

Injury Difference of the Un-Aggregated and Aggregated Calcium Oxalate Monohydrate on Human Kidney Proximal Tubular Epithelial Cells

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Abstract. Purpose: To study the injury difference of the un-aggregated and aggregated calcium oxalate monohydrate (COM) on human kidney proximal tubular epithelial (HKC) cells, and to discuss the molecular and cellular mechanism of kidney stone formation. **Methods:** The cell injury by crystals was evaluated by measuring the cell viability, malonaldehyde (MDA) release, and the expression level of osteopontin (OPN). Scanning electron microscope (SEM) was used to observe the HKC-COM crystal adhesion. **Results:** After adhesion to COM, cell vitality dropped, both MDA release and OPN expression increased. The crystal injury in HKC was in a time-dependent manner within 24 h. aggregated COM caused more serious injury than un-aggregated COM, and the adhesive capacity of aggregated COM was also obviously stronger than un-aggregated COM. **Conclusions:** Aggregated COM crystals enhanced the risk of calcium oxalate stone formation much stronger than un-aggregated COM crystals.

Introduction

Nephrolithiasis is a common disease around the world. Despite the number of researchers approaching the problem from different angles, the mechanism for the formation of the stones is not yet clearly understood. Various factors, such as oxalate [1], calcium oxalate (CaOx) crystals [2,3], and free radicals like superoxide anion ($O_2^{\bullet-}$) [4], were thought to be involved in renal tubular cell injury.

The damaged renal tubules epithelial cells is mediated by lipid peroxidation reaction through the generation of oxygen free radicals. Free radical scavengers, such as catalase and superoxide dismutase (SOD), could provide significant protection against renal tubular cell injury induced by oxalate and CaOx crystals [4,5]. Cultured epithelial cell lines have been used to examine the mechanisms in the renal epithelium leading to urolithiasis despite the clear differences among various cultured epithelial cells.

At present, studies on crystal-cell interactions in vitro focus on two aspects. The first is to investigate the injurious effect of oxalate, CaOx crystals, and free radicals on cells. For example, Lieske et al. [6] exposed LLC-PK₁ and IMCD cells to oxalate for 15 min–72 h. In response to all oxalate concentrations (0.5–2.0 mmol/L) and lengths of exposure (15 min–2 h), both cell lines generated reactive oxygen species (ROS). Khan et al. [7] exposed NRK-52E cells in vitro to synthetic and biological CaOx or brushite crystals. Results indicated that even though both synthetic and naturally produced biocrystals invoke an injurious response from renal epithelial cells, the latter were

significantly less injurious and inflammatory. Exposure to low concentrations of these crystals alone may not be sufficient to invoke an inflammatory response, cause uncovering of crystal binding molecules on epithelial cell surfaces, and promote crystal attachment and retention. The second approach is to investigate the process of crystal attachment to normal or injurious renal tubular cells. Lieske et al. [8] identified the most prominent COM crystal-binding protein (*Mr*, 37 kD) on the surface of MDCKI cells as annexin II (Ax-II). However, the impact of different crystal properties toward renal tubular epithelial cells is still not clear; even the differences between un-aggregated and aggregated known at present in urine have not been reported.

Experimental details

Reagents and apparatus

Human kidney tubular epithelial cells (HKC) was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Cell proliferation assay kit (Cell Counting Kit 8, CCK-8) (Dojindo Laboratory, Kumamoto, Japan). MDA kit and superoxide dismutase kit (Jiancheng Institute of Biotechnology of Nanjing, China). The first antibody of OPN and FITC secondary antibody (SANTA CRUZ, America). Cell culture plate (Iwaki, Japan).

The medium DMEM-F12 and newborn calf serum (HyClone, America). Penicillin and streptomycin (Beijing Pabo Biology Technology limited Co.). 30% Hydrogen peroxide (H_2O_2) and other conventional reagents were all analytically pure.

X-L type environmental scanning electron microscope (ESEM, Philips). Laser confocal microscope (LSM510 META DUO SCAN, ZEISS, Germany). Inverted fluorescence microscope (IX51) (Olympus Corporation, Japan). Enzyme mark instrument (Safire2™, Tecan, Männedorf, Switzerland).

