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2D map of proteins from human renal stone matrix and evaluation of their effect on oxalate induced renal tubular epithelial cell injury

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ABSTRACT

Purpose: Proteins constitute a major portion of the organic matrix of human calcium oxalate (CaOx) renal stones and the matrix is considered to be important in stone formation and growth. The present study evaluates the effect of these proteins on oxalate injured renal epithelial cells accompanied by a 2D map of these proteins.

Materials and Methods: Proteins were isolated from the matrix of kidney stones containing CaOx as the major constituent using EGTA as a demineralizing agent. The effect of more than 3kDa proteins from matrix of human renal (calcium oxalate) CaOx stones was investigated on oxalate induced cell injury of MDCK renal tubular epithelial cells. A 2D map of >3kDa proteins was also generated followed by protein identification using MALDI-TOF MS.

Results: The >3kDa proteins enhanced the injury caused by oxalate on MDCK cells. Also, the 2D map of proteins having MW more than 3kDa suggested the abundance of proteins in the matrix of renal stone.

Conclusion: Studies indicate that the mixture of >3kDa proteins in the matrix of human renal stones acts as promoter of calcium oxalate crystal nucleation and growth as it augments the renal epithelial cell injury induced by oxalate. The effect of promoters masks the inhibitors in the protein mixture thereby leading to enhanced renal cell injury. 2D map throws light on the nature of proteins present in the kidney stones.

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Urolithiasis; Calcium Oxalate; Madin Darby Canine Kidney Cells; Electrophoresis, Gel, Two-Dimensional

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INTRODUCTION

With its multifactor aetiology and high rate of recurrences, urinary tract stone disease provides a medical challenge. Depending on the socio-economic conditions and subsequent changes in the dietary habits, the overall probability of stone formers differs in various parts of the world: 1-5% Asia, 5-9% Europe, 13-15% USA and 20% Saudi Arabia (1). Calcium containing stones are the most common, consisting of about 75% of all urinary calculi, which may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%). The controlling influence of macromolecules in the construction of healthy biomineralised tissues is undisputed (2-4). It is now well recognised that the organic component of such tissues, which in animals include bone, shell, dentin and enamel, is crucial to the biomineralization process. Some macromolecules

in these systems are responsible for initiating mineralisation, defining its physical limits and dictating its cessation, but others provide an architectural framework upon which the inorganic salts are laid down. Less clearly defined, however, are the roles played by macromolecules in the formation of human uroliths, a process possessing all the hallmarks of uncontrolled mineralisation (5,6).

Many proteins occur in stone, but their role in urolithiasis remains unknown. Calculi contains some proteins normally present in urine, in addition to others arising from injury inflicted by the stones themselves, making it impossible to discriminate between those that bind to the stone as it grows, but play no role in its development (7); the inhibition is generally understood to arise mainly from the non-dialyzable molecules of urine, particularly acid glycoproteins, and acidic glycoproteins and glycosaminoglycans (8,9). Some inhibitor molecules have been identified, including Tamm-Horsefall Protein, uropontin (10,11), calgranulin (12), bikunin (13), and prothrombin F1 fragment (14). Thus, in order to understand the mechanism of stone genesis, it is essential to determine the characteristics of molecules constituting the urinary stone matrix. In the present study, we analysed a 2D map (2- dimensional polyacrylamide gel electrophoresis) of human renal stone matrix proteins by MALDI-TOF (Matrix-assisted laser desorption/ionization-Time of Flight) to throw light on the matrix proteins and also study their effect on oxalate injured renal epithelial cells.

MATERIALS AND METHODS

Human Renal stones collection

Stones surgically removed by Percutaneous nephrolithotomy (PNL) from the kidney stone patients were obtained from the Department of Urology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Stones were preserved at 4° C before study. Stones were of non-infectious nature and were collected from those patients who were more than 25 years of age and were suffering from no other abnormality. After FTIR (Fourier transform infrared spectroscopy) analysis, the stones with calcium and oxalate as their major components were selected for present study. Thirty stones with calcium and oxalate, as the major components were used for further studies. Thirty stone samples were randomly pooled into 5 groups, each group containing 6 stone samples.

