Urol Res (2012) 40:17–25
DOI 10.1007/s00240-011-0388-4

ORIGINAL PAPER

# Hyperoxaluria-induced tubular ischemia: the effects of verapamil and vitamin E on apoptotic changes with an emphasis on renal papilla in rat model

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Received: 20 February 2011 / Accepted: 26 April 2011 / Published online: 24 May 2011


**Abstract** An experimental study in rats was performed to evaluate the presence and the degree of both tubular apoptotic changes and crystallization at cortical, medullar and papillary regions of the kidney during hyperoxaluric phase and assess the possible protective effects of vitamin E and verapamil on these pathologic changes (particularly in papillary part of the affected kidneys). A total of 32 rats have been included into the study program. Hyperoxaluria was induced by continuous administration of ethylene glycol (0.75%). In addition to hyperoxaluria induction, animals in Groups 2 and 3 did receive a calcium channel-blocking agent (verapamil) and vitamin E, respectively. Histologic alterations of the kidneys including crystal formation together with apoptotic changes were evaluated on days 1, 14 and 28, respectively. Both apoptotic changes and the presence and degree of crystallization were assessed separately in renal cortical region, medulla and particularly papillary parts of the removed kidneys. Although verapamil did well limit the degree of crystal formation and apoptosis and brought it to the same levels observed in control group animals in all parts of the kidneys during intermediate phase, addition of vitamin E was failed to show the same protective effect during both intermediate and late phase evaluations. As demonstrated in our study, the limitation of both crystal deposition and apoptotic changes might be instituted by calcium channel-blocking agents. Clinical application of such agents in the prophylaxis of stone disease might limit the formation of urinary calculi, especially in recurrent stone formers.

**Keywords** Hyperoxaluria · Tubular ischemia · Apoptosis · Protective agents · TUNEL · Verapamil

## Introduction

Clinical and experimental studies suggested that renal tubular epithelium is the major target for oxalate-induced injury where sustained hyperoxaluria and subsequent calcium oxalate (CaOx) crystal formation/deposition may be injurious to the renal tubular cells [1–4]. The interaction between these cells and CaOx crystals and/or oxalate ions is likely to play a critical role in the formation of urinary calculi [5–7].

Related with this issue, studies on LLC-PK1 cells have clearly shown that oxalate exposure can produce a variety of changes in renal epithelial cell morphology and function, including cell necrosis at elevated concentrations. The interaction between oxalate ions with renal epithelial cells was found to elicit a programed sequence of events that can lead to renal tubular cell damage and/or dysfunction which may express itself as cell apoptosis [8, 9]. The underlying mechanism of cell injury during hyperoxaluria

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involves induced lipid peroxidation in tubular cells, which usually leads to the functional impairment of cellular components by reactive oxygen species (ROS) formation due to oxidative stress. Hyperoxaluria-induced tubular ischemia has been suggested as the main responsible factor initiating the programed sequence of events leading to cell death [9–15].

Keeping the crucial role of tubular ischemia in crystal formation in renal parenchyma in mind, the ongoing controversy about the primary site of calcium oxalate stone (CaOx) formation gains more importance than ever. Anatomical studies evaluating the pathogenesis of Randall's subepithelial plaques have clearly demonstrated a close association between the renal vasculature and renal tubules [16, 17]. It has been again hypothesized that, depending on the highly characteristic blood supply of the kidneys where smallest branches "end arteries" in nature, the exceptional papillary environment with low oxygen and high carbon dioxide is of interest in this context, and its impact on CaOx toxicity to renal cells has to be evaluated. Being mostly supplied by end arteries, low $O_2$ and high $CO_2$ pressure in papillary region of the kidney influence the metabolism of interstitial and tubular cells, and cell death may occur more commonly in these specific portions of the kidneys which promote subsequent crystal deposition and possible stone formation [18–20]. Thus, ischemia-induced apoptotic changes occurring mostly in the papillary region of the involved kidneys will cause crystal deposition in a more common manner than the other parts of the kidneys.

