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ORIGINAL PAPER

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# Effects of Tamm-Horsfall protein on the protection of MCDK cells from oxalate induced free radical injury

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**Abstract** Renal cell injury and fixed particle formation is one of the theories of urinary stone formation. The exposure of renal epithelial cells to oxalate ions and calcium oxalate monohydrate crystals can cause free radical generation and increase lipid peroxidation. Tamm-Horsfall protein (THP) has a protective effect on the production of free radicals in vitro. We aimed to show that THP (and its deglycosylated products, D-THP) could protect culture cells from free radical injury in vivo as well as the possible mechanism by which this is done. Exposure of Madin-Darby canine kidney (MDCK) cells to Ox resulted in a significant increase in the release LDH, NBT and MDA, as well as an increase in caspase 3 activity, all of which were further elevated when COM crystals were added. With the addition of THP at 500 nM, there was a significant decrease in the release of LDH and the production of MDA and NBT. A decrease in capase 3 activity was observed when 500 nM THP was added to the culture medium that reached 32.7% and 40.4% of inhibition in CaOx + THP and CaOx + COM + THP, respectively. THP decreased the adhesion of COM crystals to the MDCK cells but lost its effect when THP was deglycosylated. The results indicate that both Ox and COM crystals cause the release of LDH, MDA, NBT and increase the activity of capase 3 in MDCK cells. As a free radical scavenger, THP reduces the amount of free radicals and provides significant protection at a critical concentration of 500 nM. The deglycosylated THP decreased the effect of the protection of the MDCK cells from oxalate-induced injury and an increase of adhesion of the COM crystals to the MDCK cells. Therefore, the effects of THP on the protection of oxalate induced radical injury may be partly due to its intact glycosylation and its adhesion to the cell membrane.

**Keywords** Tamm-Horsfall protein · Free radicals · Calcium oxalate · Urolithiasis

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## Introduction

Urolithiasis is a complex disease and many theories have been proposed regarding the mechanism of crystal formation. One of the theories involves renal tubular cell injury and fixed particles [5, 19]. An excess urinary excretion of oxalate can damage renal epithelial cells [26]. Thamilselvan et al. reported that oxalate (Ox) and COM (calcium oxalate monohydrate) crystals induced free radical production, renal epithelial cell injury, and membrane lipid peroxidation [32]. The cellular damage and its debris could be a heterogeneous nucleator of both calcium oxalate and calcium phosphate [15]. Evidence from cell cultures has shown cellular injury from oxalate exposure by measuring lipid peroxidation and lactate dehydrogenase release [10, 14, 27]. Furthermore, antioxidants such as catalase and superoxide dismutase can provide protection from free radicals [32]. Therefore, hyperoxalauria might be one of the major risk factors for calcium oxalate stone disease. However, this hypothesis does not differentiate between normal individuals and stone patients.

Tamm-Horsfall glycoprotein (THP) is one of the most abundant proteins in normal human urine [14]. Glycosylation, the post-translational modification of THP, is reported to have various functions. The occurrence of a gel-like property in a solution of THP appears to be involved with an intact glycosylation [28]. The carbohydrate moiety of THP could be a specific ligand for cytokines, such as interleukin-1, interleukin-2 and tumor necrosis factor [13]. Enzymatic removal of *N*-linked glycosylation of THP destroys its ability to bind to cytokines [24]. The low sialic acid form of THP may promote crystal aggregation [11]. This suggests that the alteration of glycosylation could result in differential bioactivity of THP. Therefore, glycosylation should be taken into consideration when considering the functions involved in the crystallization of calcium oxalate. Furthermore, THP inhibited the reaction of xanthine and xanthine oxidase and was proposed to be a possible protector against free radicals [3].

Various antioxidants have been used for protection against renal cellular damage and the prevention of stone formation by free radicals [20, 32]. However, few studies have focused on the role of proteins in the urine for the prevention or stability of stone formations following cellular injury. Our previous study showed that THP has an inhibitory effect on the xanthine-xanthine oxidase reaction in vitro [3]. In order to study the protective effect of THP from free radicals in vivo, a culture of Madin-Darby canine kidney (MDCK) cells was tested.

