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Short Communication

EVALUATION OF RENAL EPITHELIAL CELL PROTEIN UNDER STRESS CONDITION

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ABSTRACT

Objective: Proteins are an important component of cells which are involved in various cellular functions. Different kind of stressing conditions has different responses in the components of the protein synthesis system. Super saturation condition in kidney environment leads to crystallization process. Crystals thus formed injure the surrounding cells and result in reactive oxygen species (ROS) formation. There might be some changes in the protein synthesis when the kidney cells enter in oxidative stress. In the present study, kidney cell lines were exposed to oxidative stress and their proteins were analyzed using Bradford analysis and SDS-PAGE.

Methods: Vero cells were obtained from NCCS Pune and cultured in DMEM (Dulbecco's Modified Eagle's Medium) and maintained in a humidified incubator at 37 °C with 5% CO_2. Calcium phosphate (CaP) crystals were prepared by the homogeneous system. After FTIR analysis crystals were used to injure Vero cell line. H_2O_2 was also used to injure the Vero cells. Intracellular protein was extracted from healthy cells and injured cells (with CaP crystals and H_2O_2). Ammonium sulfate precipitation method was used for the isolation of extracellular protein from the media of healthy and injured cells. Bradford method was used for the quantitative estimation of protein. Extracted proteins were analyzed by SDS-PAGE.

Results: Amount of intracellular and extracellular protein of normal cells was 4.84±0.004µg/ml. Intracellular protein of CaP injured and H_2O_2 injured cells were 10.59±0.003 µg/ml and 10.78±0.011µg/ml respectively. While extracellular protein of injured cells was nearly 4 µg/ml. Intracellular protein bands ranging from 14.3 to 97.4 kDa was observed in healthy cells. Protein bands of ~40kDa and ~20kDa was absent in H_2O_2 and CaP injured intracellular protein extract. Two extracellular protein bands of 66kDa and ~60kDa were present in injured cells and healthy cells.

Conclusion: When exposed to oxidative stress several proteins are oxidized decreasing the activity of many metabolic pathways. In the present study amount of intracellular protein increases when cells are injured with CaP or H_2O_2. While extracellular protein remains more or less same in both healthy and injured condition of cells. In SDS-PAGE analysis few bands were missing in the intracellular extract of injured cells. These results indicate that the amount of protein varies when cells are injured with CaP and H_2O_2.

Keywords: Oxidative stress, Kidney, Protein, Calcium phosphate, Crystal

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INTRODUCTION

Kidney stone disease is a major public health concern that affects a significant fraction of the world population. Due to super saturation crystals are formed which serves as nidus for the growth of the crystal. Large crystals retain in kidney and forms kidney stones. Calcium phosphate is the first crystal that forms in the loop of Henle or in the distal part of the distal tubule [1-4]. Adhesion of these crystals to renal epithelial cells generates excess ROS, causing injury and inflammation [5]. Different kinds of stressing conditions might have different responses in the components of the protein synthesis system. Within the cell, several targets are oxidized decreasing the activity of many metabolic pathways including translation in both prokaryotic as well as eukaryotes. Part of this inhibition might be due to the oxidative inactivation of several enzymes involved in the metabolism of energy and amino acid synthesis both of which are essential for protein synthesis [6].

Oxidative stress leads to a cascade of events in a cell including the upregulation and down regulation of various proteins. Study of proteins during the injury of renal epithelial cells by crystals will help to understand the etiology of urolithiasis. Cellular protein profile of crystal injured renal epithelial cells have not been reported so far, therefore the current research attempts to identify the protein from healthy and injured African monkey renal epithelium (Vero cell line) through SDS-PAGE analysis. Protein quantity was determined by Bradford method. Protein bands of healthy and injured cells which appeared on the gel were compared.

Triton X and PMSF (phenyl methyl sulfonyl fluoride) were obtained from Sigma, DTT (Dithiothreitol) from CDH and DMEM from Himedia, India. All other chemicals were of analytical grade.