Preparation of COM Crystals

Un-aggregated COM crystals: Approximately 0.2 g of gelatin was added to 20 mL of distilled water and heated to dissolve completely. Impurities in gelatin were removed via centrifugation using 400 mL of 0.05% (W/V) of the gelatin solution and heated to 75°C. In addition, about 20 mL of K_2Ox (20 mM) solution was added and stirred at 300 rpm for 10 min. Afterward, 20 mL of $CaCl_2$ (20 mM) solution was added, and the speed and reaction was maintained for 10 min. The prepared crystals were collected with the same processing as above.

Aggregated COM crystals: A total of 150mL of Na_2Ox (10 mM) and 150 mL of NaCl (0.5M) were mixed in 500-mL beaker, evenly stirred, and heated to 70°C. Afterward, 150 mL of $CaCl_2$ (5 mM) solution preheated to 70°C was added in the reaction mixture, and the reaction was maintained at 70°C under continuous stirring at 900 rpm for 2 min. The final solution was incubated overnight at room temperature. The prepared crystals were collected with the same processing as the synthesis of COM-elliptical.

Cell Culture

The cells were cultured in DMEM-F12 supplemented with 10% newborn calf serum (Gibco), 100 U/ml penicillin-100 µg/ml streptomycin antibiotics (Gibco), with pH 7.4 at 37°C in 5% carbon dioxide air atmosphere. Cell culture media were exchanged every two days as needed. Cell viability of HKC cells was evaluated colorimetrically with CCK-8. The cells were divided into two groups: A group was control group, and B

group was crystal group. Cells in A group were only exposed to serum-free culture medium, while cells in B group were exposed to serum-free culture media containing 200 µg/ml un-aggregated or aggregated COM.

Measurements of malondialdehyde (MDA) content

The MDA kit was used to measure the content of MDA, which was strictly conducted according to the instructions of kit.

Detection of osteopontin (OPN) expression by HKC during adhesion

Cell concentrations were adjusted to 2×10^5 cell/ml. Cell suspension was assimilated and then plated in 12-well plates (1ml/well) with cover slips at bottom and cultured in incubator at 37°C with 5% CO₂. After 24 h, cells almost covered the whole cover slips. The culture media were removed, and the cells were rinsed once with D-Hanks and then were divided into two groups (A: control group, and B crystal group) as described above.

After cells were incubated with crystals for 2, 6, 12 and 24 h, respectively, they were washed for three times with PBS (5 min each time), fixed with 4% paraformaldehyde for 10 min, washed with PBS for three times (3 min each time), blocked with sheep serum for 20 min, and incubated with primary antibody of OPN (1:100) overnight at 4°C. Cells were then rinsed for three times with PBS, incubated with FITC secondary antibody (1:100) under darkness for 0.5 h at 37°C, rinsed three times with PBS again, and stained and sealed with DAPI. The fluorescence was observed using Laser confocal microscopy (LSM). The nucleus was in blue while the OPN was in green.

SEM observation of HKC-COM adhesion before and after the injury

The cells were incubated in 6-well plates (4×10^5 cell/ml, 2 ml/well) with cover slips at bottom.

When cells were incubated for 2, 6, 12 and 24 h, respectively, the cover slips were taken out, the culture media were removed, the nonadherent crystals were washed away with D-Hanks, and the cells were fixed with 2.5% glutaraldehyde at 4°C for 24 h. Fixed cells were dehydrated in ethanol gradient (30%, 50%, 70%, 90% and 100%, respectively), refixed with isoamyl acetate, then dried under the critical point of CO₂, and treated by gold sputtering. Adhesion between cells and crystals were observed by SEM.

Results and Discussion

Characterization of COM crystals

The morphologies of aggregated and un-aggregated COM were shown in Figs. 1a and 1b. The un-aggregated COM was mainly hexagon. The dimensions of most crystals ranged from 12 µm to 16 µm, with an average size of 15 ± 2 µm. The spectra of XRD (Figs. 1c & 1d) demonstrated that these crystals were COM, the d values in spectrogram were 0.598, 0.365, 0.298 and 0.236 nm, which belong to ($\bar{1}01$), (020), ($\bar{2}02$) and (130) of the crystal planes of COM, respectively.