## Materials and methods

### Materials

The electrophoresis gels (SDS-PAGE) and silver stain plus were purchased from Bio-Rad Laboratories (Hercules, Calif., USA). Low pre-stained molecular weight markers were purchased from Electran (Poole, England). All chemical reagents used were of analytical grade and were purchased from Merck (Darmstadt, Germany). Dulbecco's modified essential medium (D-MEM) and agents used in the cell culture were purchased from Life Technologies (Rockville, Md., USA).

### Collection of urine

Twenty-four-hour urine samples were collected from healthy adult male volunteers without any history of urinary stones. Routine renal sonography was performed before the collection of urine in order to rule out possible nephrolithiasis. The sample was kept at 4°C during collection and sodium azide (0.02% W/V) together with 5 $\mu$M phenylmethane-sulfonylfluoride were added as preservatives. Test-strips confirmed the absence of hematuria. After collection, the samples were filtered through a 0.22 $\mu$m filter membrane (Millipore, Mass., USA) and pooled to 20 l for later use.

### Purification of Tamm-Horsfall protein

The purification procedure was conducted according to reports by Grover et al. and our previous studies [3, 8]. Briefly, NaCl was added to the pooled urine bringing the concentration to 0.58 M. The urine was stirred at 4°C for 48 h, then the solution was centrifuged at 10,000×*g* for 20 min at 4°C using a Beckman J21 centrifuge (Beckman Instruments, USA). The supernatant was discarded and the precipitate was redissolved in deionized distilled (dd) water. Cellular debris was then removed by centrifugation. The precipitation procedure was repeated twice and the pellet was dissolved in dd water as before. After precipitation, the material was dissolved in a minimal volume of dd water and dialyzed against dd water at 4°C for 24h. The sample solution was chromatographed on Sephrarose 4 B (4×80 cm column) and eluted with a 0.05 M Tris-HCl pH 7.4 (Pharmacia Biotechnology, Uppsala, Sweden). The elutes were pooled and dialyzed against dd water at 4°C for 24 h. The sample was then lyophilized and stored at −20°C until use. THP (2 mg/ml) was added in an equal volume of sodium acetate and acetic acid buffer (90 mmol/l, pH 5.5) containing NaCl (0.3 mol/l), calcium chloride (18 mmol/l), and 7 U/l neuraminidase (type 5 from *Clostridium perefringens*, Sigma, St Louis, Mo., USA). The solution was kept at 37°C for 1 day and dialyzed against dd water with three changes in 48 h. The deglycosylation was verified by SDS-PAGE.

### Cell culture

MDCK cells (CCRC60004) of distal tubular origin (Food Industry Research and Development Institute, Hsinchu, Taiwan) from passages from 15–45 were used. Serial cultures were maintained as subconfluent monolayers on 75 $cm^2$ Falcon T-flasks in D-MEM containing 15 mmol HEPES, 10% fetal calf serum (serum iron approximately 100 $\mu$g/dl), $Fe(NO_3)_3$ (0.1 mg/l), streptomycin (0.20 mg/ml) and penicillin ($1.0\times10^2$ IU/ml), pH 7.4 at 37°C in a 5% carbon dioxide air atmosphere. The medium was replenished two to three times weekly. Cells were grown to confluence in D-MEM medium on 12-well plates (Corning, N.Y.). In all studies, the monolayers were rested for 1 day in serum free D-MEM media after the cells became confluent, to achieve quiescence [6, 29].

### Oxalate ions and calcium oxalate crystal preparation

COM crystals were prepared as previously describes by Nakagawa et al. and the product was verified using an infrared spectrometer as in our previous work (Bio-Rad, Hercules, IR-7) [2, 25]. The amount of COM crystal load utilized varied from 10 $\mu$g/ml to 2 mg/ml while the concentration of Ox employed ranged from 0.1 to 4.0 mM [32]. Oxalate ion was prepared as potassium oxalate (KOx) in a stock solution of 10 mM in normal sterile saline. The cell cultures were exposed to BME medium containing 1.0 mM Ox or 1.0 mM Ox plus 500 $\mu$g COM crystals for 2 or 4 h. Control cultures were not exposed to either agent.

