Urol Res (2003) 31: 3–9
DOI 10.1007/s00240-002-0286-x

ORIGINAL PAPER

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Oxalate and calcium oxalate mediated free radical toxicity in renal epithelial cells: effect of antioxidants

Received: 7 June 2002 / Accepted: 10 October 2002 / Published online: 22 November 2002

Abstract In a previous study we demonstrated that oxalate induced free radical injury can promote calcium oxalate stone formation. In the present study, we tested whether the antioxidants vitamin E, superoxide dismutase (SOD), catalase and desferoxamine (DFO) can provide protection against oxalate toxicity in LLC-PK_1 cells. LLC-PK_1 cells were exposed to oxalate (1.0 mM) or oxalate + calcium oxalate monohydrate crystals (COM, 500 μgm) for 3, 6, and 9 h. Cellular injury was assessed by lactate dehydrogenase (LDH) release. Malondialdehyde (MDA) content, catalase and glutathione peroxidase activities were also measured. The effect of vitamin E (200 μM), DFO (1.0 mM), SOD (400 U), and catalase (400 U) on oxalate-exposed cells was tested. LLC-PK_1 cells exposed to oxalate showed a significant increase in LDH release and MDA content, which was further elevated when COM crystals were added. Cellular glutathione peroxidase and catalase activities were decreased on exposure to oxalate. The addition of vitamin E, SOD, catalase and DFO significantly reduced the release of LDH and restored glutathione peroxidase and catalase activities towards the control level. The increased formation of MDA on oxalate or oxalate + COM toxicity was restored towards normalization by antioxidants and antioxidant enzymes. The protection rendered by vitamin E was greater than that of SOD, catalase and DFO. We conclude that oxalate associated free radical injury may promote stone formation by providing cellular debris for crystal nucleation and aggregation and augment crystal attachment to other tubular cells. Antioxidant administration may prevent calcium oxalate nucleation and retention in the renal tubules by preventing oxalate mediated peroxidative injury.

Keywords Oxalate · Calcium oxalate · Lipid peroxidation · Vitamin E · Antioxidants · Oxygen free radicals

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Introduction

A major risk factor for calcium oxalate urolithiasis is hyperoxaluria [5], which is augmented and promoted when combined with cellular degradation products derived from renal tubular injury [8, 20, 23, 36]. Oxalate plays no vital function and most urinary oxalate appears to be produced by endogenous biosynthesis from oxalate precursors [15]. Increasing evidence supports the concept that cellular injury is a predisposing factor for calcium oxalate urolithiasis [19]. The products of cell damage can act as heterogenous nucleators of calcium oxalate crystallization both in vivo and in vitro [20]. Reports have shown that hyperoxaluria, even without crystalluria, causes increased excretion of enzymes of renal tubular origin [23], and that the cellular injury potentiates calcium oxalate crystal formation in hyperoxaluric rats [9].

Lipid peroxidation represents oxidative tissue damage by superoxide, hydroxyl radicals and hydrogen peroxide, which results in structural alteration to membranes and the functional impairment of the cellular component. The peroxidation of membrane phospholipid polyunsaturated fatty acid is a pathogenic factor in a variety of diseases [12]. The oxalate induced peroxidative injury has been implicated in the pathogenesis of calcium oxalate stone formation [46, 48]. Our earlier studies, as well as those of others, have also shown that renal injury is associated with the production of free radicals during oxalate exposure [40, 41, 48]. Further, oxalate treatment induces immediate early genes (e.g. *c-myc*, *egr-1*) to initiate DNA synthesis and cellular proliferation

[17, 27] and promotes the redistribution of phosphatidyl serine to the membrane surface [4].

The cell is endowed with several antioxidant systems including ascorbic acid, vitamin E and thiols. Antioxidant enzymes include catalase, glutathione peroxidase, and superoxide dismutase (SOD). Glutathione peroxidase detoxifies a wide variety of reactive oxygen metabolites, and thus protects against oxidative damage. It catalyses the reduction of hydrogen peroxide by reduced glutathione. SOD causes the dismutation of the superoxide anion to hydrogen peroxide. Hydrogen peroxide can be eliminated by glutathione peroxidase and catalase. We previously reported that oxalate toxicity results in altered biochemical reactions, including the alteration of the antioxidant defensive system [47]. Therefore, the aim of the present study was to examine the effect of free radical scavengers including vitamin E, SOD, catalase and iron chelators on oxalate induced free radical injury to renal epithelial cells in culture.

