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Injured Human Kidney Proximal Tubular Epithelial Cells Modulate Nucleation and Growth of Calcium Oxalate Crystals

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Abstract. Purpose: The oxidative damage of hydrogen peroxide (H_2O_2) on human kidney proximal tubular epithelial cell (HKC) and the nucleation and growth of calcium oxalate (CaOx) crystals modulated by normal and injured cells were investigated. **Methods:** The method of CCK-8 was used to detect the proliferating inhibition effect of H_2O_2 on HKC. Cells were incubated with serum-free culture media containing CaOx supersaturated solutions. The cellular morphology and crystal growth were observed by invert microscope and scanning electron microscope (SEM). **Results:** H_2O_2 could damage HKC in time-dependent (0~60 min) and concentration-dependent manners (0~1.0 mmol/L). The amount of CaOx crystals induced by injured cells was more than that induced by control cells, and the size of the crystals induced by injured cells was also larger. An oxidative damage mode for H_2O_2 was established. **Conclusions:** HKC could be damaged obviously by both H_2O_2 and high concentration CaOx solution in time-dependent and concentration-dependent manners. Injured cells promote the nucleation and growth of CaOx crystals.

Introduction

Renal calculus, the production of pathological biomineralization in organism, is always one of the clinical diseases investigated significantly because of its high incidence rate of 10%-15% and high recurrence rate, whereas its formation mechanism has not yet been elucidated clearly.

Calcium oxalate (CaOx) crystal is the main component of renal calculus. It is generally agreed that the retention of CaOx microcrystals in renal tubule is an important inducement for the formation of renal calculus. CaOx microcrystals can retain in renal tubule in two ways: first, they nucleate and grow in virtue of some matrix in urine [1]; second, they adhere to renal tubular epithelial cells. In normal conditions, there are various defensive mechanisms in living bodies against crystal retention, such as urinary inhibitors in urine, the mechanism of internalization-lysis, and so on [2]. Thus, the formation of renal stones is properly due to the disturbance of defensive mechanisms in living bodies, resulting in the retention of CaOx crystals. Wiessner et al [3] have shown that renal epithelial cell injury could induce the changes of the components, structures and functions of plasma membrane, promoting the adherence of CaOx crystals and favoring the formation of kidney stones.

Because it is difficult to investigate the adherence and retention of crystals in situ, some modes in vitro, such as animal modes and cells modes, were used to explore the pathological mechanisms of nephrolithiasis nowadays. It is more common for cells models system in vitro. Usually some investigators choose different cell lines basing on their experiment purposes, including pig kidney proximal tubular epithelial cell line (LLC-PK1), Madin-Darby canine kidney cell line (MDCK), African green monkey

epithelial cell line (BSC-1), primary cultured rat inner medullary collecting duct cell line (IMCD), and so on [3-5].

Materials and Methods

Materials and Apparatus

Human kidney proximal tubular epithelial cells (HKC) (Shanghai Institute of Cell Bank, China). Microplate Reader (Safire2, Austria). X-L type environmental scanning electron microscope (ESEM, Philips). All conventional reagents used were analytically pure and purchased from Guangzhou Chemical Reagent Factory of China (Guangzhou, China).

CCK-8 Detects Cell Proliferation inhibition Rate

The method of CCK-8 was used to measure the injury degree of HKC caused by H_2O_2. The cells were seeded in 96-well plates with the cell density of 5×10^4 cell/ml and 150 μl per well and incubated for 24 h in an incubator with 37 ℃, 5% CO_2, saturated humidity. Cells were divided into two groups. In control groups (n=4), only serum-free culture media was added. In injured groups, cells were exposed to serum-free culture media containing H_2O_2 (0.1, 0.3, 0.5, 0.8, 1.0 mmol/L) for 20, 30, 40, 60 min, respectively. Each sample at each time point and concentration point was repeated 4 wells (n=4). Then all the culture media was changed to fresh serum-free culture media following addition of 10 μl CCK-8 to each well and incubation for 24 h in an incubator at 37 ℃. Absorbance was measured by enzyme mark instrument at 450 nm, and the cell proliferation inhibition rate was calculated.

