layer of calcium oxalate crystals could then form on the matrix band, thereby increasing the likelihood of further deposition of proteins with an affinity for calcium oxalate. Immobilization of the inhibitor protein might convert them to promoter *in vivo*.

Supplementation of vitamin E normalized the promoting as well as inhibitory effect of proteins on calcium oxalate nucleation and aggregation, which may be due to restoration of thiol groups by preventing lipid peroxidation.

2.5.2.6 Crystal growth

Crystal growth studies were carried out in the presence of all the three DEAE cellulose eluted fractions. There was a substantial decrease in the $^{14}$C-oxalate concentration in the solution after the addition of seed crystals in the control in the absence of protein. The system attained
Table 7: Inhibitory activity of COM binding proteins on calcium oxalate crystal growth

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (GP I)</td>
<td>-37.2 ± 3.5 b***</td>
<td>119.0 ± 10.6 b***</td>
<td>23.8 ± 2.1 b***</td>
</tr>
<tr>
<td>KSP (GP IIa)</td>
<td>-101.3 ± 8.2 a***</td>
<td>90.2 ± 8.0 a***</td>
<td>19.8 ± 1.6 a***</td>
</tr>
</tbody>
</table>

Treated

<table>
<thead>
<tr>
<th></th>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd month (GP IIb)</td>
<td>-89.9 ± 7.4 a***</td>
<td>96.8 ± 8.1 a***</td>
<td>9.7 ± 0.8 a***</td>
</tr>
<tr>
<td>6th month (GP IIc)</td>
<td>-66.9 ± 5.5 a***</td>
<td>103.0 ± 8.9 a***</td>
<td>16.6 ± 1.3 a***</td>
</tr>
<tr>
<td>9th month (GP IIId)</td>
<td>-44.0 ± 3.8 a***</td>
<td>113.2 ± 9.5 aNSb***</td>
<td>20.0 ± 1.7 a***</td>
</tr>
</tbody>
</table>

Proteins (10mg/L) was added to the crystal growth assay system as described in materials and methods

Inhibitory activity = \(\frac{I_0}{K} = \frac{C_t - C_w}{C_1 - C_t} \times \frac{C_1 - C_a}{C_w - C_a}\)

Values are mean ± S.D. for six experiments. Statistically different when compared to a – control; b – EG when ***p<0.001, **p<0.01, *P<0.05, NS Non - Significant.
equilibrium at 20 minutes. Control F I exhibited a promoting effect of 37.23\
%, while in KSP it was almost tripled. The reduced inhibition of F II and F III
/ increased promoting activity of F I in stone formers were not observed on
treatment with vitamin E for 9 months (Table 7).

Fraction I seem to promote calcium oxalate crystal growth and
fraction II and III inhibit the same. Most naturally occurring urine proteins
when adsorbed to the crystal surface impart an increased negative charge to
the crystal (Hess et al., 1989). The repulsive effect of like charges on the
crystal surface may well prevent the aggregation of the crystals or prevent
the interactions of the crystal with anionic crystal components, thereby
preventing crystal growth.

Nephrocalcin, THP, uropontin or crystal matrix protein (CMP),
inhibit crystal growth or aggregation of calcium oxalate in urine (Atmanu
et al., 1996). The mitochondrial oxalate binding protein (Selvam and Sridevi,
1991) and histone oxalate binding protein (Selvam and Prasannalakshmi,
1996) have promoting activity on calcium oxalate crystal growth. Selvam
and Govindaraj (2002) reported that 48 kDa protein isolated from stone
matrix is a potent promoter of calcium oxalate crystal growth with high
oxalate binding activity and it is enriched in nucleus and mitochondria.