Materials and methods

Cell culture

$LLC\text{-}PK_1$ cells, (CRL 1392; ATCC, Rockville, Md.), of proximal tubular origin were used. Serial cultures were maintained as subconfluent monolayers on 75 cm^2 Falcon T flasks in 50:50 Dulbecco's modified essential medium: nutrient mixture F-12 (DMEM/F-12) containing 15 mmol HEPES, 10% fetal calf serum (serum iron approximately 100 mg/dl), $Fe(NO_3)_3$ (0.1 mg/l), streptomycin (0.20 mg/ml) and penicillin (100 IU/ml), pH 7.4 at 37°C in a 5% carbon dioxide air atmosphere. Medium was replenished two to three times weekly. For experimental studies, cells were grown to confluence in DMEM in four-well plates (Fisher, Atlanta, Ga.). Confluent monolayers of $LLC\text{-}PK_1$ cells were used on days 3–5 after seeding. The experiments were carried out in serum free, pyruvate free DMEM. We omitted sodium pyruvate during the experiments since studies have shown that it significantly reduced the hydrogen peroxide produced by the hypoxanthine/xanthine oxidase and the glucose/glucose oxidase systems in $LLC\text{-}PK_1$ cells [39].

Oxalate

A review of the literature on investigations into cell/calcium oxalate crystal (CaOx) interactions revealed that the calcium oxalate monohydrate (COM) crystal load utilized varies from 10 μg/ml to 2 mg/ml [11] while the concentration of oxalate employed ranged from 0.1 to 4.0 mmol [40]. We decided to use 1.0 mmol oxalate and 500 μg/ml COM crystals. The 1.0 mmol oxalate in the growth medium increases the free oxalate level to 0.4 mmol and relative supersaturation (determined by the computer program EQUIL II) to 13.5, and is the limit of CaOx metastability, i.e. neither the existing COM crystals dissolve nor do new crystals form during the experiment [11, 51]. Oxalate as $K_2C_2O_4$ (KOx) was added using a stock solution of 10 mmol KOx in normal sterile saline. Potassium oxalate was diluted to a final concentration of 1.0 mmol in defined media. The cell cultures were exposed to DMEM medium containing oxalate for 3, 6, and 9 h. Control cultures were not exposed to either agent. At the end of the experimental periods, the culture media and cells were used for the following analysis.

Crystal preparation

COM crystals of 1 μm were prepared by mixing equal, unbuffered volumes of 1.0 mmol $CaCl_2$ and 10 mmol KOx at room temperature. The solution was allowed to mix overnight at room temperature. The suspension was applied to a 0.2 mm filter, washed in a COM saturated solution; freeze dried and stored desiccated at –20°C. Scanning electron microscopic (SEM) examination and particle size analysis by Particle Data Incorporated Model 80XY/PC280 verified an average crystal size of 1 μm. Crystals analyzed before and after UV sterilization and equilibration in defined medium were determined to be pure COM by high resolution X-ray diffraction using a Philips APD 3720. UV-sterilized crystals were equilibrated in medium, without phenol red, containing 1 mmol 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 mmol KOx media, to individual wells at a final concentration of 500 μg/ml. Monolayers were incubated for 3, 6, and 9 h.

Lactate dehydrogenase

Cellular injury was assessed by LDH release. Other methods of determining cell injury such as trypan blue exclusion or ^{3}H-adenine release under similar conditions were used in our previous studies and demonstrated no advantage compared to LDH release [10, 11]. The media from the control and experimental wells were recovered and centrifuged to remove crystals and cellular debris. Lactate dehydrogenase (LDH) activity from the media, at all time periods, was determined with a commercial kit (Proteins International, Rochester Hills, Mich., USA) by microtiter assay [26]. All determinations were made against appropriate reagent blanks. Oxalate did not interfere with the LDH assay [11]. Enzyme activity was expressed as IU/ml media.

