Toxicity of sub-micron and micron calcium oxalate monohydrate on renal epithelial cells

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Abstract: [Objective] This study aims to synthesize calcium oxalate monohydrate (COM) crystals with a size of 300 nm and 5 μm, respectively, and to compare their damage and adhesion ability toward human kidney proximal tubule epithelial cells (HKC). [Method] The injury degree of HKC caused by COM crystals was measured by detecting the cell viability, lactate dehydrogenase (LDH) release amounts, reactive oxygen species (ROS), cell death rate, hyaluronic acid (HA) expression and the crystal adhesion amount on cell surface. [Result] HKC cell viability, LDH release quantity, cell death rate and HA expression were closely associated with the size of COM crystals. The damage ability of 300 nm COM crystals on normal cells was higher than that of COM-5 μm crystals. The adhesive amount of small size COM crystals (62.4 μg/cm2) was higher than micron COM crystals (26.7 μg/cm2). The increased adhesion amount of small size COM crystal are related to its larger specific surface area, crystal face changes and larger cell contact probability. [Conclusions] The cytotoxicity of COM crystals toward HKC cells is size dependent. This study is helpful to further clarify the formation mechanism of COM calculi, and provide inspiration for its prevention and treatment.

1 Introduction

Calcium oxalate monohydrate (COM) is the main composition of the renal stones, its incidence rate is about twice as much as the COD. COM crystals in the urine have obvious damage effect to renal epithelial cells [1].

Studies have shown that there are different sizes, different crystal phases, and different morphologies crystals in urine of normal and kidney stone patients [2-4]. The difference of crystal size will lead to different crystal aggregation degree, different cell contact area and thus different cytotoxicity. For instance, compared with micro CuO particles, nano-CuO particles exhibit higher cytotoxicity. Tamura et al. [5] studed the interaction of the different size of micron Ti with the neutrophils cells. Compared to the large Ti particles (10 μm, 45 μm, 150 μm), the small-sized Ti (2 μm) has the most cell damage, and has the higher expression of biochemical indicators (such as superoxide anion, LDH, TNF alpha and beta IL-1β).

Based on this, we compared the damage and adhesion difference of sub-micron and micron COM crystals with a size of 300 nm and 5μm on normal HKC cell. We hope to clarify the mechanism of cell damage, crystal adhesion, endocytosis with the formation of kidney stones, and it is expected to provide a new inspiration for the clinical inhibition of kidney stones from cellular and molecular level.

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2 Materials and Methods

2.1 *Materials and apparatus*

(1) Materials: HKC, a line of renal proximal tubular epithelial cells of human origin, was obtained from the Shanghai Changzheng Hospital (Shanghai, China, provided by Prof. Mei Chang-Lin). DMEM culture medium was purchased from Hyclone Biochemical Products Co., Ltd. (UT, USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Cell culture plates were purchased from Wuxi Nest Bio-Tech Co., Ltd. (Wuxi, China).

Cell proliferation assay kit (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Lactate dehydrogenase (LDH) kit and were all purchased from Shanghai Beyotime Bio-Tech Co., Ltd.(Shanghai, China).

Propidium iodide (PI), 4,6-diamidino-2-phenylindole (DAPI) staining solution, anti-fade fluorescence mounting medium and bovine serum albumin (BSA) were all purchased from Shanghai Beyotime Bio-Tech Co., Ltd. (Shanghai, China). Fluorescein FITC-Avidin was purchased from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China).

All conventional reagents used were analytically pure and purchased from Guangzhou Chemical Reagent Factory of China (Guangzhou, China).

2.2 *Preparation of calcium oxalate monohydrate (COM) nanocrystals and COM suspension*

Sub-micron and micron COM crystals with a size of 300 nm and 5μm were synthesized according to previous reference [6] by changing the concentration of reactants ($CaCl_2$ and Na_2Ox), reaction temperature, solvent and stirring speed. The size and crystal phase of the prepared crystals were characterized by SEM and XRD.