The changes of HKC cell vitality during adhesion

The nonradioactive property of cell counting kit-8 (CCK-8) assay allows sensitive colorimetric assays for the determination of the number of viable cells in cell

proliferation and cytotoxicity assays. Detection sensitivity of CCK-8 is higher than other tetrazolium salts, such as MTT, XTT, MTS, or WST-1.

Fig. 2a showed the vitality of HKC cell exposure to aggregated COM and un-aggregated COM crystals for different treatment times. As the adhesive time increased, the cell vitality of HKC in both aggregated COM group and un-aggregated COM group gradually reduced, but the vitality of HKC decreased more for aggregated COM than the un-aggregated COM ($P<0.05$). These indicated that COM crystals damaged cells, furthermore, the aggregated COM damaged much stronger, leading to declined cell vitality. For HKC in aggregated COM group, the vitality decreased rapidly in the first 2 h.

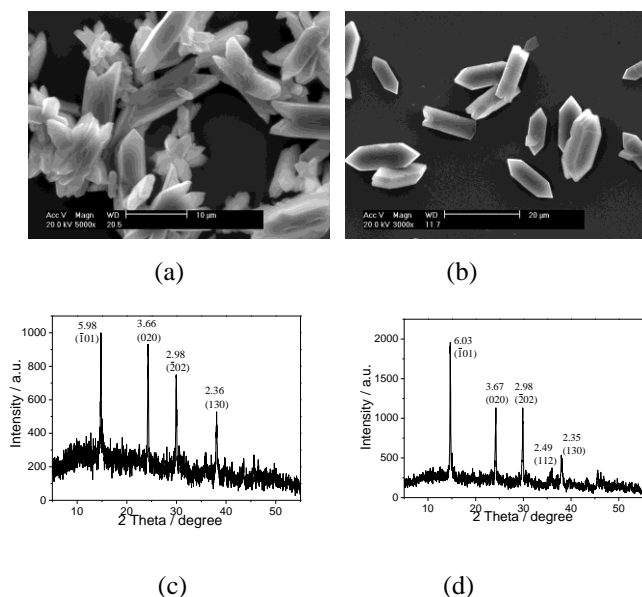


Fig. 1. Morphology and XRD of un-aggregated or aggregated COM crystals. (a,c) aggregated COM; (b,d) un-aggregated COM. The bars: (a) 10 μm ; (b) 20 μm .

The change of MDA content during adhesion

The change of MDA content usually reveals the level of lipid peroxidation *in vivo*, and indirectly reflects the degree of cell injury. The change of MDA content of HKC cell exposure to aggregated COM and un-aggregated COM crystals for different treatment times was shown in Fig. 2b, in which we could see:

The MDA content augmented as treatment time increased ($P<0.05$), indicating the degree of cell injury aggravated with time.

During the treatment process of COM crystals, the MDA content in aggregated COM was generally higher than that un-aggregated COM group, suggesting that the aggregated COM were damaged HKC more severely.

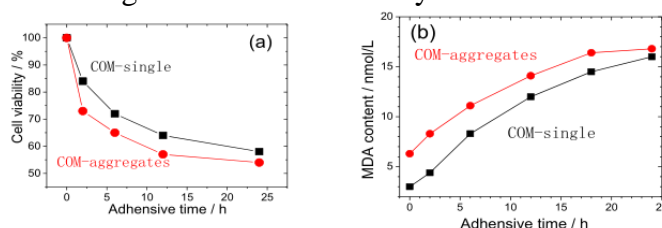


Fig. 2 Cell viability (a) and MDA content (b) assay of HKC cells after exposure to aggregated and un-aggregated COM crystals for different time. Crystal concentration: 200 $\mu\text{g/mL}$.

Expression of Crystal Adhesion Molecule OPN on HKC Surface

OPN is a crystal-binding molecule that plays an important role in cell adhesion. OPN expression in HKC cells after treatment with COM crystals is shown in Fig. 3. Only a little green fluorescence could be found around HKC in control group (Fig. 3a). However, the crystal-treated groups showed obvious green fluorescence (Figs. 3b & 3c). The fluorescence intensity of aggregated COM crystals was obviously higher than that in the un-aggregated COM crystal groups, indicating that aggregated crystals induce higher OPN expression.