Glycoprotein gp 210 isolated from human renal nuclear membrane
showed calcium oxalate crystal growth inhibitory properties (Vijaya, 1999).
Han et al. (2002) in the course of nephrocalcin cDNA cloning, identified
FKBP – 12 as an inhibitory molecule of calcium oxalate crystal growth.
Sokalingum et al. (1998) found that a S100 protein Calgranulin made by human kidney is present in human urine and is found to be a potent inhibitor of calcium oxalate growth and aggregation. Aggarwal et al. (2000) stated that both urine and serum of kidney stone patients contained higher concentration of 66 kDa, stimulator of COM crystal growth, which is different from the 66-kDa osteopontin – phosphorylated glycoprotein.

It is observed that human beings excrete in their urine biomolecules, which cannot only inhibit but also stimulate mineral phase formation. Under physiological conditions, a balance exist between the inhibitors and stimulators. The excretion of fraction II and III are inhibitors of crystal growth is low, while the excretion of fraction I is high, which creates an imbalance between the promoting and inhibitory activity of the urine.

Upon supplementation of vitamin E to stone formers the promoting effect of fraction I is found to be decreased and the inhibitory effect of fraction II and III is found to be increased.

Fan et al. (2001) studied the effect of oral intake of potassium citrate (PC) or potassium – sodium citrate (PSC) and calcium – sodium citrate (CSC) on calcium oxalate crystallization. PC and PSC lead to high urinary citrate and pH that inhibited crystallization. PC inhibited nucleation, growth, agglomeration and PSC inhibited beyond nucleation. CSC increased calculuria and crystal growth without affecting agglomeration. Even though, no such role can be assigned to vitamin E as it is involved in the crystallization process, its role is beneficial because it prevents the conformational alterations in the protein induced by hyperoxaluria to its
native form, resulting in reduced promotion or increased inhibition, when compared to stone formers.

2.5.2.7 SDS PAGE of human fraction I

Fig 3 represents the electrophoretic mobility of the fraction I protein isolated from control, KSP and vitamin E treated (9 months) groups. The molecular weight of the protein was found to be 45 kD. The protein from the various groups migrated to the same level. Proteins involved in lithogenesis exhibit a wide range of molecular weights. The existence of several oxalate binding proteins has been demonstrated in human and rat kidney (Selvam and Kalaiselvi, 2003). Oxalate binding protein isolated from nuclear pore complex has a molecular weight of 205 kDa (Selvam et al., 2003). The RBC band 3 membrane protein involved in oxalate transport has a molecular weight of 91 kDa (Baggio et al., 1986). THP, a major urinary protein has a molecular weight of 90 kDa (Kumar and Muchmore, 1990). The molecular weight of rat and human kidney mitochondrial oxalate binding proteins are found to be 62 kDa and 56 kDa respectively (Selvam and Sridevi, 1997). Uropontin is a 50 kD urinary protein similar to osteopontin (Kleinman et al., 1995). Crystal matrix protein is a 31 kD glycoprotein showing various degrees of glycosylation (Anderson, 1979). Oxalate binding histone (H1) has a molecular weight 23kD (Selvam and Kannabiran, 1996). Nephrocalcin is found to have a molecular weight of 14 kDa (Coe et al., 1991).

The molecular weight of this protein is similar to uropontin. Hence, cross-reactivity of this protein with uropontin antibody was checked. No
FIG 3: SDS-PAGE of urinary Fraction I COM binding protein

Lane I = Molecular weight markers

Lane II = Control fraction I protein

Lane III = Fraction I isolated from hyperoxaluric patients

Lane IV = Fraction I isolated from hyperoxaluric patients treated with vitamin E for 9 months
cross reactivity of this protein was observed (data not given) with uropontin antibodies suggesting that this protein is different from the latter.