UV-sterilized crystals were equilibrated in medium containing 1.0 mM KOx at a concentration of 10 mg/ml at 37°C for 24 h before addition to cultures. Aliquots of stock slurry were added, along with 1.0 mM KOx media, to individual wells at a final concentration of 500 $\mu$g/ml. Monolayers were incubated for 2 or 4 h.

### Lactate dehydrogenase

The media from the control and experimental wells were recovered and centrifuged to remove crystals and cellular debris. The lactate dehydrogenase activity from these media was determined with a commercial kit (Proteins International, Rochester Hills, Mich., USA) by microtiter assay. All determinations were made against appropriate reagent blanks.

### Malondialdehyde

Lipid peroxidation was measured by the amount of malondialdehyde (MDA) as described by Wong et al. [33] with the following modifications. At the completion of the experimental treatment

periods, the cells were rinsed three times with phosphate buffered saline (PBS). A total of 0.5 ml of 100 mM KCl solution containing 3 mM EDTA was added to the wells and the cells were scraped off with a rubber baton. The cell suspension was removed with a pipette and placed into a 2-ml test tube on ice. The cell suspension was sonicated three times on ice from 100 W to 20KHz for 45 s. After centrifugation at 5,000 rpm for 5 min at 4°C, the supernatants were used for the determination of MDA. The reaction mixture contained 80.3 $\mu$l of cell homogenate and 1 ml of reagent (15% trichloroacetic acid; 0.375% thiobarbituric acid; 0.25 N HCl) containing butylated hydroxytoluene (0.03 volume of 2% in ethanol). The mixture was then heated to 95°C for 30 min in a boiling water bath. The samples were cooled and centrifuged at 13,000 rpm for 10 min and extracted by 70% methanol. After extraction, the samples were centrifuged at 13,000 rpm for 5 min. The MDA contents were then measured by spectrophotometer at $OD_{532}$.

### Nitroblue tetrazolium

We monitored oxalate-induced changes in the reduction of nitroblue tetrazolium (NBT, Merck, Germany) as described previously [4]. Briefly, confluent cultures of MDCK cells in 12-well plates were exposed for varying periods (0, 2, and 4 h) to serum-free DMEM medium containing 25 $\mu$g/ml NBT, a dye that reacts with superoxide (44, McPhail 45). Where indicated, 1 mM Ox, 1 mM Ox + 500 $\mu$g COM crystals, and/or THP (100, 200, and 500 nM) were also added. At 2 and 4 h, the medium was removed and the reaction was stopped by adding 70% methanol. The monolayers were washed with four changes of 100% methanol to remove non-reduced NBT, then air dried, and solubilized with a mixture of 2 M KOH and dimethyl sulfoxide (DMSO) (1:1.167). Samples were then centrifuged for 4 min at 15,000 rpm and read at 700 nm (density $OD_{700}$) against a blank cuvette containing KOH and DMSO.

### Apoptotic assay

The early stage of apoptosis was assessed by measuring the activity of caspase 3 using the immunosorbent enzyme assay. The concentration used for the THP or D-THP co-treatment was 500 nM. After the treatment of MDCK cells for 4 h, the MDCK cells were treated with the lysis buffer for 30 min at room temperature. The MDCK cell plates from the control and treatment wells were centrifuged to remove COM crystals and cellular debris. Caspases 3 activity in the cytosol was determined with a commercial kit (Promega, Madison, Wis., USA). The activity of caspase 3 was measured at 405 nm.