2.3 *Cell injury and analysis of cell viability*

Cell viability of HKC cells was evaluated colorimetrically with CCK-8. After cells were trypsinized, 100 μl of cell suspension (1×10^4 cells/ml) was plated per well in 96-well plates (Corning Costar, Badhoevedorp, The Netherlands) and cultured in DMEM/F12 containing 10% newborn calf serum for 24 h. Then, medium was aspirated and replaced with serum-free DMEM/F12 medium. Cells were kept in serum-free medium for 12 h to achieve quiescence. Subsequent experiments were carried out in serum-free DMEM/F12 medium. At the indicated time points, the medium was aspirated, cells were washed twice with D-Hanks, and freshly cultured medium was added. Then, 10 μl of CCK-8 solutions was added to each well. After 4 h incubation at 5% CO_2 air atmosphere and 37° C, absorbance at 450 nm of each well was measured using the microplate reader. Cell viability was calculated with $A_{exp}/A_{con}\times100\%$, where A_{exp} is the absorbance of cells exposed to reagents and A_{con} is the absorbance of control cells.

2.4 *Intracellular reactive oxygen species (ROS) assay*

Two milliliters of cell suspension with a cell concentration of 1×10^5 cells/mL was inoculated per well in six-well plates. After establishment of quiescence and treatment of cells with culture medium, as described above, medium was aspirated at the indicated timepoints to be added to the ROS detection kit.

2.5 *Fluorescence microscopy observation of cell apoptosis and necrosis*

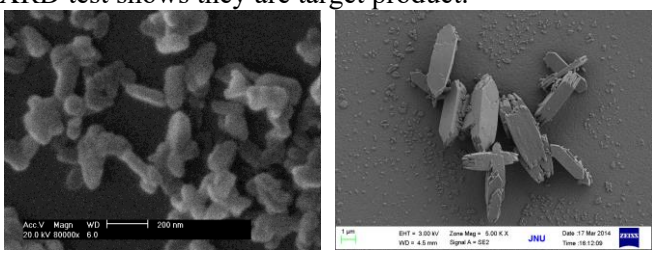
The density of seeded cells and experimental grouping were the same as those in Section 2.2.3. Afterward, the culture media were discarded and the cells were rinsed with PBS and then incubated in serum-free culture media with COM crystals for 6h. Then the cells were washed with PBS for three times and stained using 5 μL of Hoechst 33342/PI for 20 min at 4℃. The Hoechst 33342 exhibited blue fluorescence and PI exhibited red fluorescence.

2.6 *HA expression assay*
One milliliter of cell suspension with a cell concentration of 1×10^5 cells/mL was inoculated per well in 12-well plates. After synchronization, the cells were grouped. HA expression and cell nuclei were stained green and blue, respectively.

2.7 *SEM observation of adhered COM crystals on cell surface*
The density of seeded cells and experimental grouping were the same as those detected by HA. After reaching the adhesion time, the supernatant was removed by suction, washed three times with PBS, fixed in 2.5% glutaraldehyde at 4 °C for 24 h, fixed with 1% OsO_4, washed three times with PBS, dehydrated in gradient ethanol (30%, 50%, 70%, 90% and 100%, respectively), dried under the critical point of CO_2, and treated with gold sputtering. The crystal adhesion was observed by SEM.

3 Results

3.1 *Synthesis and characterization of sub-micron and micron COM*
Fig. 1 shows the scanning electron microscope (SEM) photos of sub-micron and micron COM crystals. The crystal size is about 300 ±20 nm and 5.0 ± 1.0 μm respectively. We define them as COM-300nm and COM-5μm. XRD test shows they are target product.



(a) (b)

Fig 1. SEM images of sub-micron and micron COM crystals. (a) COM-300nm; (b) COM-5μm.

3.2 *Cell vitality and lactate dehydrogenase (LDH) release amount change caused by sub-micron and micron COM crystals*
CCK 8 method is used to detect the oxidative damage of sub-micron and micron COM crystals on normal HKC cell. As shown in Table 1. the damage of COM-300nm in cells was significantly greater than COM-5μm under the 200 μg/mL crystal concentration.