Last but not least, taking the injurious effects of hyperoxaluria causing crystal deposition in renal parenchyma and apoptotic changes in renal tubular epithelium (as a result of ischemia-induced ROS production) into account, studies focused on the possible protective effects of some agents in an attempt to prevent or at least limit ischemia formation and related pathologic alterations [21–25]. Among these agents, while verapamil and nifedipine (calcium channel-blocking agents) were found to limit the histologic changes as well as crystal deposition induced by certain renal trauma [26–30], potassium citrate was found to limit stone recurrence after SWL [31–33], and allopurinol [27, 34–36] and vitamin E have been used as antioxidant agents to minimize the effects of lipid peroxidation in certain tissues [37–40].

In this present animal study, we aimed to evaluate the presence and degree of intratubular crystal formation as well as cell apoptosis in different sections but especially in the papillary region of the involved kidneys and possible protective effect of some definite agents (verapamil and vitamin E) on these changes induced by hyperoxaluria in rat model.

## Materials and methods

A total of 32 Sprague–Dawley rats (350–400 g of each) were included into the study program after obtaining the ethical committee approval from the animal laboratory of the medical faculty. All animals were kept in special cages under normal room conditions (with temperatures of 23 ± 1°C and humidity of 55% ± 5%) with 12 h of light and dark periods. Following a complete physical examination, all animals underwent biochemical evaluation including blood and urine analyses and stool examination for parasitic infections which may affect the renal parenchymal alterations induced by hyperoxaluria. No pathologic anatomical findings and/or infections of the urinary tract were found. Apart from the study protocol, no specific treatment has been applied.

Animals ($n = 32$) were then divided into four main groups: while three study groups contained nine animals (3 animals were evaluated separately during early, intermediate and late follow-up for pathologic alterations), control group was consisted of five animals. In *Group 1* (EG study group) hyperoxaluria was induced by 0.75% EG containing drinking water. In addition to hyperoxaluria induction, animals in *Group 2* were given verapamil (1 mg/kg, through feeding tube); in *Group 3* they were given vitamin E (150 mg/kg through feeding tube). Subgroup animals ($n = 3$) in all study groups were evaluated during early (24 h), intermediate (14 days) and late (28 days). Animals in *Control group* received no specific medication.

Animals were euthanized, and bilateral flank incision was performed to remove both kidneys for histopathologic evaluation. Histologic alterations including crystal formation (under light microscopy) together with apoptotic changes (by using the TUNEL method) were evaluated on days 1 (short term), 14 (intermediate term) and 28 (late term), respectively. During all evaluations, both apoptotic changes and the presence and degree of crystallization were assessed separately in renal cortical region, medulla and particularly papillary parts of the removed kidneys.

### Evaluation of apoptotic changes

Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling assay (TUNEL) has been used to evaluate the tubular apoptotic changes. For detection of apoptotic nuclei in frozen kidney sections from male Sprague–Dawley rats treated with ethylene glycol (EG) alone or ethylene glycol and vitamin E (EGE), or ethylene glycol and the drug verapamil (EGV), TUNEL was used to mark cells fluorescently with DNA fragmentation. An In Situ Cell Death Detection Kit (Roche Applied Science, USA) was used according to manufacturer's suggestions. Briefly, slides with kidney sections were air

dried at 37°C for 20 min, fixed with 4% paraformaldehyde, washed in PBS, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate and pre-treated with microwave irradiation in 0.1 M citrate buffer for 1 min. The TUNEL reaction was carried out for 1 h at 37°C with deoxynucleotidyltransferase (TdT) and FITC-labelled dUTP; negative control samples were incubated without the TdT enzyme. Samples were then counterstained with nuclear dye DAPI in order to visualize all the nuclei and mounted with anti-fade solution (Sigma). DAPI- and TUNEL-positive nuclei in kidney cortex, medulla and papilla were visualized by a Nikon Eclipse fluorescence microscope by capturing five images for each kidney region for a total of 15 images per experimental sample. For quantification, the Image Analysis program Scion Image (National Institute of Health) was used to analyze the total number of cells which was given by DAPI-stained nucleus and that of apoptotic cells which were positively stained with the TUNEL labeling. The percentage of apoptotic cell death was calculated by the number of TUNEL-stained nuclei per total number of DAPI-stained nuclei multiplied by 100.