### Crystal retention

Attached CaOx crystals were observed with a phase contrast microscope (Nikon, Japan) equipped with a camera (Nikon FX-35A). First, the MDCK cells were seeded on the cover glasses for 2 days and treated serum free for 1 day. The concentration used for the THP co-treatment was 500 nM. After the treatment, the cells on the cover glasses were washed several times with PBS. The cover glasses were then soaked in 2.5% glutaldehyde at room temperature for 30 min before observation.

## Results

The MDCK cells exposed to Ox for 2 h showed a significant release of LDH (Table 1). Cells treated for 4 h continued to release LDH. The presence of COM crystals in the medium significantly increased the production of LDH in cells treated for 2 and 4 h. When serial concentrations of THP (100, 200, and 500 nM) were added to the cells co-treated with Ox, the results showed that 100 and 200 nM THP did not decrease LDH significantly. The data suggest that THP decreased Ox-induced free radical production only at high concentrations. At a suitable concentration, THP provided protection against superoxide radical generation induced by exposure to Ox. Similar observations were noted in the treatment of Ox + COM.

When THP was deglycosylated by sialidase, the protein did not decrease the amount of LDH released either at high or low concentrations for either 2 or 4 h exposure. With the addition of COM to cells, D-THP

**Table 1** Effect of Tamm-Horsfall protein (THP) on superoxide production in MDCK cells exposed to oxalate (Ox) or Ox + calcium oxalate monohydrate crystals (COM) by measuring the amount of LDH released

| Exposure time (OD) | 2 h (490 nm) | | 4 h (490 nm) | |
|---|---|---|---|---|
| | Mean ± SD (% inhibition) | | Mean ± SD (% inhibition) | |
| Control | 0.17 | 0.05 | 0.38 | 0.03 |
| Ox | 0.69 | 0.05 | 1.18 | 0.01 |
| Ox + THP 100 nM | 0.58 | 0.06 | 1.14 | 0.06 |
| Ox + THP 200 nM | 0.64 | 0.07 | 0.97**[a] | 0.06 |
| Ox + THP 500 nM | 0.31***[a] | 0.02 | 0.70***[a] | 0.08 |
| Ox + COM | 0.73 | 0.02 | 1.06 | 0.01 |
| Ox + COM + THP 100 nM | 0.66 | 0.02 | 1.05 | 0.04 |
| Ox + COM + THP 200 nM | 0.47***[b] | 0.06 | 0.88*[b] | 0.10 |
| Ox + COM + THP 500 nM | 0.32***[b] | 0.02 | 0.65***[b] | 0.04 |
| Control | 0.30 | 0.02 | 0.32 | 0.04 |
| Ox | 0.54 | 0.02 | 1.06 | 0.09 |
| Ox + THP–D 100 nM | 0.52 | 0.03 | 0.93 | 0.03 |
| OX + THP-D 200 nM | 0.52 | 0.06 | 0.87*[a] | 0.02 |
| Ox + THP-D 500 nM | 0.58 | 0.01 | 0.77**[a] | 0.02 |
| Ox + COM | 0.58 | 0.07 | 0.99 | 0.06 |
| Ox + COM + THP-D 100 nM | 0.60 | 0.08 | 0.91 | 0.07 |
| Ox + COM + THP-D 200 nM | 0.60 | 0.06 | 0.88 | 0.05 |
| Ox + COM + THP-D 500 nM | 0.65 | 0.08 | 0.88 | 0.04 |

also had no effect on the release of LDH. These results suggest that glycosylation is an important process and plays a key role in inhibiting Ox-induced superoxide radical production in MDCK cells.

### Lipid peroxidation

Table 2 shows the effects of THP and D-THP on the production of MDA in MDCK cells. Exposure of MDCK cells to Ox led to a significant increase in MDA production and the addition of COM crystals (Ox + COM) resulted in a further increase of MDA. When cells were co-treated with Ox and THP, the MDA significantly decreased at 200 nM and 500 nM THP for both the 2 and 4 h treatments. When MDCK cells were co-treated with Ox and COM, only 500 nM of THP had a significant inhibitory effect. We noted that, regardless of the concentration of D-THP, there was no inhibitory effect on MDA production. The inhibitory effect of THP on the production of NBT was similar to MDA, which showed a significant reduction at concentrations of 200 and 500 nM (Table 3). The inhibitory effect of D-THP was observed only at a concentration of 500 nM.