Nucleation and Growth of CaOx Crystals Induced by Injured HKC

The cells were seeded in 12-well plates with the cell density of 4×10^5 cell/ml and 1000 μl per well where was placed with, and then the plates were placed in an incubator with 37 ℃ and 5% CO_2 for 24 h incubation. The media was changed to serum-free culture media for 12 h incubation to achieve synchronization. Cells in the plate were divided into two groups (n = 6): group A and group B. group A was control group and group B was injured group. Then the plates were placed at 37 ℃ in a 5% CO_2 air atmosphere incubated for 0.5 h, 1 h, respectively. After that, culture media was aspirated prior to rinsing twice with D-Hanks. Serum-free culture media containing 0.50 mmol/L CaOx was added into group A and group B following incubation for 2, 12 and 24 h in an incubator with 37 ℃ and 5% CO_2, respectively.

Observation of Crystals on Cells by Scanning Electron Microscope

After all the glass coverslips in 12-well plates were taken out, the cells on coverslips were rinsed twice with D-Hanks and fixed with 2.5 % glutaraldehyde for 24 h. Afterward the cells were rinsed thrice with D-Hanks, dehydrated in gradient ethanol (30, 50, 70, 90, 100%, respectively), dried under the critical point of CO_2 and embedded through gold sputtering. Finally, cell morphology and crystals growth were observed by X-L environmental scanning electron microscope.

Results

Effect of Concentration of H2O2 on the Injury of HKC Cells

The injure degree of HKC caused by H_2O_2 was assessed by proliferation inhibition rate. CCK-8 was used to measure the inhibition effect. As shown in Table 1, after exposure

of HKC to 0.1 mmol/L H_2O_2 for 20 min, proliferation of HKC was significantly inhibited, and the proliferation inhibition rate was 16.7 % (P<0.05), showing that H_2O_2 had induced significant oxidative damage of HKC cells. As the concentration of H_2O_2 increased from 0.1 mmol/L to 1 mmol/L, the proliferation inhibition rate was increased to 44.1 %, suggesting that the damage effect of H_2O_2 on HKC was concentration-dependent in this concentration range.

As the cells exposed to H_2O_2 for 30 min, 40 min, and 60 min, respectively, the results were approximately consistent with the concentration-dependent curve shown by the exposure time of 20 min.

Effect of Exposure Time of H2O2 on the Injury of HKC Cells

It was also significant that the injury of H_2O_2 on HKC was in time-dependent manner (Table 1). As the exposure time increased from 20 min to 60 min, the proliferation inhibition rate of H_2O_2 to HKC increased from 16.7 % to 49.9 % (P<0.05), while the proliferation inhibition rate of 1.0 mmol/L H_2O_2 to HKC cells increased from 44.1% to 78.2% (P<0.05); and the other concentrations points such as 0.3, 0.5 and 0.8 mmol/L H_2O_2 presented the similar changes as mentioned above. However, when HKC was exposed to 0.8 mmol/L H_2O_2 for 30 min, 40 min, and 60 min, the proliferation inhibition rate was 50.9%, 62.4%, and 77.3% respectively, it was 53.5% (30 min), 65.0% (40 min), and 78.2% (60 min) while HKC were exposed to 1.0 mmol/L H_2O_2, respectively. Multiple comparison tests showed no significant differences (P>0.05), suggesting that there were no significant differences in the exposure of HKC to 0.8 mmol/L and 1.0 mmol/L H_2O_2 for 30-60 min.

We chose the exposure of HKC to 0.3 mmol/L H_2O_2 for 30 min to set up an oxidative damage mode for HKC.

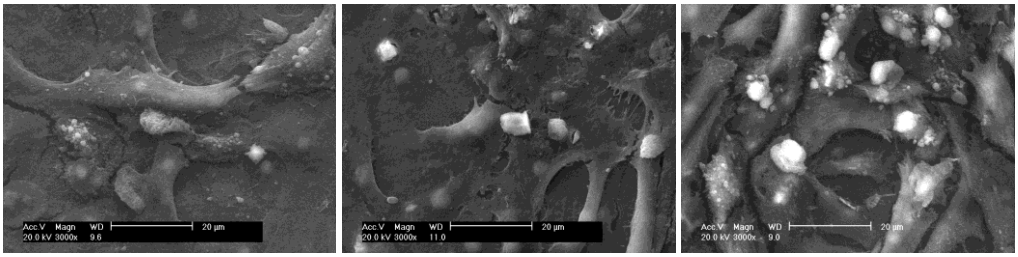
Table 1. Effect of concentration and exposure time of H_2O_2 on the proliferation inhibition rate of HKC