Protein extraction from Human Renal stones

Proteins were isolated from the matrix of kidney stones containing calcium oxalate (CaOx) as the major constituent using EGTA as a demineralising agent. Stones were washed in 0.15 M sodium chloride (NaCl) and were then dried and pulverized with a mortar and pestle. For extraction of the organic matrix of powdered stone, each gram of stone was suspended in 10 mL of 0.05 M EGTA (ethylene glycol tetraacetic acid), 1 mM PMSF (phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride) and 1% β-mercaptoethanol. The extraction was carried out for 4 days at 4° C with constant stirring. The suspension was centrifuged for 30 minutes at 10,000 g and at 4° C. The supernatant of EGTA extract was filtered through Amicon ultra centrifugal filter device (Catalog UFC 800324) with a molecular weight cut off 3kDa at 4° C and concentrated to a known volume. The excess of the 3kDa fractions were stored at -20° C for further studies (15).

Protein determination

Total protein concentration was determined by Lowry's method using BSA as a standard (16).

Assay to measure inhibitory activity of protein w.r.t Calcium Oxalate (CaOx) crystal nucleation

The method used was similar to that described by Hennequin et al. with some minor modifications (17). Solutions of calcium chloride ($CaCl_2$) and sodium oxalate ($Na_2C_2O_4$) were prepared at the final concentration of 3 mmoL/L and 0.5 mmoL/L, respectively, in a buffer containing Tris 0.05 moL/L and NaCl 0.15 moL/L at pH 6.5. Both solutions were filtered through a 0.22 µm filter; 1.5 mL of $CaCl_2$ solution was mixed with different concentrations of extracted proteins. Crystallization was started by adding 1.5 mL of $Na_2C_2O_4$ solution. The final solution was stirred at 37° C repeatedly after an interval of 60 sec for 8 min. The

absorbance of the solution was monitored at 620 nm after every 60 sec. The percentage inhibition produced by the protein extract was calculated as [1-(Tsi/Tsc)] X 100, where Tsc was the turbidity slope of the control and Tsi the turbidity slope in the presence of the inhibitor.

Assay to measure activity of protein w.r.t. CaOx crystal growth

Activity against CaOx crystal growth was measured using the seeded, solution-depletion assay, according to the method described by Nakagawa et al. (18).

Cell Culture

Madin-Darby Canine kidney (MDCK) cells were obtained from National Centre of Cell Sciences (NCCS, Pune). The cells were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) with 2.0 mM L-glutamine adjusted to contain 3.7 g/L sodium bicarbonate, 4.5 g/L glucose. Media was supplemented with 1% Penicillin (100 units/mL)-Streptomycin (10,000 µg/mL) and 10% fetal bovine serum. Cells were cultured in 25 cm^2 tissue-culture treated flasks at 37° C and 5% CO_2 in humidified chambers (19).

Oxalate-induced Cell Injury

MDCK cells were incubated in DMEM containing 1 mM sodium oxalate in the presence of different concentrations of protein samples for 48 hours (20). Cell injury was assessed by measuring the cell viability through MTT and LDH (Lactate dehydrogenase) Assay.

Preparation of the protein samples

For cell culture studies, the proteins was dialysed through Millipore Amicon Ultra Centrifugal Filters, 3kDa and desalted by ReadyPrep 2-D Cleanup Kit (catalog 163-2130) and it was reconstituted in 0.22 µm filtered distilled water using Millipore Millex GV Filter Unit 0.22 µm (Catalog SLGU033RS).