### Initiation of cell apoptosis

Because 500 nM THP has the maximum inhibitory effect, we chose it to measure the inhibitory effect on the MDCK cells exposed to Ox and Ox + COM crystals for 4 h. The activity of caspase 3 was inhibited significantly by 500 nM THP when co-treated with Ox or Ox + COM (Fig. 1). D-THP had no effect on the inhibition of caspase 3 activity in MDCK cells

### CaOx crystal retention

Figure 2b shows COM crystals attached to the cell membrane. When MDCK cells were co-treated with Ox + COM crystals and 500 nM THP for 4 h, the relative amount of crystals attached to the cell membrane significantly decreased, as can be seen by comparing with Fig. 2b and c. We also observed that MDCK cells co-treated with Ox + COM crystals and 500 nM D-THP for 4 h showed serious cell damage and crystal adhesion (Fig. 2d).

## Discussion

The THP in the crystal-cell interaction reacted in many ways to inhibit cell damage induced by exposure to Ox. Our previous studies of THP purified from healthy subjects showed that it promotes the nucleation of COM crystals and inhibits the growth and aggregation of COM crystals in vitro [2]. We also demonstrated that THP plays an important role in the inhibition of xanthine-xanthine oxidase reactions in vitro [3]. The effects were less significant when sialic acid was removed from THP. This indicates that a post-translation modification of THP may alter its function. In this study, it is clear that THP exerts a protective effect against free radical induced cell injury in vivo. This protective effect was reduced when THP was deglycosylated.

Khan et al. reported that hyperoxaluria in rats was associated with enzymuria and membranuria [17]. This hyperoxaluria-induced injury was due to lipid peroxidation in the renal cell membrane [30]. Thamilselvan et al. supported the suggestion that Ox can induce free radical damage to the renal epithelial cell [31]. The studies reported here provide direct evidence that exposure of MDCK cells to Ox or Ox + COM results in a significant increase in free radicals and injury to renal epithelial cells.

Our data are in agreement with results of Scheid et al. who demonstrated free radical production by LLK-$PK_1$ cells exposed to Ox [26]. Grases et al. reported that the generation of superoxide radicals occurred in endothelial cells exposed to COM crystals [7]. The results presented here demonstrate that exposure of MDCK cells to Ox

**Table 2** Effect of Tamm-Horsfall protein (THP) on superoxide production in MDCK cells exposed to oxalate (Ox) or Ox + calcium oxalate monohydrate crystals (COM) by measuring the amount of MDA released

| | 2 h (532nm) | | 4 h (532nm) | |
|---|---|---|---|---|
| | Mean ± SD (% of inhibition) | | Mean ± SD (% of inhibition) | |
| Control | 0.019 | 0.001 | 0.038 | 0.003 |
| Ox | 0.134 | 0.004 | 0.183 | 0.009 |
| Ox + THP 100 nM | 0.129 (3.96) | 0.003 | 0.182 (1.45) | 0.004 |
| Ox + THP 200 nM | 0.115 (14.11)*[a] | 0.009 | 0.177 (3.27) | 0.004 |
| Ox + THP 500 nM | 0.071 (47.02)*** | 0.005 | 0.066 (63.63)***[a] | 0.010 |
| Ox + COM | 0.151 | 0.004 | 0.191 | 0.004 |
| Ox + COM + THP 100 nM | 0.141 (7.03) | 0.002 | 0.185 (3.13) | 0.005 |
| Ox + COM + THP 200 nM | 0.135 (10.55) | 0.004 | 0.173 (9.74)**[b] | 0.009 |
| Ox + COM + THP 500 nM | 0.062 (40.04)***[b] | 0.016 | 0.068 (64.17)*** | 0.002 |
| Ox + THP–D 100 nM | 0.093 (3.83) | 0.004 | 0.161 (-2.77) | 0.004 |
| OX + THP-D 200 nM | 0.097 (-2.09) | 0.004 | 0.155 (1.06) | 0.011 |
| Ox + THP-D 500 nM | 0.085 (14.98) | 0.011 | 0.156 (0.42) | 0.006 |
| Ox + COM + THP-D 100 nM | 0.111 (5.38) | 0.003 | 0.170 (1.53) | 0.003 |
| Ox + COM + THP-D 200 nM | 0.108 (8.21) | 0.007 | 0.163 (5.57) | 0.008 |
| Ox + COM + THP-D 500 nM | 0.102 (13.31) | 0.005 | 0.163 (5.77) | 0.014 |