Malondialdehyde

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) as described by Wong et al. [53] with the following modifications: at the completion of the experimental period, cells were rinsed three times with phosphate buffered saline. Next, 0.5 ml of 100 mM potassium chloride solution containing 3 mM EDTA was added to the plate and the cells were scraped off with a rubber baton. The cell suspension was removed with a Pasteur pipette and placed into a 2 ml test tube on ice. The cellular suspension was sonicated on ice (MSC sonifier at 100 W power to 20 KHz for 30 s) and after centrifugation at 500 *g* for 10 min at 4°C, the supernatants were used for the determination of MDA. An aliquot was removed for the measurement of protein content. The reaction mixture contained 0.2–0.4 ml of cell homogenate, 1.0 ml of 0.8% thiobarbituric acid containing butylated hydroxytoluene (BHT) (15 μl of 2% BHT in ethanol) and 1.0 ml of 1% phosphoric acid. The addition of BHT is to prevent the iron-catalyzed decomposition of lipid hydroperoxides and the formation of MDA during the boiling step of the assay [3]. The mixture was then heated to 95°C for 60 min in a boiling water bath. The tubes were cooled and centrifuged at 2,700 *g* for 10 min and assayed by high performance liquid chromatography using a Water's M-Bondapak C-18 column, eluting with a phosphate-methanol (65%–35%) mobile phase. The eluted MDA complex, which has a retention time of 8 min, was measured at 532 nm. 1,1,3,3-tetramethoxy propane was used as a standard. The results are expressed as nmol MDA/mg protein.

Experiments with free radical scavengers

To evaluate the protective effect of free radical scavengers, we determined the glutathione peroxidase and catalase activity on exposure to oxalate or oxalate + COM crystals in the presence or absence of 200 μM vitamin E in the incubation medium. Glutathione peroxidase activity was measured by the spectrophotometric method of Paglia and Valentine [35]. Catalase was measured using the method of Sinha [43]. To evaluate an association between free radical generation and lipid peroxidation and injury, the cells with or without vitamin E (200 μM), SOD (400 U), catalase (400 U), and DFO (1 mM) were incubated with oxalate or oxalate + COM crystals, and changes in

MDA content and LDH release were investigated. Heat inactivated SOD with catalase was used as a control. Protein was measured by the method of Lowry et al. [28] using a microplate reader.

Statistics

Results are given as the mean ± SD. Our observations come from a minimum of six separate experiments performed in duplicate. Data were analyzed by three way analysis of variance (ANOVA) with Tukey's multiple comparisons.

Results

Free radical scavengers significantly decrease MDA production

As shown in Fig. 1, the incubation of LLC-PK$_1$ cells with 1.0 mmol oxalate time dependently increased MDA level. The maximum increase was observed at 9 h (3.3 ± 0.25; $P < 0.05$; $n = 6$) when compared with 3 h (1.75 ± 0.19) and 6 h (2.46 ± 0.22). Pre-treatment of cells with the free radical scavengers SOD (400 U), catalase (400 U), DFO (1 mM) and vitamin E (200 μM) time dependently decreased the oxalate induced MDA level ($P < 0.05$; $n = 6$). When compared to SOD, catalase and DFO, vitamin E provided maximum protection against oxalate toxicity.

Oxalate time dependently increases LDH release

Figure 2 shows the effect of antioxidants on oxalate induced LDH release in LLC-PK$_1$ cells. Oxalate (1.0 mM) significantly increased LDH release at all time points when compared to the control. At 3 h (9.8-fold ± 0.95), 6 h (13-fold ± 1.2), and 9 h (15-fold ± 1.5). The antioxidants SOD, catalase, DFO and vitamin E significantly prevented oxalate induced LDH release. When compared with all of the other antioxidants studied, vitamin E (60.0% inhibition) provided significantly more protection against oxalate toxicity than SOD (17%), catalase (31.4%) and DFO (30.1%).

Vitamin E significantly reduces oxalate + COM induced LDH release and MDA production

Oxalate (1.0 mM) induced LDH release and MDA production was further enhanced when COM (500 μg/ml) crystals were added to LLC-PK$_1$ cells (Figs. 3, 4). Oxalate + COM crystals significantly and time dependently increased LDH release and MDA production in LLC-PK$_1$ cells. Vitamin E significantly reduced oxalate + COM crystals inducing LDH release (57.4% inhibition) and MDA production (56.5% inhibition) when compared with oxalate + COM treated cells. The protection rendered by vitamin E was greater than SOD and catalase.