### Evaluation of tubular crystallization

Evaluations of renal crystal deposition were performed under light microscopy. The tissues were fixed in 10% formalin for 24 h. After routine tissue processing, the tissues were embedded in paraffin. 4-μm-thick sections were stained with hematoxylin and eosin for histopathological evaluation. Crystal deposition and calcification were evaluated under light microscopy by calculating the percentage of crystal granules and/or calcification in tubules of cortical area, medullar area and renal papilla, respectively.

### Statistical analysis

All statistical analyses have been made with NCSS 2007 package program. During evaluation of the data obtained, in addition to the definitive statistical methods [mean values, standard deviation, median value, interquartile range (IQR)], Kruskal–Wallis test has been used to make comparison between the groups, and finally, Dunn's multiple comparison test has been used to compare the data between subgroups. $p < 0.05$ value was accepted as statistically significant.

## Results

### Tubular crystallization at papillary region

Although no significant crystallization could be demonstrated in all study groups during early phase evaluation (24 h) (Table 1), verapamil was found to be effective

**Table 1** Evaluation of the presence and the degree of crystallization during early, intermediate and late phase evaluation in different parts of the affected kidneys

| | Cortex | | Medulla | | Papilla | |
|---|---|---|---|---|---|---|
| EG group | | | | | | |
| Early | 0 ± 0 | 0 (0–0) | 1 ± 1.73 | 0 (0–3) | 2 ± 2 | 2 (0–4) |
| Intermediate | 48.33 ± 50.08 | 45 (0–100) | 38 ± 28.93 | 50 (5–59) | 54.67 ± 23.35 | 50 (34–80) |
| Late | 58.67 ± 52.21 | 76 (0–100) | 36.67 ± 40.42 | 30 (0–80) | 15.33 ± 21.46 | 5 (1–40) |
| *p* value | 0.230 | | 0.166 | | 0.079 | |
| EGV group | | | | | | |
| Early | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) | 0.33 ± 0.58 | 0 (0–1) |
| Intermediate | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) |
| Late | 60.33 ± 52.35 | 80 (1–100) | 29.33 ± 22.94 | 40 (3–45) | 22.33 ± 17.79 | 30 (2–35) |
| *p* value | **0.022** | | **0.022** | | **0.035** | |
| EGE group | | | | | | |
| Early | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) | 1 ± 1 | 1 (0–2) |
| Intermediate | 12.67 ± 4.51 | 13 (8–17) | 23 ± 7.21 | 25 (15–29) | 7.33 ± 1.53 | 7 (6–9) |
| Late | 56.67 ± 45.09 | 60 (10–100) | 53.33 ± 45.09 | 50 (10–100) | 18 ± 5.29 | 20 (12–22) |
| *p* value | **0.046** | | 0.055 | | **0.027** | |
| Control group | | | | | | |
| Early | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) |
| Intermediate | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) |
| Late | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) | 0 ± 0 | 0 (0–0) |

Bold values are statistically significant at $p < 0.05$

*EG* ethylene glycol, *EGV* ethylene glycol + verapamil, *EGE* ethylene glycol + vitamin E

enough to limit crystal formation at this particular level during intermediate phase (14 days) being similar to the findings obtained during early phase ($p > 0.05$). During intermediate phase, however, while statistically significant crystallization (up to 80%) was present in Group 1 (Fig. 1; Table 1), both verapamil (Fig. 2) and vitamin E did tend to limit the degree of crystal formation during this period. However, this difference was statistically significant in animals receiving verapamil (Group 2) ($p < 0.05$; Fig. 3).

Finally, while late phase (28 days) evaluation did show the persistence of crystal formation in Group 1 animals to some extent (40%, which was statistically different from control group animals) ($p < 0.05$; Table 1) unlike to the expected values, similar degree of crystallization has been observed both in Group 2 and 3 animals which was significantly higher than control group animals ($p < 0.05$; Fig. 3).

## Tubular crystallization at medullar region

During early phase evaluation, no statistically significant crystal formation could be seen at medullar level in all groups ($p = 0.261$; Fig. 3). In Group 1 animals evident crystallization has been noted during all phases (early, intermediate and late phase) which varied between 0 and 80% ($p = 0.166$; Table 1). In Group 3, however, there was no significant difference between crystal formation during all phases, and vitamin E application did not seem to be effective to limit crystal formation ($p = 0.055$; Table 1).