2.5.2.8 Light microscopic studies

COD crystals were formed in the presence of citrate and in the absence (Fig 4 a) of any protein. However, fraction I isolated from control group promoted formation of individual COM crystals (Fig 4 b) and KSP fraction I induced for aggregation of the formed COM crystals (Fig 4 c). The extent of aggregation of COM crystals was reduced, when vitamin E was given to patients for 9 months (Fig 4 d). Control 45 kDa protein forms COM crystals and hyperoxaluric 45 kDa protein enhances aggregation of COM crystals. COM, is the thermodynamically stable hydrate and most abundant crystal found in kidney stones (Coe and Parks, 1988), while COD is more frequently observed in asymptomatic crystalluria (Robertson et al., 1969; Elliot and Rabinowitz, 1980). Formation of COD in preference to COM may be protective against stone disease by reducing attachment to renal tubule cells (Weissner et al., 1986; Wesson et al., 1995). The formation of COD crystals may be important for two reasons 1) they present a higher positive charge than COM crystals. This is due to the fact that they display more calcium ions per unit of cell. 2) There are repulsive charges between crystals, which decrease the formation of aggregates. As aggregation is one of the main factors of lithogenesis, formation of COD crystals will lead to decreased incidence of stone formation (Cerini et al., 1999). Urinary inhibitor proteins from normal persons preferably form COD formation over COM (Wesson et al., 2000). As control 45 kDa promoter protein forms COM
**FIG.4**

\[ A = \text{CONTROL COD CRYSTALS} \]

\[ B = \text{COM CRYSTALS FORMED IN THE PRESENCE OF CONTROL FRACTION I} \]

\[ C = \text{AGGREGATED COM CRYSTALS FORMED IN THE PRESENCE OF HYPEROXALURIC FRACTION I} \]

\[ D = \text{LESS AGGREGATED COM CRYSTALS FORMED IN THE PRESENCE OF FRACTION I DERIVED FROM VITAMIN E TREATED HYPEROXALURIC INDIVIDUALS (9 MONTHS)} \]
EMBRYOLOGICAL CHANGES INDUCED ON ALCHEMIA, A TUMOR FORMED IN THE PRESENCE OF ABSENCE OF FRACTION F DERIVED FROM A VARIOUS EXPERIMENTAL CONDITIONS.
crystals, it increases the risk of stone formation, but since aggregates are not formed and the percentage distribution of this protein is less when compared to other inhibitor proteins (20 and 23 kDa) and also there is lack of supersaturation of calcium oxalate, the effect of 45 kDa protein might be insignificant in \textit{in vivo} condition. But, in stone formers, the decreased excretion of other inhibitory proteins and enhanced excretion of defective 45 kDa protein, which is modified by excessive free radical production will favour the formation of COM aggregates, that can block the tubules, damage the urothelium get retained in the urinary tract and the process and ultimately retention, a process that finally culminates into with the formation of macro / microliths

Upon supplementation of vitamin E, the aggregation of COM crystals seems to be less pronounced. Thus, the salient feature of vitamin E is not only to upgrade the antioxidant status of the cell, but also to protect the proteins from oxidative damage, which indirectly aids in protection of stone formation.

2.6 CONCLUSION

Vitamin E therapy as assessed by the routine urinary risk factors reveal that it is successful. Apart from these factors, the macromolecules present in the urine are capable of altering the crystallization rate In order for a molecule to take part in the lithogenic event, it should have the capacity to bind crystals. Hence, the Calcium oxalate monohydrate binding proteins were isolated from these patients and studied. It was found that hyperoxaluric proteins are thiol depleted and aggravated promoters/
subnormal inhibitors. On fractionation, three COM binding proteins were obtained among which the one eluted in the buffer alone designated as fraction I was comparatively high in stone formers than non-stone formers. This fraction I aggravates calcium oxalate crystallization *invitro* and also favours the formation of COM crystals, which retains within the kidney rather than COD. Stone formers fraction I exhibits higher promoting activity and forms COM aggregates which worsens the situation. Upon vitamin E supplementation, the excretion of COM binding protein was well within the non-stone formers range. The kinetic behavior was also similar to that of non-stone formers.

The adverse effects of oxalate/oxalate induced oxidative stress to the protein is reverted by Vitamin E.

2.7 QUESTIONS THAT ARISE AND FUTURE DIRECTIONS TO WORK

1. Is this protein present in the kidney of all species?

2. If so, what is the nature of the protein in hyperoxaluric kidneys?

3. Will pretreatment of Vitamin E prevent the oxidative damage to the protein?