Crystal adhesion and internalization of sub-micron COM and COD crystals on Human kidney proximal tubular epithelial cells

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Abstract: [Objective] This study aims to compare the internalization of sub-micron calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) crystals in human kidney proximal tubular epithelial cells (HKC) so as to explore the mechanism of renal epithelial cell injury caused by urine calcium oxalate crystals. [Methods] Cell viability, malondialdehyde (MDA) content, and propidium iodide (PI) staining were determined to examine cell injury during adhesion. The internalization of COM and COD crystals to HKC cells was observed through scanning electron microscopy. [Results] The injury effect of COM was stronger than that of COD on cells under the same conditions. Both of them injured HKC in a dose-dependent manner. The ability of COM to adhere to the injured HKC cells was higher than that of COD crystals. Compared to the COD group, the crystal adhered on cell surface appeared obvious aggregation in COM treatment group. [Conclusions] Cytotoxicity of sub-micron COM was greater than sub-micron COD. The attached COM crystals aggregated on cell surface. HKC cells swallow COD crystals more easily than COM. These findings provide further insights into kidney stone formation.

1 Introduction
Calcium oxalate (CaOx) crystals are the major constituents of most human kidney stones worldwide. Ansari et al [1] studied the spectrum of stone composition of upper urinary tract calculi by X-ray diffraction crystallography technique. Of the 1050 stones analyzed, 977 (93.04%) were calcium oxalate stones, out of which 80% were COM (COM) and 20% were COD. Grases et al [2] applied to 2500 renal calculi to achieve the percentage of each category. And results indicated 12.9% calculi corresponded to calcium oxalate monohydrate papillary calculi, 16.4% to COM unattached calculi, 33.8% to COD calculi, 11.2% COD/hydroxyapatite mixed calculi.

However, the mechanism of how a CaOx stone is formed is complex, and many factors are believed to be involved [3,4]. Urinary supersaturation with respect to stone-forming materials is the driving force for nucleation and crystal growth. However, crystalluria may not always lead to urolithiasis[5].

The study have shown that not only large amount of micron crystals exist in the urine [6], but also there is a large number of nano/sub-micron crystallites [7], which is the basis for forming the micron and millimeter stone. However, is mainly focused on the adhesion of micron COM and COD on renal epithelial cells and crystals

It has been shown that high levels of urinary oxalate cause renal epithelial cell injury and crystal retention. Lin [8] studied in vitro toxicity of silica nanoparticles in human lung cancer cells and indicated that cell viability decreased significantly as a function of nanoparticle dosage (10–100μg/ml). Low
concentration (≤0.2 μg/μL) nano-SiO$_2$ and normal SiO$_2$ had no significant toxicity to Hela cells (p > 0.05), while in the high concentration, nano-SiO$_2$ (≥0.4 μg/μL) and normal SiO$_2$ (≥0.8 μg/μL) had significant toxicity to Hela cells (p < 0.01), and the cytotoxicity increased with the increase of concentrations [9]. Therefore, this study investigated the adhesion of COD microcrystals with a size of 100-200 nm and human kidney proximal tubular epithelial cell (HKC) in order to further understand the molecular and cellular mechanism of the kidney stone formation.

2 Materials and Methods

2.1 Materials and apparatus
Human kidney proximal tubular epithelial cells (HKC) were purchased from Cell Bank of Chinese Academy of Sciences (China). The other conventional reagents were all purchased from Sinopharm (China) and were all analytically pure.

X-L type environmental scanning electron microscope (ESEM, Philips), confocal laser scanning microscope (LSM510 META DUO SCAN, ZEISS, Germany), inverted fluorescence microscope (IX51) (Olympus Corporation, Japan), and flow cytometer (Beckman Coulter, Brea, CA).

2.2 Preparation of calcium oxalate monohydrate (COM) nanocrystals and COM suspension
Sub-micron COM and COD crystals were prepared as previously described [10] by changing the concentration of reactants, reaction temperature, solvent, and stirring speed. The details would be reported elsewhere.

2.3 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. Then, 10 μl of CCK-8 solutions was added to each well. After 4 h incubation at 5% CO2 air atmosphere and 37°C, absorbance at 450 nm of each well was measured using the microplate reader. Cell viability was calculated with Aexp/Acon× 100%, where Aexp is the absorbance of cells exposed to reagents and Acon is the absorbance of control cells.