Vitamin E significantly restores glutathione peroxidase and catalase activity

The effect of vitamin E on glutathione peroxidase and catalase activity in oxalate and oxalate + COM treated

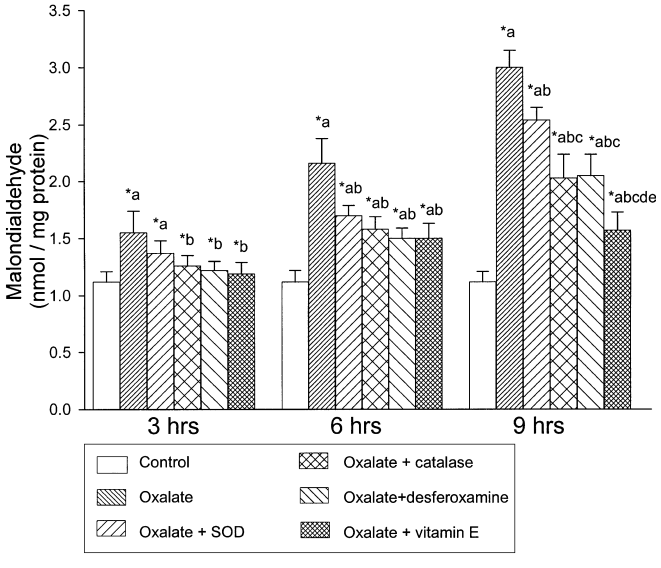

Fig. 1 Malondialdehyde production was significantly increased in LLC-PK$_1$ cells exposed to oxalate at 1.0 mM. Compared with cells treated with oxalate, the free radical scavengers SOD (400 U), catalase (400 U), DFO (1 mM) and vitamin-E (200 μM) provided a marked protection against oxalate toxicity ($n = 6$; $P < 0.05$). Comparisons showed: *a* significant vs control; *b* significant vs oxalate; *c* significant vs oxalate + SOD; *d* significant vs oxalate + catalase; *e* significant vs oxalate + vitamin E

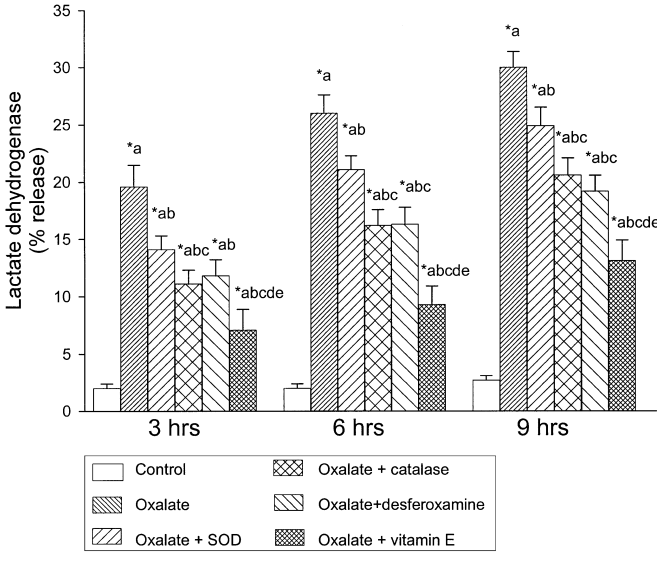

Fig. 2 Exposure of LLC-PK$_1$ cells to oxalate (1.0 mM) resulted in a significant increase in LDH release at 3 h with increase up to 9 h when compared to the control. SOD (400 U), catalase (400 U), DFO (1 mM) and vitamin E (200 μM) significantly decreased LDH release by preventing oxalate cytotoxicity ($n = 6$; $P < 0.05$). Comparisons showed: *a* significant vs control; *b* significant vs oxalate; *c* significant vs oxalate + SOD; *d* significant vs oxalate + catalase; *e* significant vs oxalate + vitamin E