**Table 3** Effect of Tamm-Horsfall protein (THP) on superoxide production in MDCK cells exposed to oxalate (Ox) or Ox + calcium oxalate monohydrate crystals (COM) by measuring the amount of NBT released

| Exposure time (OD) | 2 h (700 nm) | | 4 h (700 nm) | |
|---|---|---|---|---|
| | Mean ± SD (% of inhibition) | | Mean ± SD (% of inhibition) | |
| Control | 0.103 | 0.002 | 0.113 | 0.002 |
| Ox | 0.177 | 0.001 | 0.188 | 0.004 |
| Ox + THP 100 nM | 0.172 (2.63) | 0.006 | 0.174 (7.43) | 0.006 |
| Ox + THP 200 nM | 0.124 (29.89)***[a] | 0.001 | 0.163 (13.45)** | 0.005 |
| Ox + THP 500 nM | 0.118 (33.27)***[a] | 0.001 | 0.145 (22.65)*** | 0.004 |
| Ox + COM | 0.183 | 0.003 | 0.200 | 0.008 |
| Ox + COM + THP 100 nM | 0.174 (4.90)*[b] | 0.003 | 0.188 (5.98) | 0.004 |
| Ox + COM + THP 200 nM | 0.137 (25.23)*[b] | 0.003 | 0.167 (16.61)*** | 0.006 |
| Ox + COM + THP 500 nM | 0.126 (31.22)*[b] | 0.003 | 0.149 (25.58)*** | 0.003 |
| Ox + THP–D 100 nM | 0.158 (-0.42) | 0.006 | 0.284 (3.07) | 0.003 |
| OX + THP-D 200 nM | 0.152 (3.59) | 0.002 | 0.282 (3.52) | 0.005 |
| Ox + THP-D 500 nM | 0.14 (11.39)**[a] | 0.003 | 0.252 (13.99)** | 0.013 |
| Ox + COM + THP-D 100 nM | 0.163 (0.61) | 0.004 | 0.286 (4.25) | 0.004 |
| Ox + COM + THP-D 200 nM | 0.162 (6.12)*[b] | 0.002 | 0.277 (4.59) | 0.005 |
| Ox + COM + THP-D 500 nM | 0.153(14.69)***[b] | 0.004 | 0.276 (8.05)* | 0.004 |

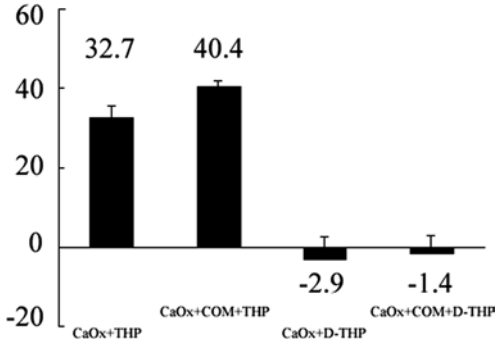


**Fig. 1** The percentage of inhibition by Tamm-Horsfall protein in the calcium oxalate inducing caspase 3 activity on MDCK cells. *CaOx*: calcium oxalate, *COM*: calcium oxalate monhydrate, and *D-THP*: siladase treated Tamm-Horsfall protein. The activity of caspase 3 was measured by OD405 nm by photospectrometer. Control = 0.0153 ± 0.055, CaOx = 0.218 ± 0.021, CaOx + COM = 0.287 ± 0.010, 500nM THP = 0.0276 ± 0.00565, CaOx + THP = 0.1466 ± 0.006, CaOx + COM + THP = 0.17 ± .0.004 THP-D = 0.041 ± 0.005, CaOx + THP-D = 0.224 ± 0.012, and CaOx + COM + THP-D = 0.283 ± 0.0128