2.4 Measurement of MDA levels
Lipid peroxidation was assessed through MDA levels. After establishment of quiescence and treatment of cells with culture medium containing 0.1, 0.3, 0.5, 1.0, and 2.0 mmol/L of H2O2 for 0.5, 1, 1.5, and 2 h, as described above, medium was aspirated at the indicated timepoints to be added to the MDA detection kit. Absorbance was measured at 532 nm.

2.5 Propidium iodide (PI) staining assay
One milliliters of cell suspension with a cell concentration of 1×10⁵ cells/mL was inoculated per well in twelve-well plates. Cells were grouped after synchronized, and then varying sizes of COM crystals were added into the wells. After 6h incubation, the supernatant was removed by suction and the cells were washed three times with PBS, then incubated at 37°C for 10 min after adding 4μmol/L PI solution. The cells were washed with PBS three times again followed by observing the dead cells under the fluorescence microscope.

2.6 SEM analysis
Cells were cultured and treated with H2O2 as discussed above. At the indicated time points, 0.5 mmol/L CaOxa solutions were added to the cells, followed by incubation at 37°C for 6 h. After re-
moval of the culture medium, cells with crystals were rinsed twice with D-Hanks solution and fixed in 2.5% glutaraldehyde solution for 2 h. They were washed in 0.05 mmol/L cacodylate buffer, postfixed with 1% OsO4 for 2 h, and washed in cacodylate buffer thrice again. Then, samples were dehydrated through a graded series of ethanol solutions, and critically point-dried with CO2. After gold sputtering, they were examined with ESEM at 20 kV.

3 Results

3.1 Characterization of sub-micron COM and COD crystals
The SEM images of the prepared sub-micron COM and COD crystals were shown in Fig. 1. both the mean sizes of COM and COD crystals were about 500 nm. XRD and FT-IR results revealed that both of the COM and COD crystals prepared were pure forms of the target products.

Fig 1. SEM images of sub-micron COM and COD crystals. (a) COM; (b) COD. Scale bars: 500 nm.

3.2 Changes in cell viability and malondialdehyde (MDA) content
The extent of oxidative stress in HKC cells after treatment with COM and COD crystals is indicated in Fig. 2. The decreased cell viability (Fig. 2a) and the increased lipid peroxidation products (MDA content; Fig. 2b) reflect oxidative stress and cell injury. The injury effect of COM was stronger than that of COD on cells under the same conditions.

3.3 Cell death detection by propidium iodide (PI) staining
The results of PI staining in HKC cells after interaction with COM and COD for 6 h were shown in Fig. 3. The number of PI-stained nuclei in COM group was stronger than in the COD group, thereby indicating that the COM crystals induced higher cell death than the COD crystals.

Fig 2. Change in cell viability (a) and MDA content (b) of HKC cells after exposure to varying crystal concentration of sub-micron COM and COD crystals for 6 h.
Fig 3. PI staining detection by fluorescence microscope of HKC cells after exposure to 200 μg/mL sub-micron COM and COD crystals for 6 h. (a) Control; (b) COM; (c) COD. Magnification: ×600.

3.4 SEM observation of crystal adhesion on HKC

In order to observe the microscopic changes of cells and crystals after sub-micron COM or COD crystals interacted with HKC more intuitively, the cells were characterized by SEM after adhered with sub-micron COM or COD crystals (Fig. 4). After 6 h of exposure to crystals, the COM crystals could bind to the HKC cells much more readily than COD under same condition.

Compared to the COD group (Figs. 4c & 4d), the crystal adhered on cell surface appeared obvious aggregation in COM treatment group (Figs. 4a & 4b). Crystal aggregation on cell surface will further damage the cell. This suggests that the formation of COM crystals in the urine will cause great damage to the kidney epithelial cells and increase the risk of stone formation, but the equivalent amount of COD has significantly less damage to cells than COM.

Figure 4. SEM images after the adhesion of sub-micron COM or COD crystals with HKC for 6 h. (a, b) sub-micron COM; (c, d) sub-micron COD. The bar: 10 μm.
3.5 SEM observation of crystal internalization by HKC

Fig. 5 shows the SEM images of sub-micron COM and COD crystals endocytosed by HKC cells. It can be seen that HKC cells had stronger phagocytic ability to COD. Their ability to uptake COM crystals weakened. That is, HKC cells swallow COD crystals more easily than COM.