Although verapamil administration has been found to be significantly effective in limiting crystal formation during intermediate phase (14 days), this effect has not been observed during late phase assessment ($p = 0.022$; Table 1), where crystal formation persisted during this phase when compared with early phase. Thus, the protective effect was not significant during late phase assessment ($p > 0.05$; Fig. 3).

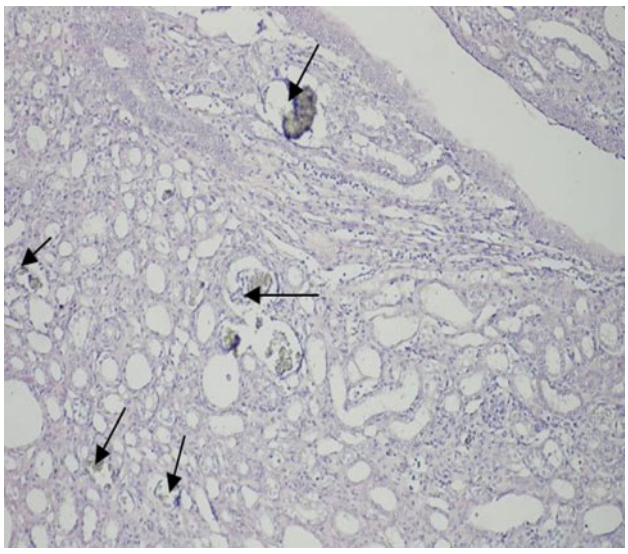

**Fig. 1** Pathology: marked crystal deposition (*arrows*) in the tubules of papillary areas after ethylene glycol alone during intermediate phase

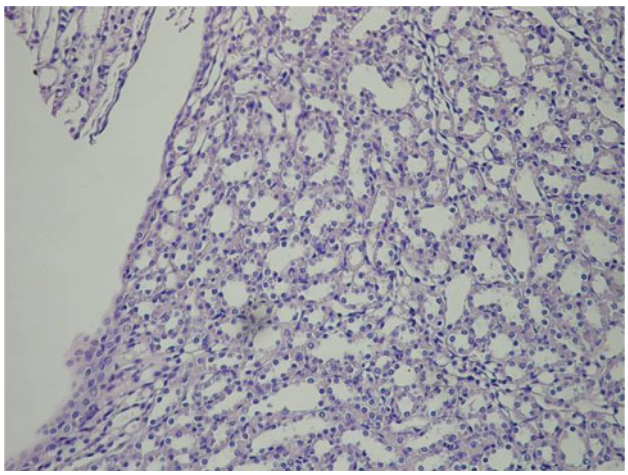

**Fig. 2** Pathology: crystal deposition is limited by verapamil, especially in the papillary tubules during intermediate phase

In summary, evaluation of crystallization at medullar level during late phase (28 days) did reveal significant changes (similar to EG only group) in Groups 2 and 3 indicating the lack of efficacy with two medications ($p < 0.05$; Fig. 3).

## Tubular crystallization at cortical region

During early phase evaluation, no statistically significant crystal formation could be seen at cortical level in all groups ($p > 0.05$; Fig. 3). In Group 1 animals, evident crystallization has been noted during all phases (early, intermediate and late phase) which varied between 0 and 100% (Table 1). In Group 3 again, there was no significant difference between crystal formation during both intermediate and late phases, and vitamin E application did not seem to be effective to limit crystal formation (Fig. 3).

Evaluation of tubular crystallization in animals receiving verapamil, however, did reveal a statistically significant difference between early, intermediate and late follow-up ($p = 0.022$; Table 1). While there was a significant crystallization during late phase with a significant difference when compared with both early and intermediate phases, there was no statistically significant difference between early and intermediate phases in terms of tubular crystallization ($p > 0.05$; Table 1).

Evaluation of tubular crystallization during late phase showed significant crystallization in all three study groups when compared with control one ($p < 0.05$; Fig. 3), indicating the lack of efficacy of both medications given.