and COM crystals is associated with: (1) the production of superoxide radicals, (2) lipid peroxidation as determined by MDA. (3) release of LDH which indicates cell injury, (4) apoptosis, and (5) COM crystal retention. We also demonstrated that THP acts as a free radical scavenger by reducing the production of superoxide radicals. The inhibition of cell injury by THP can be measured by the amount of LDH released and membrane peroxidation can be measured by MDA content induced by Ox and/or Ox + COM crystals in MDCK cells. Furthermore, THP inhibited MDCK apoptosis induced by Ox and COM, and prevented the retention of COM crystals on the MDCK cells.

The fact that only 500 nM of THP reduced the production of free radicals suggests that it must remain at a stable concentration in the urine to effect the prevention of Ox-induced cellular damage. Thus, healthy subjects probably have higher concentrations of THP than patients with urinary stones. Hess et al. demonstrated that the concentration of THP in the urine of patients with urinary stones was only 100–200 nM [12]. Kumar and Muchmore found that the concentration of THP above 600 nM in urine resulted in self-aggregation and promotion of COM crystal growth [22]. Therefore, the concentration of THP in urine may play an important role in CaOx crystallization. In our studies, there was a significant reduction in MDA content and LDH release when MDCK cells were cultured with 500 nM THP and Ox + COM crystals. These data suggest that THP protects MDCK cells against Ox induced cellular damage and that 500 nM is a critical concentration.

Koul et al. have shown that both Ox and COM crystals can induce DNA synthesis in renal epithelial cells [21]. The nature of the induction depends upon the concentration of Ox and the presence or absence of COM crystals. Occasional COM crystals are passed as crystalluria fixed particles or are endocytosed by renal epithelial cells [16]. Moderately high levels of oxalate, within the limit of CaOx metastability, can provoke an inflammatory response. This inflammatory response may also be mediated by THP. Renal tubular epithelial cells undergo mitosis and produce additional crystallization modulators such as ostopontin, fibronectin, and THP [23]. Constant exposure to high levels of oxalate and calcium oxalate crystals injures renal epithelial cells [9]. Many of the renal cells become apoptotic and their surfaces become receptive to crystal attachment [1, 18]. Our results show that THP prevented apoptosis of MDCK cells that were induced by Ox and COM crystals, and inhibited the retention of COM crystals on MDCK cell surfaces. Ox-damaged cells detach from the basement membrane and their degradation products are released into the urine. Membranes in the cellular degradation products can also promote the nucleation of