MTT Assay

Cell viability studies via MTT test were conducted by the method described by Fulya Karamustafa et al. with slight modifications (21). MDCK cells were suspended in DMEM with serum and plated into the microwells of 96-well tissue culture plates. Plates were incubated for 24 h at 37° C in a humidified incubator containing 5% CO_2. Then the medium was removed from wells. 200 µL DMEM (without serum) containing different concentrations of proteins with and without sodium oxalate were added into the wells. After 48 hours, the medium was removed. Each well was treated with 100 µL medium and 13 µL MTT solution, and incubated for a further 3 hours. Then, plates were emptied and 100 µL isopropanol was added to dissolve the formazan precipitate. The developed colour was read at a wavelength of 570 nm with spectrophotometer.

LDH Leakage Assay

MDCK cells were suspended in DMEM with serum and plated into the microwells of 96-well tissue culture plates. Plates were incubated for 24 h at 37° C in a humidified incubator containing 5% CO2. Then the medium was removed from wells. 200 µL DMEM (without serum) containing different concentrations of proteins with and without sodium oxalate were added into the wells for 48 hours. LDH leakage assay was performed by the LDH Cytotoxicity Assay Kit (Cayman 10008882) according to the manufacturer's instructions (22).

Statistical analysis

Data were expressed as mean values of three independent experiments (each in triplicate) and analyzed by the analysis of variance ($p < 0.05$) to estimate the differences between values.

2-D Gel Electrophoresis

The samples were desalted using ReadyPrep 2-D Cleanup Kit and dissolved in 125 µL of sample rehydration buffer containing 8 M urea, 2% w/v CHAPS, 50 mM DTT, 0.2% w/v Ampholytes and 0.0002% bromophenol blue. IEF was first carried out using Bio-Rad IPG strip (pH 3-9; 7 cm) in Bio-Rad protean IEF cell according to manufacturer's instructions, followed by equilibration for 15 minutes each in equilibration buffer I (6 M Urea, 2% SDS, 0.375 M Tris HCl (pH 8.8), 20% Glycerol, 130mM DTT) and equilibra-

tion buffer II (6 M Urea, 2% SDS, 0.375 M Tris HCl (pH 8.8), 20% Glycerol, 135mM Iodoacetamide). Equilibrated IPG strips were loaded onto a 10% polyacrylamide gel sealed with overlay agarose, and electrophoresed at a constant voltage of 100 V. The gel was stained by silver staining and analysed using Biorad PD Quest Advanced 2D Analysis Software. The spots of interest were manually excised from the gel and were destained using destainer provided in the ProteoSilver™ Plus Silver Stain Kit (PROTSIL2, Sigma-Aldrich Co.) followed by in-gel digestion using Trypsin profile IGD kit (PPO100, Sigma-Aldrich Co.). The proteins were identified by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS followed by MASCOT database search.

Tryptic in-gel digestion of purified protein

Single band detected after molecular-sieve chromatography was excised from the gel and was destained with destainer provided in the ProteoSilver™ Plus Silver Stain Kit (PROTSIL2, Sigma-Aldrich Co.). Trypsin profile IGD kit (PPO100, Sigma-Aldrich Co.) was used for in-gel digestion of purified protein. Destained gel piece was dried for approximately 15 to 30 min. Trypsin solubilised in 1 mmoL/L HCl and mixed with 40 mmoL/L ammonium bicarbonate and 9% acetonitrile was added to the destained gel piece. Gel piece was fully covered by the addition of 40 mmoL/L ammonium bicarbonate and 9% acetonitrile (pH 8.2) solution and was incubated for 5 hours at 37° C. After the incubation, liquid was removed from the gel piece and transferred to a new labeled Eppendorf tubes and was preserved for mass spectroscopic analysis (23).

Peptide mass fingerprinting by MALDI-TOF-MS

Peptides were extracted into the extraction solution and dried by speedvac. Dried spots samples were spotted onto the MALDI plate with thorough mixing with matrix at 1:1 concentration and analyzed by MALDI TOF/TOF ULTRALFLEX III (Bruker Daltonics).

