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Kidney stone matrix proteins ameliorate calcium oxalate monohydrate induced apoptotic injury to renal epithelial cells

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ABSTRACT

Aims: Kidney stone formation is a highly prevalent disease, affecting 8–10% of the human population worldwide. Proteins are the major constituents of human kidney stone's organic matrix and considered to play critical role in the pathogenesis of disease but their mechanism of modulation still needs to be explicated. Therefore, in this study we investigated the effect of human kidney stone matrix proteins on the calcium oxalate monohydrate (COM) mediated cellular injury.
Main methods: The renal epithelial cells (MDCK) were exposed to 200 μg/ml COM crystals to induce injury. The effect of proteins isolated from human kidney stone was studied on COM injured cells. The alterations in cell-crystal interactions were examined by phase contrast, polarizing, fluorescence and scanning electron microscopy. Moreover, its effect on the extent of COM induced cell injury, was quantified by flow cytometric analysis.
Key findings: Our study indicated the antilithiatic potential of human kidney stone proteins on COM injured MDCK cells. Flow cytometric analysis and fluorescence imaging ascertained that matrix proteins decreased the extent of apoptotic injury caused by COM crystals on MDCK cells. Moreover, the electron microscopic studies of MDCK cells revealed that matrix proteins caused significant dissolution of COM crystals, indicating cytoprotection against the impact of calcium oxalate injury.
Significance: The present study gives insights into the mechanism implied by urinary proteins to restrain the pathogenesis of kidney stone disease. This will provide a better understanding of the formation of kidney stones which can be useful for the proper management of the disease.

1. Introduction

Nephrolithiasis, the deposition of stones in kidney is one of the most painful diseases affecting mankind since ages. The earliest historical evidence dates back to 4800 BC when a stone was found in the pelvic region of a mummified Egyptian [1]. Due to its multifactorial etiology and high rate of recurrence, this disease poses a major health burden to society. Contingent upon the various factors including socio-economic conditions, climate, lifestyle and diet, the overall probability of stone formation varies markedly in various parts of the world: 1–5% Asia, 5–9% Europe, 13–15% USA, 20% Saudi Arabia and with 50% rate of reoccurrence in 10 years [2,3]. Kidney stones are majorly composed of calcium and oxalate (70–80%), some are calcium phosphate (10%) or mixture of both (40–50%) [4,5]. In addition to this inorganic composition, kidney stones also consist of organic matrix accounting for 2–5% of the total stone weight [6,7]. Proteins constitute a major portion (64%) of the organic matrix of human calcium oxalate (CaOx) renal stones and are considered to play an important role in the modulation of kidney stone formation [8]. The disruption in the equilibrium between the inhibitor and promoter proteins causes de-regulation of the bio-mineralization process in the kidney, thereby leading to the formation of kidney stones. The role played by these human kidney stone matrix proteins in the course of crystallization process and stone formation is still not clearly understood. In addition, it has been reported that certain proteins can have dual activity; they can either promote or inhibit a process depending upon the urinary conditions at the time of crystallization or retention [9,10].

Till date no permanent cure for kidney stone disease in recurrent stone formers is available, and this can majorly be attributed to the fragmentary knowledge about the molecular events taking place at the time of stone formation. Therefore, it is necessary to identify biomolecules that might be of etiological importance and also get an insight into the mechanism of stone formation at the molecular level. In addition, various studies have emphasized on the significance of the interaction between CaOx crystals and renal epithelial cells in kidney stone formation. Proteins being predominant modulators in stone formation could

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regulate this cell-crystal interaction to some extent [11,12]. Although, several proteins have been identified from the renal stone matrix, function of only a few proteins is reported [6,10,13,14]. Deep insights into the kidney stone's molecular make-up and its role in pathogenesis are the prerequisites for the proper management of the disease. Thus, in the present study we evaluated the effect of human kidney stone matrix proteins on calcium oxalate monohydrate (COM) injured renal epithelial cells and their impact on cell-crystal interactions.

2. Materials and methods

2.1. Protein extraction from human kidney stones

The ethical clearance for the present study was obtained from Institutional Ethical Committee of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Surgically removed human kidney stones were Fourier transform infrared spectroscopy (FTIR) analyzed for calcium oxalate content, the stones with calcium and oxalate as their major component were collected from the Department of Urology, PGIMER, Chandigarh. The selected calcium oxalate stones were washed in 0.15 M NaCl solution with gentle stirring for 48 h to remove adhered blood and tissue at 4 °C. For extraction of matrix proteins, the stones were pulverized to fine powder and extracted with a solution containing 0.05 M EGTA, 1 mM PMSF and 1% β-mercaptoethanol for 4 days at 4 °C. The collected whole extract was then dialyzed further to remove salts by using 3 kDa cut-off ultracentrifugation Amicon filter units [13]. The protein concentration was determined through Bradford's method [15].