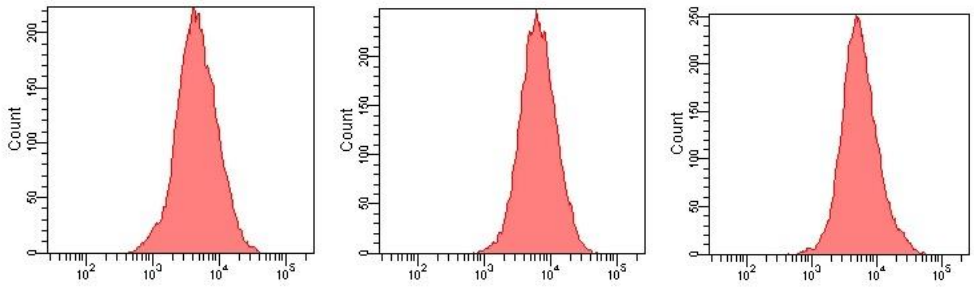
The LDH release quantity can be used as a sign of cell membrane integrity. The more LDH release quantity there is, the more the cell damage is [7]. LDH release amount of HKC cells after exposuring to sub-micron and micron COM are greater than the control group (2.79%) (Table 1), and the addition of LDH release of COM-300 nm is more obvious. It shows that the cell damage of sub-micron COM is greater than that of micro crystal. This result is consistent with that of cell vitality detection.

Table 1 Changes in cell viability, LDH release amount, ROS and adhesion amount of HKC cells after exposure to sub-micron/micron COM crystals

		Control group	COM-300 nm	COM-5 μm
Cell vitality using CCK8	COM	100	61.2	78.1
LDH release amount / %	COM	2.79	12.7	7.28
ROS level	COM	5960	7950	6780
Crystal adhesion amount using ICP /μg/cm^2	COM	0	62.4	26.7

3.3 *Reactive oxygen species (ROS) change caused by sub-micron and micron COM crystals*
In order to further clarify the toxicity influence of COM size on HKC cell, we detected the intracellular ROS change (Table 1). The content of ROS decreased in the order: COM-300 nm > COM-5 μm > the control group (Fig. 2B). The larger ROS level means more serious cell damage.

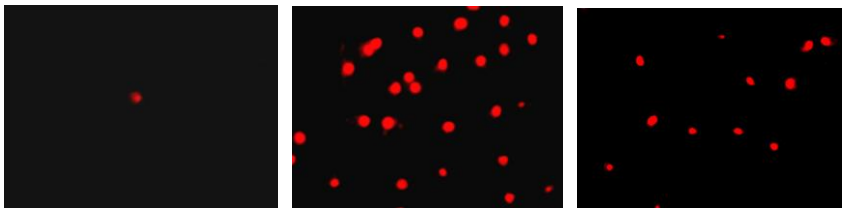
There is a direct relationship between crystal surface area and ROS level. Under same mass concentration of the crystal, the small-sized crystal has a larger surface area, leading to more atoms or molecules be exposed on the surface. That is, the small-sized COM crystals have more active sites on surface, these active sites can capture oxygen molecules, and thus produce superoxide free radicals and other ROS by disproportionation reaction or Fenton reaction [8].



(a) (b) (c)

Fig. 2 Intracellular ROS level of HKC cells after exposure to sub-micron and micron COM crystals for 6 h. (a) Control group; (b) sub-micron COM; (c) micron COM.

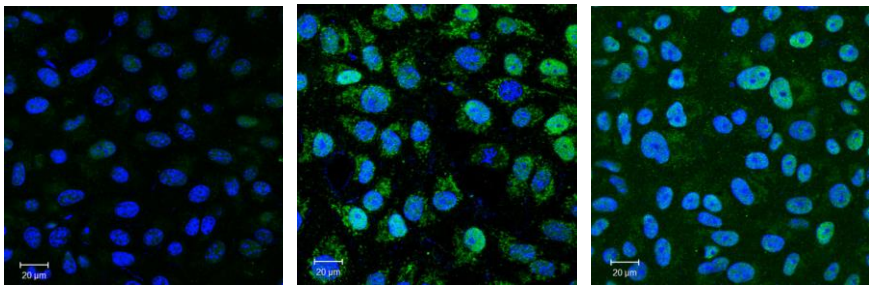
3.4 *Detections of the iodide c organism (PI) staining after COM crystals adhering to HKC cells*
The cell death by PI staining after HKC adhering to sub-micron and micron COM crystals was compared by PI staining, the greater fluorescence intensity indicates that the HKC mortality rates increased constantly. As shown in Fig. 3, the control group was dyed only a small amount of the nucleus, COM-300 nm group was dyed the most, and the PI staining number of COM-5 μm group decreased significantly. It showed that smaller COM caused largest HKC cell mortality [9].