Mascot Protein Identification

The mass/charge spectra obtained were searched in MASCOT search engine (http://www.matrixscience.com) using all the 3 databases (MSDB, SwissProt, NCBInr). For search, peptides were assumed monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. An Homo sapiens taxonomy restriction was used, only one missed cleavage was allowed, and peptide mass tolerance of 1.2 kDa was used for peptide mass fingerprinting.

RESULTS

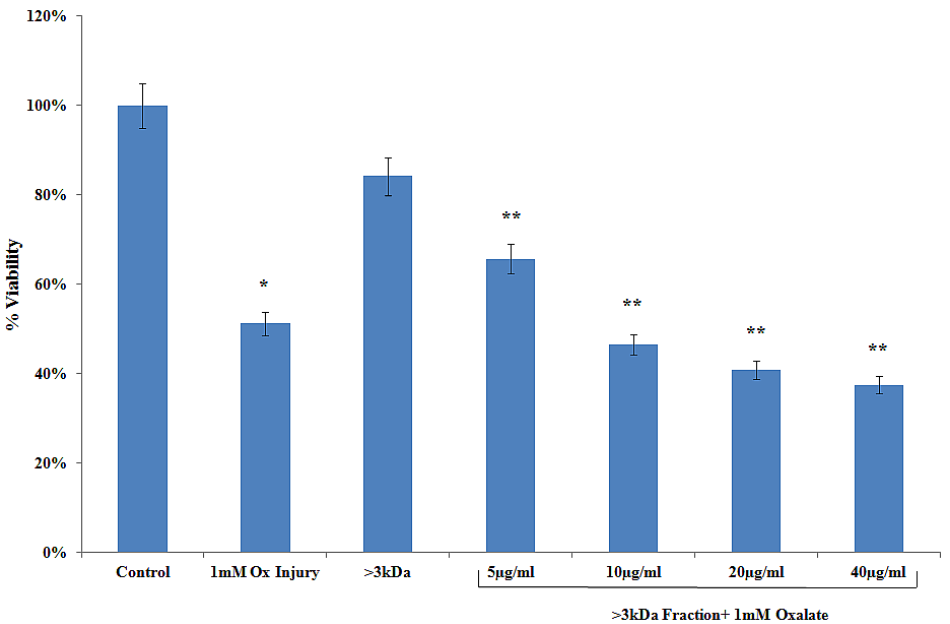
Activity study of proteins from human renal stone on CaOx assay system

Whole EGTA extract and >3kDa were assayed to measure the activity against CaOx crystal nucleation and growth. Different concentrations of whole extract showed both promoter and inhibitory activity against nucleation of CaOx crystal as well as CaOx growth.