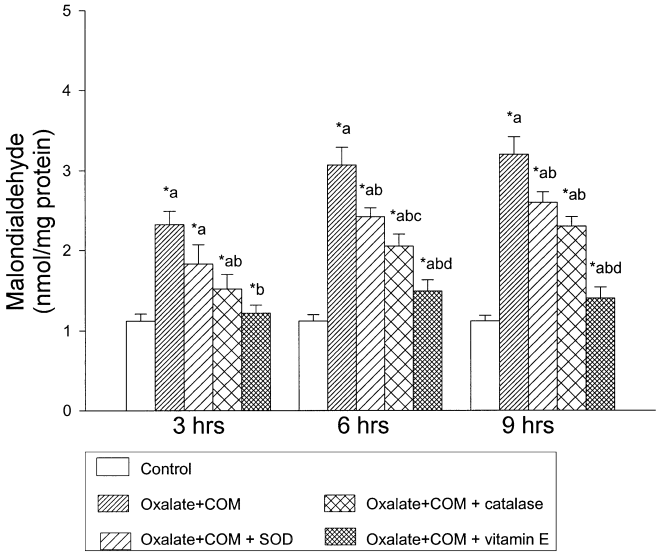

Fig. 3 Oxalate + COM crystals significantly increased MDA production after 3 h when compared to the control. Formation of MDA was significantly elevated even 9 h after oxalate + COM exposure. Cells treated with antioxidant enzymes, SOD and catalase and vitamin E had a significantly decreased MDA production. Vitamin E provided maximum protection ($n = 6$; $P < 0.05$). Comparisons showed: *a* significant vs control; *b* significant vs oxalate; *c* significant vs oxalate + SOD; *d* significant vs oxalate + catalase; *e* significant vs oxalate + vitamin E

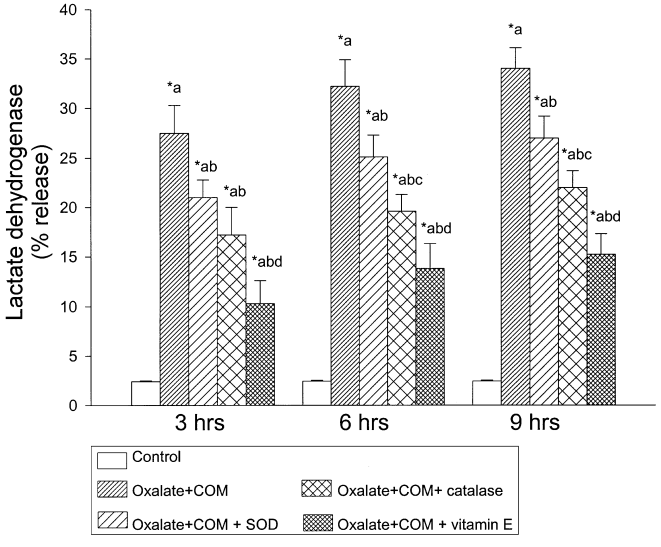

Fig. 4 Oxalate along with COM crystal exposure produced a time dependent augmentation in cellular injury as LDH leakage. Free radical scavengers SOD, catalase and vitamin E significantly lowered the LDH release when compared to LLC-PK_1 cells exposed to oxalate + COM ($n = 6$; $P < 0.05$). Comparisons showed: *a* significant vs control; *b* significant vs oxalate; *c* significant vs oxalate + SOD; *d* significant vs oxalate + catalase; *e* significant vs oxalate + vitamin E

LLC-PK_1 cells is shown in Figs. 5 and 6. Oxalate and oxalate + COM significantly decreased glutathione peroxidase and catalase activity in a time dependent manner when compared to the control. Oxalate + COM significantly decreased glutathione peroxidase and catalase activity when compared to oxalate. Vitamin E pretreatment significantly restored both glutathione peroxidase and catalase activity, when compared with oxalate and oxalate + COM. Vitamin E provided protection against oxalate and oxalate + COM induced toxicity and restored glutathione peroxidase and catalase activity towards normal ($P < 0.05$; $n = 6$) level.