Concentration / mmol/L	Time / min				
	0	20	30	40	60
0.1	0	16.7	28.9	39.8	49.9
0.3	0	26.0	39.3	47.5	57.6
0.5	0	36.0	44.8	54.0	63.1
0.8	0	34.5	50.9	62.4	77.3
1.0	0	44.1	53.5	65.0	78.3

Normal and Injured HKC Modulate the Nucleation and Growth of CaOx Crystals

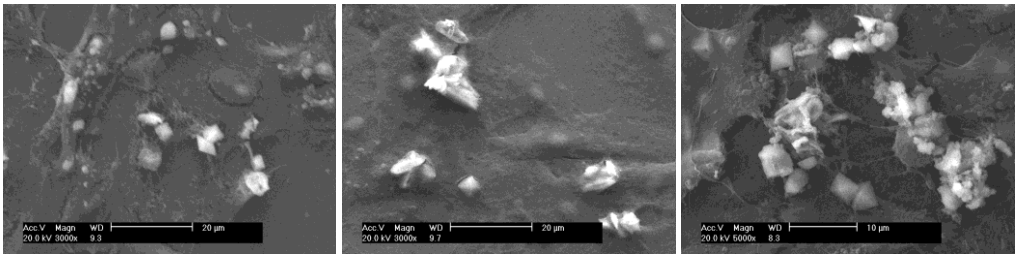
When the cells were exposed to 0.5 mmol/L CaOx supersaturated solution for 2 h, both control group and injury group could induce CaOx crystals formed on the HKC cellular surface. As the time of incubation increased, the size of crystals increased, suggesting that the cells modulated the nucleation and growth of CaOx in the whole process. The crystal surfaces were gradually covered by cell materials, which presented the trend of internalization of crystals as the time of incubation increased further (Figure 1).

Compared with the normal HKC cells, the size and number of the crystals induced by injured cells increased (Figure 2). That means that there was some differences between the mechanisms of H_2O_2-injured cells and non-injured cells in the induction of the formation of crystals, but the exact reason was not yet clear.



(a) (b) (c)

Figure 1. SEM images of the formation of CaOx crystals induced by normal HKC in control group. Crystallization time: (a) 2 h, (b)12 h, (c) 24 h. c(CaOx)=0.5 mmol/L. Bar: 20 μm.



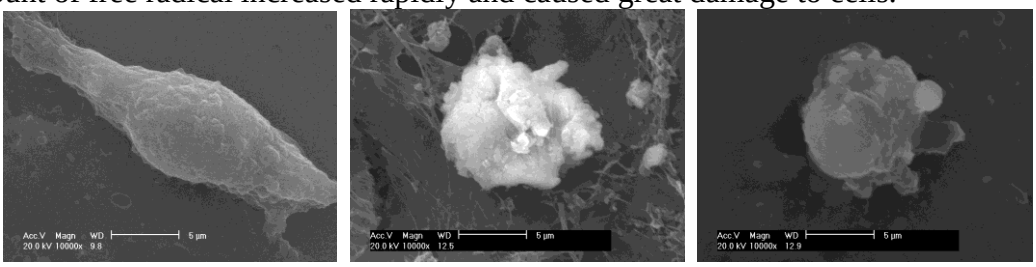
(a) (b) (c)

Figure 2. SEM images of the formation of CaOx crystals induced by HKC in injured group. Crystallization time: (a) 2 h, (b)12 h, (c) 24 h. c(CaOx)=0.5 mmol/L. Bar: 20 μm.

Injury of CaOx Supersaturated Solution on HKC Cells

The normal HKC cells were long strip, well-spread, plump with intact cilia and synapse (Figure 3a). As the exposure time of HKC to 0.50 mmol/L CaOx supersaturated solution increased to 12 h, the cells present shrinkage, subround shapes, rough surfaces, shedding cilia and synapse mostly, and existing a mass of cell debris (Figure 2b). Cells were damaged aggravatingly as the time increased to 24 h with more obviously shrinkable and round morphology (Figure 2c).

That is, exposure of HKC to CaOx supersaturated solution induced cell injury, and the injury aggravated as time increased. Some studies had shown that the cultured renal tubular epithelial cells could be injured by oxalate and CaOx crystals, which belonged to free radical injury [6]. Free radical reaction was a chain reaction. Little free radicals at the starting only caused little damage to cells. But as the reaction proceeded, the amount of free radical increased rapidly and caused great damage to cells.