OPN was a negatively charged noncollagenous matrix protein with a molecular weight of about 44 kD. It had 300 amino-acid residues, among which negatively charged aspartate, serine and glutamic acid had a high proportion^[9]. Therefore, OPN could not only adsorb the calcium ions in solution, but also adhere to the positively charged COM crystals^[10]. Indeed, it has been confirmed that OPN has the capability to bind calcium ions, calcium oxalate crystals, and hydroxyapatite in urine^[11].

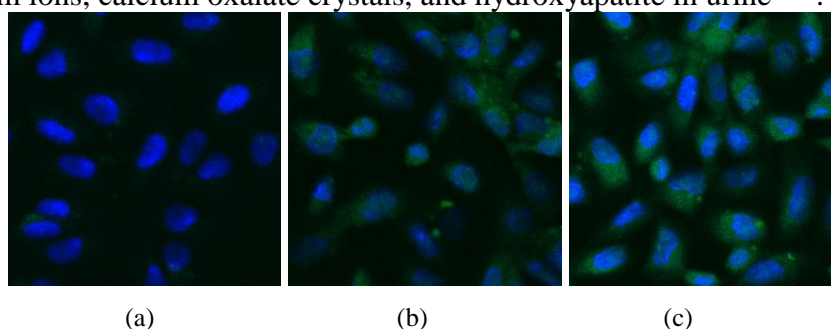


Fig. 3. Laser scanning confocal microscope images ($\times 630$) showing fluorescent changes of OPN on HKC cells after exposure to aggregated and un-aggregated COM crystals. a) Control group; (b) COM-single; (c) COM-aggregates. $c(\text{COM})=200 \mu\text{g/ml}$. The nucleus was in blue and the expressed OPN was in green.

SEM Observation of Adhesion Between COM Crystals and HKC Cells

The adhesion between HKC cells and COM crystals was detected by SEM (Fig. 4). The morphology of control cells (Fig. 4a) was plump with intact and smooth surface. However, after treatedb with crystals, the HKC shrank with rough surface, and the cell debris were attached on cells, and most of the flagella and synapses of HKC were fractured and shed. It suggested that the cells had been seriously injured by crystals, and the surface and intracellular structure were destroyed, thus producing a large amount of cell debris.

The aggregated COM crystals also presented evident aggregation after these crystals adhered to the cell surface (Fig. 4c). However, the un-aggregated COM crystals were almost individual particles (Fig. 4b).

The above results indicated that the interaction of HKC with COM-aggregates caused further injury to HKC, thus enhancing the risk of kidney stone formation.

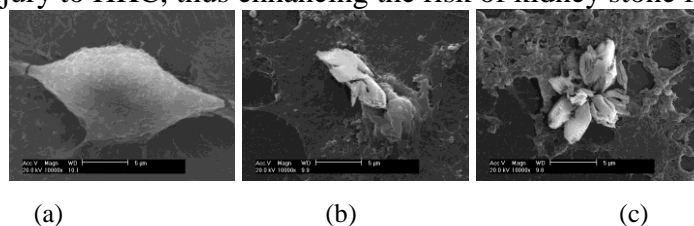


Fig. 4. SEM images of HKC cells after exposure to aggregated and un-aggregated COM crystals. (a) Normal HKC cells; (b) COM-single; (c) COM-aggregates. $c(\text{COM})=200 \mu\text{g/ml}$; Incubated time: 12 h. The bars: $5 \mu\text{m}$.

Conclusions

Aggregated COM caused more serious injury than un-aggregated COM. The crystal injury in HKC was in a time-dependent manner within 24 h. The adhesive capacity of aggregated COM was also obviously stronger than un-aggregated COM. After injured by COM crystals, cell vitality dropped, both MDA release and OPN expression increased. Aggregated COM crystals enhanced the risk of calcium oxalate stone formation much stronger than un-aggregated COM crystals.

Acknowledgements

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