Bioactivity of >3kDa fraction on Oxalate Injured MDCK cells

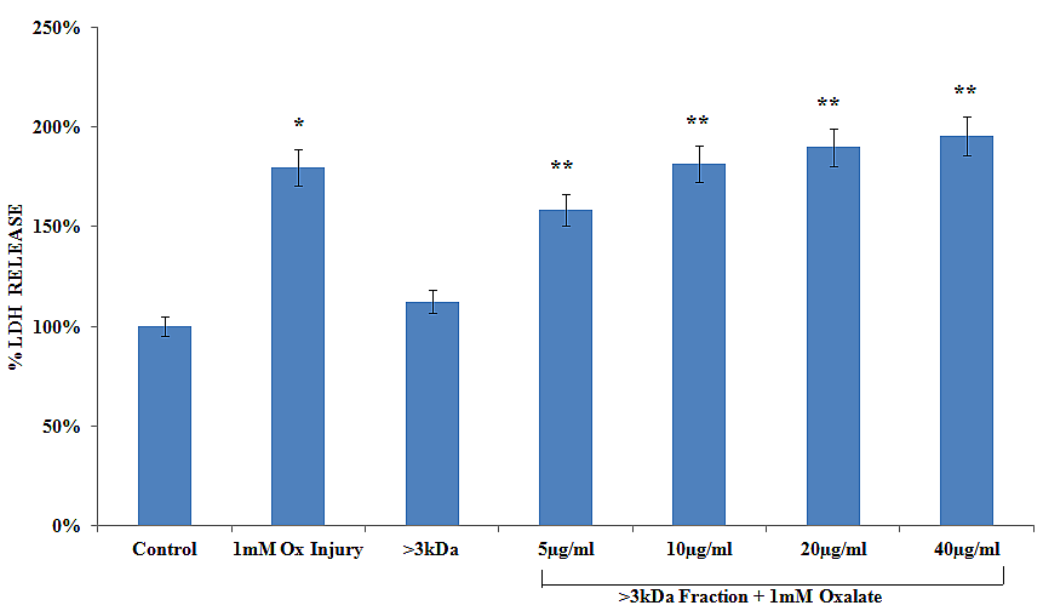
Activity of >3kDa fraction was analyzed to measure the effect of these proteins on oxalate injured MDCK cells. MTT (Figure-1) and LDH (Figure-2) assays were used for analyzing the effect of the proteins on oxalate injured MDCK renal epithelial cells. The oxalate induced a significant injury to the cells which could be ascertained by a decrease in viability in MDCK cells. The protein extract alone (40 µg/mL) had no effect on the cell injury in the absence of oxalate indicating that even at the highest concentration of protein extract used there was no cytotoxicity to the cells. >3kDa fraction showed to both prevent and enhance the injury caused by oxalate on MDCK cells in a dose dependent manner. Concentrations of 5 µg/mL, 10 µg/mL, 20 µg/mL and 40 µg/mL were used. Concentration of 5 µg/mL showed protective effect, thereby increasing the cell viability to the oxalate injured cells. But as the concentration increased from 5 µg/mL to 40 µg/mL the cell viability decreased in a dose dependent manner reflecting the enhanced injury caused by the proteins present. The concentration dependent percentage viability was observed in both the assays i.e. MTT and LDH. Both MTT and LDH assays showed the same effects, thus confirming the activity of the proteins. A significant increase in LDH release was seen when the cells were exposed to

Figure 1 - Effect of >3kDa fraction on MTT assay. Data are mean ± SEM of three independent observations.



*P < 0.01 versus untreated control. **P < 0.05 versus oxalate control (the experiment was done thrice in triplicates each time).

Figure 2 - Effect of >3kDa on the % LDH release. Data are mean ± SEM of three independent observations.



* p < 0.05 versus untreated control, ** p < 0.05 versus oxalate control.

oxalate alone (from 100% of control to 179.92% of oxalate injured).

MTT assay also reflected the same pattern. % viability of cells decreased in cells from 100% to 51.23% injured with oxalate. By these tests it can be suggested that the proteins that lead to cell injury by oxalate mask the activity of the proteins that inhibit the same.

2D-PAGE

2D PAGE of >3kDa proteins revealed the abundance of proteins in the matrix of human CaOx containing kidney stones (Figure-3). A total of 66 spots were detected using Biorad PD Quest Advanced 2D Analysis Software. 2D-PAGE revealed that the proteins present in the stone matrix were of both high and low molecular weight. The proteins were distributed throughtout the gel indicating the presence of both cationic and anionic proteins. Out of the 66 spots, 7 most prominent spots spread across the isoelectric points (pI) were further analysed using MALDI-TOF MS and MASCOT server. The identified proteins are given in Table-1.