(a) (b) (c)

Figure 3. SEM images of normal HKC exposed to CaOx supersaturated solution for different time. (a) 2 h, (b) 12 h, (c) 24 h. c(CaOx)=0.50 mmol/L. Bar: 5 μm.

Discussion

Supersaturation of calculi salt in urine is the chemical driving force of formation of urolithiasis, which determines a series of chemical thermodynamics and dynamics related to the formation of urolithiasis including the nucleation, growth, aggregation and phase transition of calculi salt. Stones can form only when crystals adhere to cells,

or else they would be swept out by renal tubular liquid at once, that is, only free crystals turn to fixed particles could result in the formation of stones. Therefore, the adhesion between crystals and renal tubular epithelial cells is the initial event and a key step of formation of nephrolith.

In normal physiological conditions, the surface of perfect polarized renal tubule epithelial cells went against the adherence of crystals. In addiction, inhibitors in the tubule lumen fluid hold back the attraction of crystals to cells by covering on the potential binding sites of crystal surfaces. Only pathological changes occurred in kidney, such as the injury of kidney tubule epithelial cells, could enhance the adherence between crystals and epithelial cells and result in the formation of kidney stones [7].

In recent years, injury of renal epithelial cells induced by free radicals, which favors the adhesion between crystals and cells, has become one of the key researches. Production of many components in kidney stones, including uric acid, oxalic acid, and phosphoric acid, demands various oxidative processes. Oxidation is enhanced along with the production of those substances increase, hence more free radicals are produced inevitably. Meanwhile, oxalic acid can inhibit the activity of catalase, reducing the capability of elimination of free radicals in live system and resulting in extensive injury to renal tubular epithelial cells by a large number of free radicals.

In the present study, H_2O_2 caused obviously injury to HKC. Exposure of HKC to the concentration of 0.1 mmol/L H_2O_2 for 20 min caused a proliferation inhibition rate of 16.7 % (Table 1). The result showed that the injury was enhanced when the reaction time increased, but went mild at 40 min (Table 1). The reason was that there were more normal cells at the beginning which were available for injury, hence the inhibition rate grew fast. As the reaction time increased, the amount of injured cells increased and proliferating cells decreased resulting in the inhibition rate decreased.

The injury of the H_2O_2 on cells is extensive, whereas the basic mechanism is that OH was created via the reaction of Haber-Weiss or Fentons. H_2O_2 mainly caused lipid peroxidation to cell membrane, resulting in change of ultrastructure of cell membrane [8].

In control groups or in injured groups, after incubation with 0.50 mmol/L supersaturated CaOx solution for a certain time, HKC could induce the formation of CaOx crystals with definite morphology and size (Figures 2 & 3). It suggests that the nucleation and growth of CaOx was not an isolated process, but modulated by HKC, resulting in differences in morphology and size between crystals formed in normal solution.

Not only could H_2O_2 injure the HKC, but also CaOx supersaturated solution incubating HKC for a certain time could. As the incubation time increased to 24 h, we can see cellular shrinkage, detachment of most cilia and synapses, and being attached by mass cellular debris (Figure 3b), suggesting that CaOx solution could produce damage on HKC, inducing change of cellular morphology, which was in direct proportion to reaction time. As the reaction time reached 24 h, cellular morphology changed more obviously with more severely shrinking and rounder shape (Figure 3c).

Cell injury brought on changes of morphology and function of cell membrane, and the cell surface would express some crystal binding molecules like hyaluronic acid, favoring the adhesion of crystal to cells [9]. Compared Figure 1 with Figure 2, both the amount and size of CaOx crystals induced by injured HKC increased than those by normal HKC cells.

Conclusions

The oxidative injury of H_2O_2 on HKC cells and the nucleation and growth of CaOx crystals induced by injured cells were investigated. H_2O_2 could decrease the survival rate of cells in time-dependent manner within 0~60 min and in concentration-dependent manner within 0~1.0 mmol/L. Both H_2O_2-injured cells and non-injured cells could induce the nucleation and growth of CaOx crystals. However, after exposure of cells to 0.50 mmol/L CaOx supersaturated solution for a period of time, more CaOx crystals with bigger size were induced by injured groups than those induced by control group, indicating that injured cells could promote the nucleation and growth of CaOx crystals much easier than non-injured cells. CaOx supersaturated solution also could cause damage effect on HKC, resulting in cell shrinkage. The nucleation and growth of CaOx crystals was modulated by HKC.

Acknowledgements

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