CaP crystals were prepared by mixing 5 mmol $CaCl_2$ and 5 mmol KH_2PO_4 with Tris buffer [pH 7.4] and distilled water. The mixture was incubated at 37 °C for 30 min and CaP crystals were harvested by centrifugation at 4000 rpm for 15 min [7, 8]. Supernatant was discarded and crystals were washed twice in distilled water. The pellets were then dried and were analyzed by FTIR (Spectrum BX-II FTIR spectrophotometer, Perkin Elmer) and SEM (Zeiss EVO40).

Renal epithelial cells derived from green African monkey (Vero Cells) were obtained from National Centre of Cell Sciences (NCCS, Pune). Cells were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and antibiotics streptomycin (100 mg/ml), penicillin(60 mg/ml), gentamycin (100 mg/ml) in 25 cm^2 tissue culture flask and maintained in a humidified incubator at 37 °C with 5% CO_2 [9].

The cells were seeded in six-well plates, incubated at 37 °C for 24 h, with 5% carbon dioxide in a humidified incubator. Media were changed to serum-free culture media and the cells were incubated for 12 h for acclimatization purpose. The cells in control group were exposed to serum-free culture medium only, whereas the cells in injury group were injured separately with 0.3 mmol hydrogen peroxide and with 10µg/ml of CaP crystals [10]. After 24 h of incubation, intracellular proteins were extracted from cells and extracellular proteins were extracted from the culture media.

Proteins were extracted from the cells injured with H_2O_2 and cells injured with CaP crystals as well as healthy cells and from the culture media in which they were grown. Protein extraction buffer (1M HEPES Buffer, 100% Glycerol, 5M NaCl, 1M $MgCl_2$, 500 mmol EDTA, 100% Triton-X 100, 1M PMSF, 1M DTT, 1M Protein Inhibitor

cocktail) was used for intracellular protein of injured and control group. After trypsinization, cells of each group were suspended in the extraction buffer and incubated in ice for 1 hr with intermittent tapping at an interval of 10 min. The suspensions were centrifuged at 10000 rpm for 18 min at 4 °C and the extracted protein present in the supernatant was aliquoted immediately into labelled microfuge tubes and stored at-80 °C.

Extracellular proteins present in the serum-free media of each group were extracted using ammonium sulfate precipitation method. The proteins were quantitated through Bradford method [11].

For SDS-PAGE each extracted protein sample was reconstituted with loading buffer containing β-mercapto ethanol. Samples were heated to 95 °C for 5 min and were submitted to electrophoresis using 5% stacking and 10% separating gels. Medium range molecular weight markers were used as standards. Protein bands were stained with silver staining.

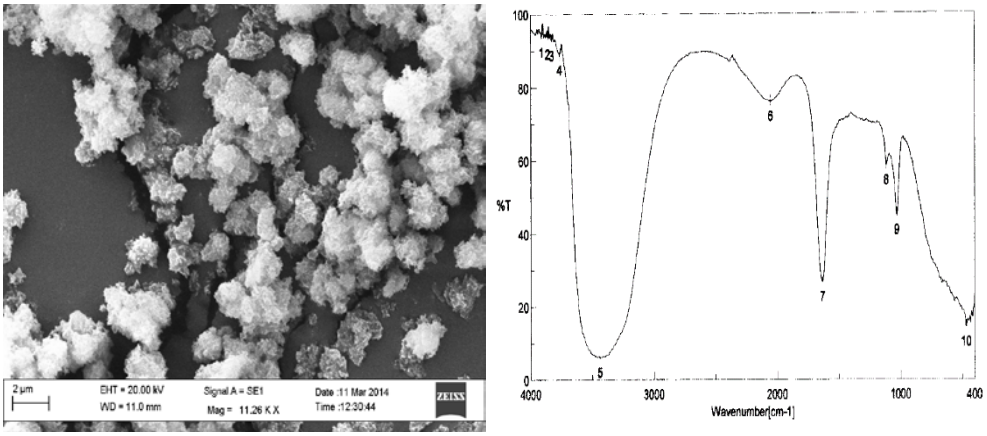
FTIR graph has peaked at a wave number of 1000-1100/cm, 3600-3900/cm, 1460-1700/cm which correspond to phosphate, hydroxide and carbonate respectively. These peaks indicated the presence of CaP crystals. Scanning electron micrograph and FTIR analysis of CaP crystals are shown in fig. 1.