DISCUSSION

The role played by the proteins present in the human CaOx renal stones in the course of crystallization is yet not clear. Some of these proteins promote crystal formation, growth, aggregation and retention, while others inhibit these processes. Their activity is often complex and depends on the urine conditions prevailing at the time of crystallization or retention. The same protein can both promote as well as inhibit a process. Under normal conditions, the crystals of calcium oxalate that form are small and well protected from crystal growth and crystal aggregation by a cover of inhibitory macromolecules. If inhibitors of crystal formation were not able to act and control their size, the final result will be nephrolithiasis (24,25). Proteins which cover crystal surface and may lead to inhibition of its growth or ability to aggregate while the same proteins bound to a surface may act to accumulate salt ions and forms a template for the first nucleus. The latter will play a role when stone formation involves processes at cell surfaces and in the sub-epithelial space (26). The purpose of the present study was to explore the 2D map of human renal stone matrix proteins by MALDI-TOF-MS to throw light on the matrix proteins and also to study their effect on oxalate injured renal epithelial cells.

Proteins have a strong affinity for CaOx crystals (27). The role of matrix compounds is different in the formation of the stone center and in the subsequent build-up of the stone (28). The same protein which inhibits crystal formation might promote

Figure 3 - 2D page of >3kDa proteins from human renal CaOx stones.

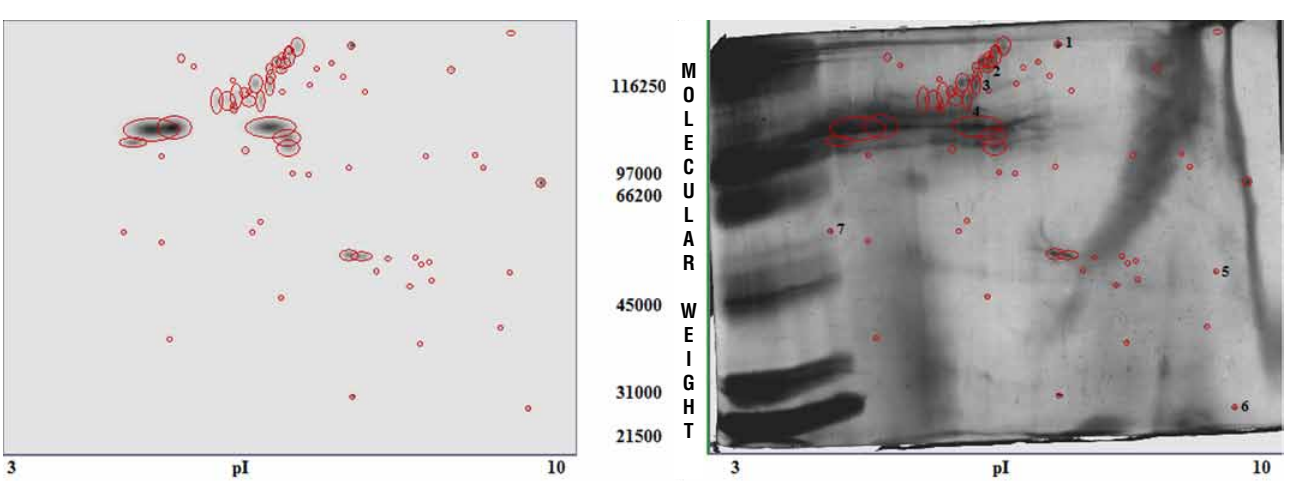


Table 1 - Details of proteins identified by MALDI-TOF MS and MASCOT database searching.