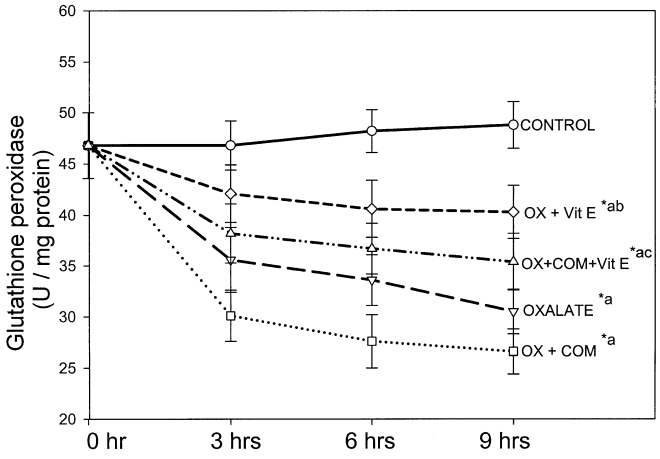

Fig. 5 A significant decrease in the activity of glutathione peroxidase was observed in oxalate (1.0 mM) and oxalate + COM (500 μg/ml) treated LLC-PK_1 cells. The treatment of cells with oxalate or oxalate + COM along with vitamin E (200 μM) resulted in a significant restoration of activity towards the control level ($n = 6$; $P < 0.05$). Comparisons showed: *a* significant vs control; *b* significant vs oxalate; *c* significant vs oxalate + COM

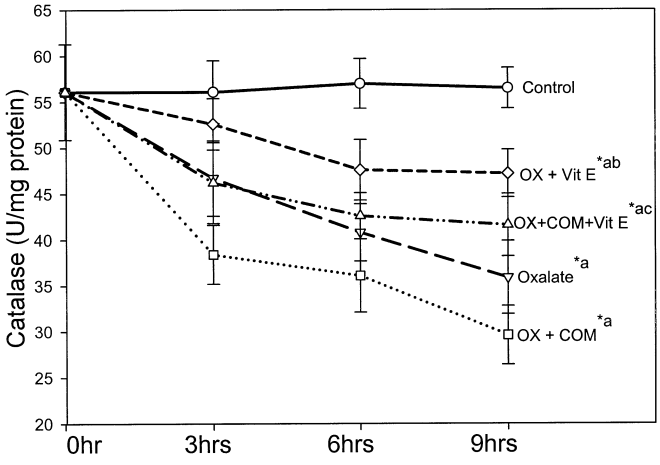

Fig. 6 LLC-PK_1 cells in the presence and absence of vitamin E were subjected to 1.0 mM oxalate or oxalate + COM crystals. Catalase activity was determined at the end of 3, 6, and 9 h. Catalase activity showed a progressive decline during the course of oxalate or oxalate + COM exposure. Pre-treatment with vitamin E (200 μM) showed a marked reversal in catalase activity ($n = 6$; $P < 0.05$). Comparisons showed: *a*- significant vs control; *b*- significant vs oxalate; *c*- significant vs oxalate + COM

Discussion

The interaction between renal epithelial cells and oxalate or oxalate + calcium oxalate crystals plays a significant

role in the formation, retention and development of calcium oxalate stone disease [20]. Recent evidence from our studies [46, 47, 48], as well as those of others [42], suggests that lipid peroxidation may play an important role in stone disease. Tissue culture studies showed that LLC-PK_1 and MDCK cells were injured when exposed to metastable levels of oxalate with or without added calcium oxalate crystals [10, 11]. Scheid et al. reported increased free radical production in renal culture cells exposed to oxalate, ranging from 0.1 mM to 4.0 mM [40]. Our earlier studies demonstrated that hyperoxaluria led to increased peroxidative damage to renal epithelial cells and the release of their contents into the urine. In addition, it also aids in the aggregation and growth of calcium oxalate crystals within the nephron [46]. This is supported by several lines of evidence including enzymuria and mebranuria [23, 41, 46], injury generated by gentamycin treatment [8] as well as the redistribution of phosphatidyl serine on oxalate or calcium oxalate exposure [1, 4]. Calcium oxalate kidney stone formation can provoke an inflammatory process [16, 21, 22, 25] and the development of stone involves cellular injury in the presence of hyperoxaluria [24]. Exposure to oxalate and calcium oxalate crystals injures the renal epithelial cells [2, 45, 47]; eventually cells detach and their degradation products can promote nucleation, aggregation and growth of calcific crystals [20].