2.2. Aggregation assay of calcium oxalate (CaOx) crystals

Solutions of calcium chloride and sodium oxalate were prepared at the final concentration of 5 mM and 0.5 mM respectively, in a buffer containing 10 mM sodium acetate and 200 mM NaCl at pH 5.7. Both solutions were filtered through a 0.22 μm filter (Millipore). Crystallization was initiated by adding 1.5 ml of sodium oxalate solution to the 1.5 ml of calcium chloride solution. Calcium chloride solution (1.5 ml) was mixed with 1.5 ml of sodium oxalate & 100 μl desalted extract of renal matrix proteins at different concentrations of 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml and the temperature was maintained at 37 °C. The final solutions were stirred at 37 °C for 60 min and the absorbance was monitored after every minute at 620 nm. The percentage inhibition of CaOx nucleation followed by its aggregation was calculated as [1 − (Tsi / Tsc)] × 100, where Tsc was the turbidity slope of the control and Tsi the turbidity slope in the presence of renal calculi proteins [16,17].

2.3. Cell culture

The canine kidney epithelial cell line MDCK was procured from the National Centre of Cell Sciences (NCCS) Pune, India. The cells were cultured and maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin G (100 units/ml)–streptomycin (10,000 μg/ml) and 10% fetal bovine serum (FBS), at 37 °C and 5% CO_2 in humidified incubator (Eppendorf, New Brunswick- Galaxy 170S) [13].

2.4. Preparation of COM crystals

Calcium oxalate monohydrate (COM) crystals were prepared by mixing 10 mM calcium chloride with 1 mM sodium oxalate to make final concentrations of 5 mM and 0.5 mM, respectively, in Tris buffer containing 90 mM NaCl (pH 7.4) [18]. The mixture was incubated at 25 °C overnight and COM crystals were harvested by centrifugation at 3000 rpm for 10 min. Supernatant was discarded, followed by re-suspension of the crystal pellet in methanol. This suspension was centrifuged again at 3000 rpm for 10 min, methanol was discarded and the crystal pellet was dried to fine powder at 37 °C overnight. COM crystals were then sterilized by UV irradiation for 30 min. The morphology of COM crystals was confirmed by observing under phase contrast microscope. The COM crystals were then added to DMEM to achieve the final concentration of 200 μg/ml.

2.5. Cell viability assay

Cell viability study was conducted using MTT cell viability assay [19]. The MDCK cells were seeded in the microwells of 96-well tissue culture plates at the seeding density of 10^4 cells/well in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Gibco) and 1% penicillinG/streptomycin (Gibco). The plates were incubated in a humidified incubator maintained at 37 °C and 5% CO_2. At semi-confluent level the medium was removed from wells and 200 μl serum free media having different concentrations of proteins with and without COM crystals were added into each well. After the incubation period of 24 h, Thiazolyl Blue Tetrazolium Bromide (MTT) dye (Sigma-Aldrich) at the working concentration of 0.5 mg/ml was added into each well and the plate was incubated for further 4 h. Following this, 200 μl of Dimethyl sulfoxide (DMSO) was added to dissolve the formazan precipitate. The developed color was read at a test wavelength of 570 nm and reference wavelength of 630 nm in a microplate reader (Bio-Rad 680 microplate reader). The cell viability was determined by the following formula:

$$\%\text{cell viability} = \frac{\text{Mean O.D.of Sample}}{\text{Mean O.D.of Control}} \times 100$$

2.6. Cell-crystal interaction study

To assess cell-crystal interaction, 10^5 MDCK cells were seeded onto coverslips placed in individual wells of a 6 well plates, containing DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin G/streptomycin [20]. The cultured cells were maintained in a humidified incubator at 37 °C with 5% CO_2 for 24 h. At semi-confluent level the media of the cells was replaced by serum free media for control group, 200 μg/ml COM crystal containing media for COM injury group, and 200 μg/ml COM crystal + 50 μg/ml of desalted matrix protein extract for test group and the cells were incubated for 24 h and the cells images were acquired by using various microscopy techniques:

2.6.1. Imaging by phase contrast microscopy

Following treatment for a period of 24 h, the medium was removed and the cells were washed with PBS twice and then observed under phase contrast and polarizing microscope (Olympus CH2i, Olympus Co. Ltd. Tokyo, Japan) at 20× magnification.

2.6.2. Fluorescence microscopy (Hoechst staining)

After the incubation of 24 h, the medium was removed and the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature. After washing twice with 1X PBS, cells were stained with 5 μg/ml of Hoechst 33258 dye (Sigma-Aldrich) for 10 min at room temperature in dark. Finally, the cells were washed twice with 1X PBS and the stained nuclei were observed under DAPI fluorescence filter of Olympus microscope at 20× magnification.

2.6.3. Scanning electron microscopy (SEM)

To visualise the cells subjected to various treatments by SEM, the medium was removed and the cells were fixed with 4% paraformaldehyde and 1% gluteraldehyde for 30 min at room temperature. The coverslips were then washed twice with PBS, dehydrated and air-dried overnight. The dried coverslips were then mounted on aluminum stubs and coated with gold particles. The crystal morphology was then examined under a scanning electron microscope (ZEISS EVO HD, Germany) at 2000× magnification.