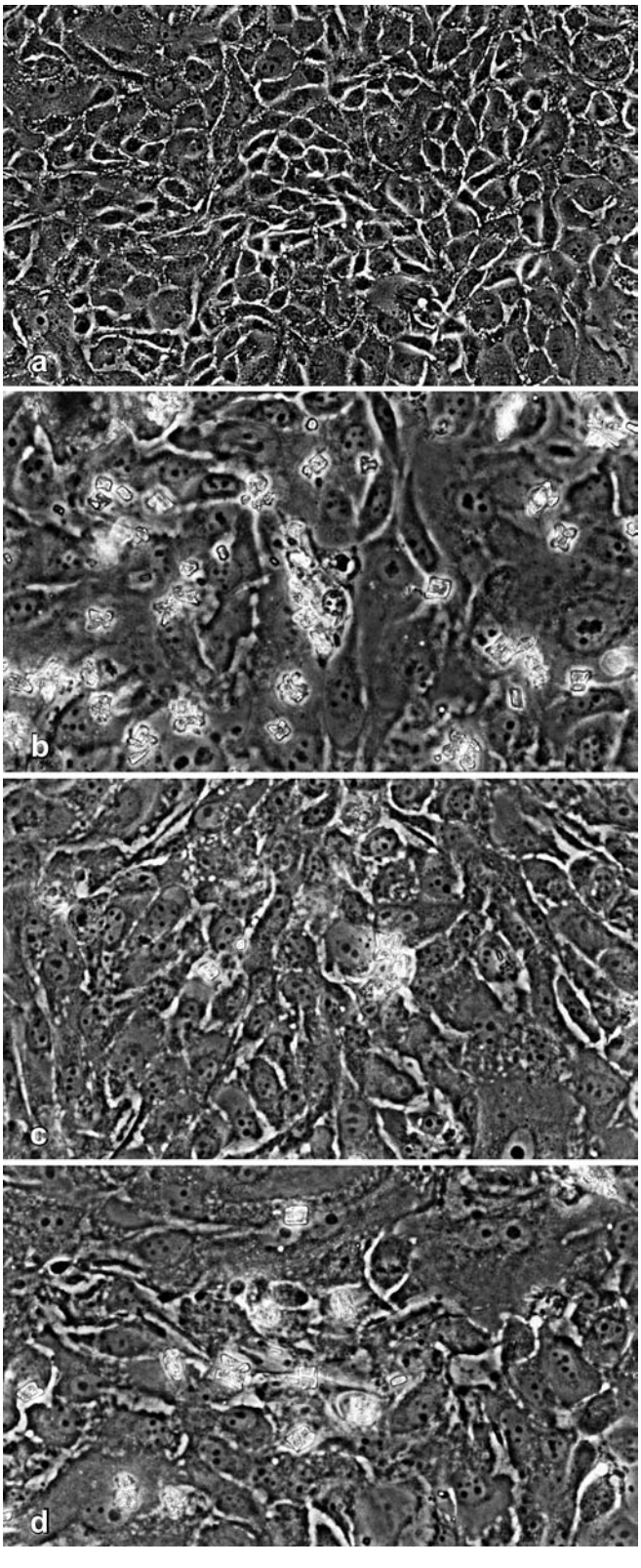


**Fig. 2** **a** Phase contrast image of MDCK cells (400×) before treatment with oxalate ion (control). **b** Phase contrast image of MDCK cells (400×) after treatment with 1.0 mM oxalate and 500 $\mu$g calcium oxalate monohydrate (COM) crystals at 37°C for 4 h. Primary aggregated and retained COM crystals on the membrane of MDCK cells are clearly visible. **c** Phase contrast image of MDCK cells (400×) after treatment with 1.0 mM oxalate, 500 $\mu$g calcium oxalate monohydrate (COM) crystals and 500 nM Tamm-Horsfall protein at 37°C for 4 h. A few COM crystals retained on the MDCK cells can be seen. **d** Phase contrast image of MDCK cells (400×) after treatment with 1.0 mM oxalate, 500 $\mu$g calcium oxalate monohydrate (COM) crystals and 500 nM deglycosylated Tamm-Horsfall protein at 37°C for 4 h. Retained COM crystals on the MDCK cells can be seen

CaOx crystals and also aid in their aggregation and eventual retention within the nephron. Crystal retention is also accomplished by attachment to the exposed basement membrane. Exposure of retained CaOx crystals to concentrated urine can lead to the development of kidney stones. Therefore, THP may be as an inhibitor of stone formation.

In our studies, the theory of stone formation has focused on the renal epithelium and the role of injury in crystal retention. The results have shown that oxalate exposure per se can damage renal epithelial cells and enhance crystal binding. In MDCK cells, oxalate exposure produces marked effects on membranes and causes elevation of LDH and MDA in the culture medium, all of which are parameters of cellular injury. Apoptosis was also observed in this study during long-term exposure to oxalate. THP can decrease the production of cellular injury parameters, possibly as a result of its antioxidant properties. In summary, THP protects MDCK cells from exposure to Ox ions and COM crystals, suggesting that it plays a crucial role in stone disease in vivo.

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