(a) (b) (c)

Fig. 3 PI staining results of HKC cells after exposure to COM crystals for 6 h. (a) Control group; (b) sub-micron COM; (c) micron COM. Magnification ×600

3.5 *Hyaluronic acid (HA) expression amount*
Normal renal epithelial cells generally express less HA. HA express only when the cells are damaged [10]. Fig. 4 showed the HA expression after the adhension COM crystals on HKC surface using laser confocal microscope. The HA fluorescence intensity was COM-300 nm > COM-5 μm > control group.



(a) (b) (c)

Fig. 4 HA expression in HKC cells after exposure to sub-micron/micron COM crystals for 6 h. (a) Control group; (b) sub-micron COM; (c) micron COM.

3.6 *SEM observation and adhesion amount detection*

Fig. 5 showed the SEM images after the COM crystals interacted with normal HKC of 6 h. There is an obvious aggregation after the small-sized COM-300nm crystals adhering to cell (Fig. 5a); but micron-sized COM-5 μm crystals only adhered as single crystal on cell surface.

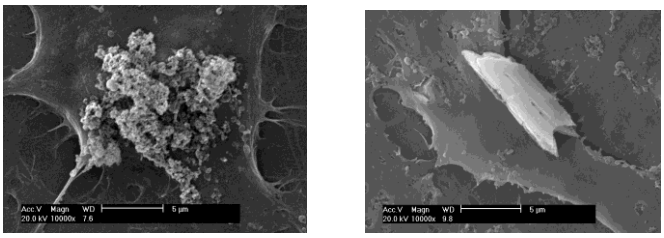
The crystal adhesion amount of COM on cell surface was measured using inductively coupled plasma emission spectrometer (ICP) and the result was also list in Table 1. The adhesive capacity of sub-micron COM (62.4 μg/cm^2) was greater than the micron COM (26.7 μg/cm^2).

4 Discussion

The COM crystals adhesion on renal epithelial cell can induce the dysfunction of mitochondria, oxidative stress and cell damage, eventually leading to the formation of kidney stones[11]. COM crystals can activate p38 cytokinins protein kinase (MAPK), increase the production of reactive oxygen species in the mitochondria, leading to the antioxidant depletion and cell oxidative damage[12]. Sub-micron COM-300 nm crystals had larger damage capacity and adhesion ability on HKC cell than icron COM-5 μm crystals. the reasons are as follows:

1) In the same concentration of crystal (200 ug/mL), the small-sized COM crystals have more active sites, higher surface energy, larger specific surface area, thus leading much serious damage on HKC cells. The surface of damaged cells expressed a large number of negatively-charged molecules such as HA (Fig. 4), these molecules can adsorb with Ca^{2+} ions on the surface of the COM crystal by electrostatic interactions.

2) Compared with COM-5 μm, small crystals have aggregated easily so as to reduce their surface energy and achieve stability, this aggregation was also observed by SEM (Fig. 5a).



(a) (b)

Fig. 5 SEM images of sub-micron/micron COM crystals adhered to HKC cells for 6 h. (a) sub-micron COM; (b) micron COM.

5 Conclusions

The cell damage and cell adhesion of COM-300nm and COM-5μm on HKC cells were comparatively studied. Both the crystals can reduce the cell vitality, increased LDH release amount, ROS level and HA expression quantity. The damage capacity of COM-300 nm is greater than that of COM-5 μm. Be-

cause there are different sizes of COM crystals in urine, this study can help to further clarify COM calculi formation mechanism, and provide inspiration for its prevention and treatment.

Acknowledgment
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