In the present study, oxalate exposure resulted in a significant increase in LDH release (an indicator of cellular injury) and cellular MDA content (an indicator of lipid peroxidation). LDH release and MDA content were increased further when exposed to oxalate along with COM crystals. We performed experiments using oxalate along with calcium oxalate monohydrate crystals, since calcium oxalate crystals in the intact organisms can not exist in the absence of oxalate. Oxalate exposure to LLC-PK_1 cells led to increased lipid peroxidation, suggesting that membranes, including plasma membrane, were an important target for injury [38], further suggested by the cellular release of LDH. Lipid peroxidation is a direct indicator of membrane damage. The degradation of membrane lipids through lipid peroxidation or in association with phospholipase, can contribute to cell injuries. These include alteration in membrane permeability as well as direct toxicity from the accumulation of free fatty acids and lysophospholipids in the membrane [29, 49, 50]. It is also important to remember that although lipid peroxidation is strong evidence that free radicals have been present, tissue injury can also result from free radical reaction with proteins, carbohydrates and DNA. Important consequences of oxidant injury in the kidneys include structural alterations (cytoplasmic blebbing, nuclear chromatic clumping), alteration in the amino acid transport in the renal brush border membrane, etc. [6]. In a previous study using a rat model, hyperoxaluria and calcium oxalate nephrolithaisis was associated with an increase in renal MDA content and the urinary excretion of MDA and LDH [46].

Catalase, SOD and extra cellular SOD are regarded as efficient scavengers for peroxide, hydroxyl radicals, superoxide anions and oxy radicals [13]. The present study demonstrated that the treatment of renal epithelial cells with catalase or SOD markedly decreased MDA content and LDH release, providing protection against oxalate toxicity. A protective effect of SOD and catalase on oxalate induced superoxide and hydroxyl radical formation and cell death has also been reported [40, 48]. The iron chelator, desferoxamine (DFO) prevented an increase in the LDH release and MDA content in renal epithelial cells. Taken together, these data suggest that catalytic iron plays an important role in the pathogenesis of oxalate induced kidney stone. DFO has been widely used to prevent iron-overload in human disease [7], and has been shown to prevent adriamycin toxicity in which oxidant mechanisms have been implicated [37]. However, by chelating iron, DFO also prevents the generation of the OH radical during oxidant toxicity [14]. In this study, we found that vitamin E is an effective scavenger of reactive oxygen species. Vitamin E at 200 μM was more effective than catalase or SOD in scavenging free radicals. It is liposoluble and its high capability to diffuse into membranes could explain its ability to reduce the MDA levels both inside the cells and in the culture medium. However, another explanation for the efficiency of vitamin E in reducing lipid peroxidation could be its properties of scavenging the peroxy radical ROO and singlet oxygen, suggesting that this scavenger was also effective by acting on a different step of lipid peroxidation [31].

Evidence of the reactive oxygen species influx/imbalance was observed with the significant decrease in the cellular antioxidant enzymes. Our results indicate that renal epithelial cells exposed to oxalate or oxalate + COM crystals significantly decreased cellular glutathione peroxidase and catalase activity. The observed changes in renal antioxidant enzymes suggest that the renal epithelial cells are influenced by oxalate induced free radical stress, and that the antioxidant defense system tries to dispose of the enhanced influx of reactive species in order to reduce oxalate induced free radical injury. Vitamin E significantly restored these antioxidant enzymes (glutathione peroxidase and catalase) towards the control level, suggesting that the antioxidant defense system of the renal epithelial cells reacts positively to combat oxalate toxicity giving the tissue more resistance against free radical attack. It is generally accepted that vitamin E in eukaryotic cells functions as an inhibitor of lipid peroxidation. Vitamin E donates a hydrogen atom to the chain propagating lipid peroxyl radicals; giving rise to phenoxyl radicals of the antioxidants [44, 52]. The possibility that vitamin E protects the membrane from lipid peroxidation by a concerted mechanism involving its recycling by non-enzymatic and enzymatic intracellular reductants has been discussed [30, 32, 33, 34]. The greater antioxidant potency of vitamin E was due to its higher recycling efficiency of short hydrocarbon chains [18].

In conclusion, oxalate exposure increases cellular susceptibility to lipid peroxidation and antioxidant imbalance. Vitamin E effectively restored the cellular antioxidants and prevented lipid peroxidation. This suggests that vitamin E treatment may be a promising approach to prevent calcium oxalate nucleation and retention in the renal tubules by preventing oxalate induced peroxidative injury.

Acknowledgement This work was supported by a grant from the National Institutes of Health RO1 DK56249.

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