Spot	Protein match	MW (Da)	PI	% Sequence coverage	Function
1	Disheveled-associated activator of morphogenesis	1,23,976	6.81	9%	Binds to disheveled (Dvl) and Rho, and mediates Wnt-induced Dvl-Rho complex formation. May play a role as a caffolding protein to recruit Rho-GDP and Rho-GEF, thereby enhancing Rho-GTP formation. Can direct nucleation and elongation of new actin filaments.
2	Glutamate receptor delta-1 subunit	113046	6.23	17%	Receptor for glutamate. L-glutamate acts as an excitatory neurotransmitter at many synapses in the central nervous system. The postsynaptic actions of Glu are mediated by a variety of receptors that are named according to their selective agonists.
3	Caspase recruitment domain-containing protein	114880	5.65	13%	Activates NF-kappa-B via BCL10 and IKK. Stimulates the phosphorylation of BCL10. Contains 1 CARD domain. Contains 1 guanylate kinase-like domain. Contains 1 PDZ (DHR) domain.
4	ALBU_BOVIN	71279	5.82	3%	Serum albumin, the main protein of plasma, has a good binding capacity for water, Ca(2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood. Major zinc transporter in plasma, typically binds about 80% of all plasma zinc.
5	VP7 glycoprotein precursor	37523	4.68	34%	Outer capsid protein involved in attachment and possibly entry into the host epithelial cell. It is subsequently lost, together with VP4, following virus entry into the host cell. The outer layer contains 780 copies of VP7, grouped as 260 trimers. Rotavirus attachment and entry into the host cell probably involves multiple sequential contacts between the outer capsid proteins VP4 and VP7, and the cell receptors
6	Chymotrypsinogen A	26230	8.52	14%	Chymotrypsinogen is a precursor (zymogen) of the digestive enzyme chymotrypsin.
7	Plasminogen	15782	8.73	54%	Plasminogen is th inactive precursor of the trypsin-like serine protease Ipasmin. It is normally found circulating through the blood stream. When plasminogen becomes activated and is converted to plamin, it unfolds a potent enzymatic domain that dissolves the fibrogen fibers that entangle the blood cells in a blood clot.

the growth of the crystal. Thus, the same protein acts differently at different stages of stone formation.

In the present study, when the >3kda fraction was examined for its effect on oxalate injured MDCK cells, it majorly reflected promoter activities thereby leading to an increased cell death in a dose dependent manner. These results suggest that the proteins which are promoters of crystallization in nature mask the activity of the proteins which are inhibitory in nature, thereby leading to an enhanced cell injury, and consequently cell death. Our observations are in conformity with the observations that that renal epithelial damage can lead to increased crystal attachment (29).

The 2D map suggests that abundant proteins are present in the matrix proteins which are of both high and low molecular weight. Also both anionic and cationic proteins are present. Solution depletion (30) and examination of crystals incubated in protein solutions by transmission electron microscopy (31) tested the theory of physical adsorption of urine proteins on surfaces of CaOx crystals. Results showed proteins have a strong affinity for CaOx crystals. Adsorption of anionic proteins was sensitive to calcium ion concentration, whereas cationic protein adsorption depended upon the oxalate ion concentration with temperature and pH playing only a minor role.

A pathological crystallization leading to stone formation might be the net result of one or several abnormalities or defects in the control of this process. Low concentrations or structural abnormalities of crystallization modifying macromolecules or small molecules will cause increased growth and aggregation of crystals (32). Therefore, any crystallization that occurs most certainly is facilitated by promoters and it has been suggested that lipoprotein membranes from the brush border of proximal tubular cells might serve this purpose (33).

CONCLUSIONS

Studies indicate that the mixture of >3kDa proteins in the matrix of human renal stones augment the renal epithelial cell injury induced by oxalate and thereby may act as promoters of calcium oxalate crystal nucleation and growth. The effect of promoters masks the inhibitors in the protein mixture thereby leading to enhanced renal cell injury. 2D map throws light on the nature of proteins present in the kidney stone matrix, however, their exact role in the mechanism of kidney stone formation warrant further investigations.

ABBREVIATIONS

CaOx: calcium oxalate
MDCK: madin darby canine kidney
DMEM: dulbecco's modified eagle's media
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
LDH: lactate dehydrogenase
2D PAGE: 2 dimensional polyacrylamide gel electrophoresis
MALDI-TOF: Matrix-assisted laser desorption/ionization- Time of Flight
FTIR: Fourier transform infrared spectroscopy

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CONFLICT OF INTEREST

None declared.

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