Crystal internalization is a common and important metabolic process. It is a self-protection mechanism for cells, it inhibits the aggregation and nucleation of CaOx crystals in urine and decreases the risk of kidney stone formation. Lieske et al has also observed that African green monkey BSC-1 or parental MDCK cells rapidly took up calcium oxalate crystals [11]. These internalized crystals are likely dissolved, forming Ca$^{2+}$ and Ox$^{2-}$ ions, and excreted extracellulary, this process has little injury to cells [12].

![Fig 5. SEM images of sub-micron COM and COD crystals endocytosed by HKC cells. The arrow A indicates crystals under the ruptured membrane. Arrow B shows that the cell membrane surface has obvious bumps, crystals were swallowed inside the cells. Adhesion time 6 h. Bars: 2 μm.](image)

4 Discussion

4.1 Cytotoxicity and damage of sub-micron COM and COD crystals on HKC cells

Figure 2a shows that the cell survival activity decreased with the increased concentration of sub-micron COM and COD crystals. In the same concentration, sub-micron COM has greater toxicity to cells than sub-micron COD group.

Studies have shown that when calcium phosphate crystals dissolved quickly in the lysosome, the released of Ca$^{2+}$ may interfere the osmotic pressure balance of the lysosome, and lead to lysosomal fracture and cell death. With the increased concentration of calcium phosphate crystals, the rate of cell necrosis or apoptosis increased [13]. Research has shown that the interaction of renal epithelial cells with crystals can cause cell oxidative injury, destroy the close connection between cells and reduce the expression of claudin and ZO-1 which will migrate from the top of the cell membrane to the basolateral membrane leading to cell death and kidney stone formation [14]. Renal epithelial cells injury is a major risk factor for kidney disease and cell membrane injury caused by oxidative stress induces attachment of crystals to renal epithelial cells.

4.2 Cell apoptosis caused by sub-micron COM and COD crystals

Figure 3 shows that PI staining nuclei increased with the concentration increase of COM and COD crystals. COM crystals adhesion induced cell damage and apoptosis and cell damage induced by COM crystals can promote the adhesion of crystal.

After treated with COM crystals and be stained, condensed nuclei and apoptotic bodies can be observed. The interaction of COM crystals and mitochondria can decrease the mitochondrial membrane potential and inhibit cell proliferation by oxidative pressure and p38MAPK/JNK signaling pathway. Heat shock protein(Hsp90) is sensitive to stress and it will be partial degradation and weaken the defensive function of cells when renal epithelial cell contact with COM crystals, which may lead to cell death caused by COM crystals[15]. Oxalate or calcium oxalate crystals can induce oxidative stress in
the kidney accompanied by the production of free radicals, leading to cell damage and even cell apoptosis and dead cell fragments will promote the nucleation and aggregation of crystals [16].

Kidney stone formation involves a cascade of process, including urinary supersaturation, crystal nucleation, growth, and aggregation, retention of crystals in the renal tubules or interstitium [10]. We can see from Fig. 5 that the adhesion of sub-micron COM and COD crystals to HKC cell is obviously different and it showed Positive correlation between aggregation degree and the concentration of crystal.

According to reports, calcium phosphate is also the component of kidney stone, 20% kidney stones contain calcium phosphate which also presents concentration-dependent effect for cell adhesion. Furthermore, ten times as many cells bound crystals of COM as compared with calcium phosphate when the same quantity of each was added. For MDCK cells, COM also presents concentration-dependent effect [12]. There is only neutral phospholipids components existing outside the cell membrane, the negatively charged phosphatidylinerine is located in the cell membrane. Renal epithelial cells membrane structure change after injury caused by calcium oxalate crystals, the negatively charged phosphatidylinerine within the cell membrane will become valgus, and CD44 and hyaluronic acid will be expressed on the cell surface. These changes will provide effective sites for the nucleation and growth of crystal, to combine with positively charged Ca$^{2+}$, promote the formation of stones in early and adhesion of crystal cell membrane, accelerate the formation of kidney stones [17]. Crystals adhered to cells could induce cells to produce free radicals, which further damaged the cells through lipid peroxidation, thereby promoting their crystal-binding ability and increasing the adhesion and aggregation degree of crystals.

5 Conclusions

The attached sub-micron COM and COD crystals have toxicity and damage effect on HKC cells. These crystals can decrease cell activity and rise MDA content in a dose-dependent, and increase the number of PI-stained nuclei. The injury effect of COM was stronger than that of COD. HKC cells swallow COD crystals more easily than COM. The results in this paper showed that a large number of COM crystals formed in the urine will cause great damage to the kidney epithelial cells, and thus promote stone formation. This study could help to elucidate the formation mechanism of calcium oxalate stones.

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