The protein content of total cellular extract of healthy cells (4.84 µg/ml) was less as compared to the injured group. CaP crystals injured cells was 10.59 µg/ml and H2O2 injured cells was 10.78 µg/ml. The protein content of culture media extracted protein of healthy cells (4.94 µg/ml), CaP injured cells (4.31µg/ml), H2O2 injured cells (3.87µg/ml) had less amount of protein.

The total cellular and media protein extract of healthy and injured cells presented different profiles of separate protein bands on the gel after silver staining (fig. 3 and 4).

SDS-PAGE depicts that bands of molecular weights such as 97.4kDa, approximately 70kDa, 66kDa, multiple bands between 66kDa and 43kDa, 29kDa, 14.3kDa were observed in the case of total cellular protein extract of healthy and injured cells. In the case of CaP and H2O2 injured cell extract, bands of molecular weights of 39kDa, 37kDa, 36 kDa and 35kDa were missing (fig. 3).

Multiple bands of molecular weights ranging from 97.4kDa to 14.3kDa were observed in the case of healthy cells culture media protein extract obtained from cells grown in complete media. Whereas, in case of CaP and H_2O_2 injured cell culture media extract as well as an extract obtained from cells grown in incomplete



A **B**

Fig. 1: **Morphology and composition analysis of calcium phosphate crystals. A. Scanning electron microscopy (scale bar 2 µm) B. Fourier transform infrared spectroscopy**

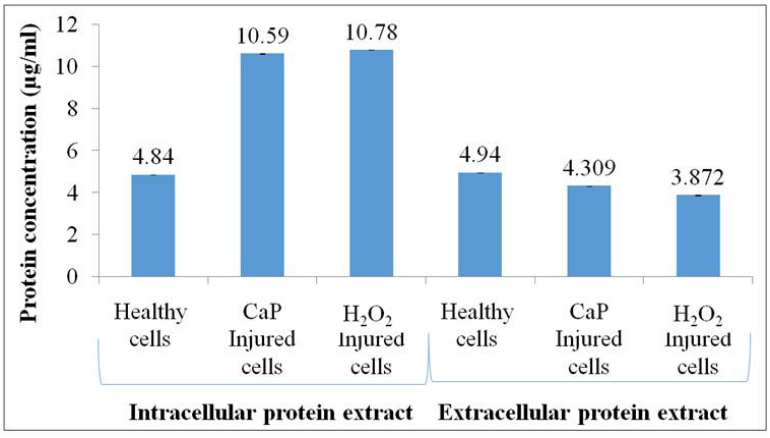


Fig. 2: Protein concentration present in different samples

Whereas, in the case of CaP and H_2O_2 injured cell culture media extract as well as an extract obtained from cells grown in incomplete media, two bands of molecular weight 66kDa and approximately 60kDa were observed as shown in fig. 4.

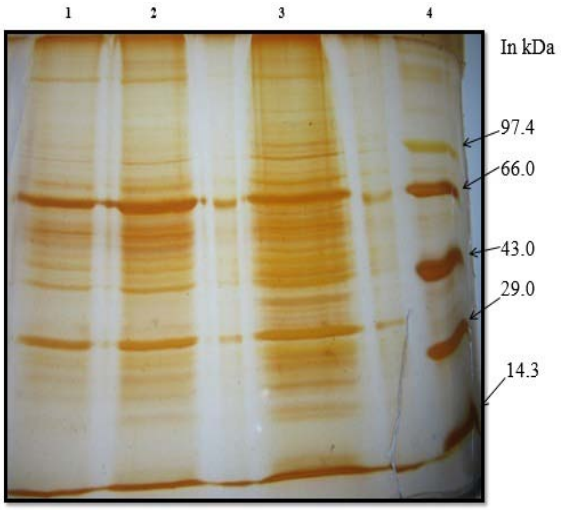


Fig. 3: SDS-PAGE analysis of Vero cell extract. Lane 1 CaP crystal injured cell extract, Lane 2–H_2O_2 injured cell extract, Lane 3–Normal cell extract, Lane 4–Medium range protein marker. In the injured group various protein bands are missing which are present in healthy cell extract

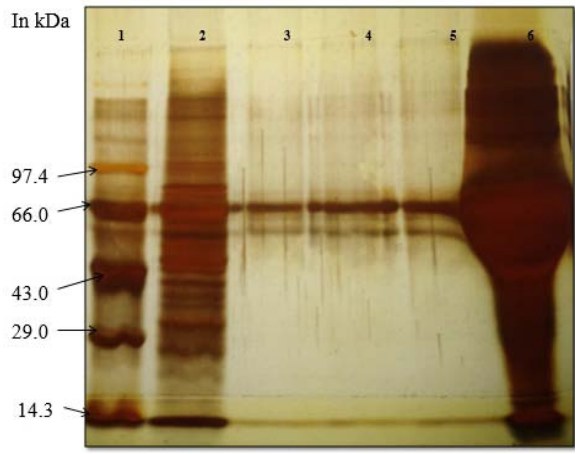


Fig. 4: SDS-PAGE analysis of cells culture media protein extract. Lane 1–Medium range protein marker, Lane 2–Extracellular protein of healthy cells grown (24 h) in complete media, Lane 3–H_2O_2 injured cell supernatant extract, Lane 4–CaP crystal injured cell supernatant extract, Lane 5–Extracellular protein of healthy cells grown (24 h) in incomplete media, Lane 6–Fetal Bovine Serum (FBS). Two bands of 60 and ~66kDa were observed in healthy and injured group in cell culture media extract

One of the major aspects in stone formation is reactive oxygen species (ROS), when generated uncontrollably they provide the surface for attachment of crystals, leading to the formation of stones [1-3]. Injury of the renal epithelial cells results in alteration of various cellular activities including protein synthesis. Proteins are a vital part of the organism and participate in almost every process in the cell. Interference in its synthesis process will lead to upset cellular fitness, cause disease phenotypes. In the present study, we have studied the protein of healthy and injured renal epithelial cells of Vero cell line.

The amount of cellular protein was increased from healthy cells (4.84µg/ml) to injured cells (~10.5 µg/ml) whereas in extracellular protein there was not much difference in the amount of protein secreted.

In our experiment, we observed that in the intracellular protein of injured group several protein bands of approximately 39kDa, 37kDa, 36 kDa and 35kDa molecular weight were missing, while they were present in the healthy group. Protein bands of 97.4kDa and 70kDa were present in both healthy and injured cells [12].

African green monkey renal epithelial cells release a glycoprotein 39kDa protein that blocks adhesion of COM crystals to the apical cell surface. CAI (crystal adhesion inhibitor) is a constituent of human urine; it is likely that this glycoprotein is present in sufficient quantities in the tubular fluid *in vivo* to coat crystals and prevent their adhesion to renal epithelial cells [13]. The absence of this protein might lead to the adhesion of crystals to the injured cells and growth thereof.

37kDa proteins from MDCK (Madin-Darby canine kidney) cell line were reported as laminin binding protein related to the 37/67 kDa laminin receptor family involved in cell attachment, spreading and polarization [14].

35 kDa and 38kDa protein are also reported as glycosyl-phosphatidylinositol-anchored proteins from renal epithelial cells of MDCK cell line [15]. These proteins were restricted to the apical domain of the plasma membrane indicating their role in polarization of the epithelial cells. Another 35 kDa protein is reported to be a splicing factor involved in the removal of introns from strings of messenger RNA [16].

In summary, we demonstrated that amount of proteins in Vero epithelial cells vary when they are injured with CaP crystals or H_2O_2 while the amount of extracellular proteins remains same. Proteins which were absent in the injured group were found to be significant in crystal and epithelial cell interaction, cell attachment, in the polarization of cells and in splicing activity. These results suggest that injury of epithelial cells either with crystals or H_2O_2 is interfering with protein synthesizing mechanism. Future studies will help us to identify and characterize these proteins and their role in urolithiasis.

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CONFLICTS OF INTERESTS

Declared none

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