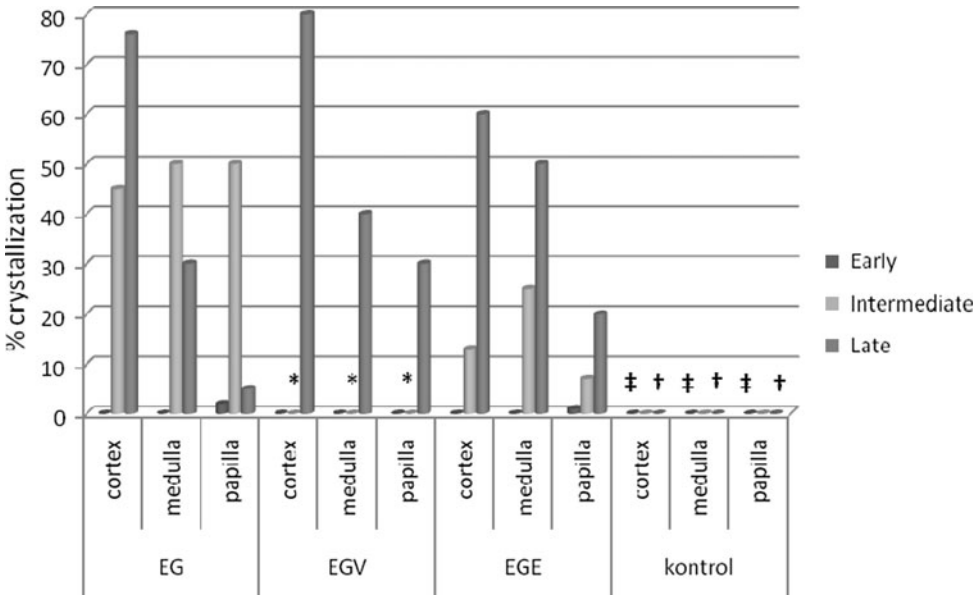


**Fig. 3** Early, intermediate and late findings of crystallization according to renal anatomy. *In animals receiving verapamil, the degree of crystallization was significantly limited in cortex medullar and papillar region, and the findings were similar to the early phase with limited or no crystallization. †During late phase follow-up, crystallization was found to be similar to EG only group in both group of animals receiving medication (verapamil and vitamin E), and crystallization was significantly higher than control group animals. ‡During early phase, however, similar to control group animals, there was no crystallization in all the three study groups

However, the degree of crystallization was significantly lower ($p < 0.05$) than the animals receiving EG only or additional vitamin E (similar to control group animals) in animals receiving verapamil during intermediate phase. These findings have clearly indicated the efficacy of verapamil in limiting crystallization during this phase (Fig. 3).

Evaluation of the apoptotic changes

In animals receiving hyperoxaluric diet (EG only), similar degree of tubular apoptosis has been observed in cortical, medullar as well as papillar region of the kidneys during early phase ($p = 0.670$) (Table 2). During intermediate and late phase, however, while tubular apoptosis was more evident in papillary region than that of medullar and cortical region ($p = 0.048$, $p = 0.045$; Table 2), there was no significant difference between the degree of tubular apoptotic changes in cortical and medullar regions during these phases.

*Verapamil* administration did not change the presence and the degree of apoptotic changes in cortical, medullar and papillar region during early phase evaluation, and there was no statistical difference between these regions ($p = 0.099$) (Table 2). Although the degree of apoptosis was found to be significantly lower in cortical region than that of medullar and papillar region ($p = 0.027$) (Table 2), when compared with the other groups the degree of apoptosis in this region did not show any significance at all ($p > 0.05$). During intermediate and late phases, similar to the control group findings, papillar apoptotic changes was found to be significantly lower in animals receiving verapamil than the ones receiving EG only and vitamin E (Fig. 4).

Last but not least, evaluation of the specimens obtained from animals receiving vitamin E did show no significant difference in apoptotic changes between cortical, medullar and papillar regions during early phase ($p = 0.587$; Table 2). During intermediate and late phase, however, the degree of papillar tubular apoptosis has been found to be higher than cortical and medullar apoptotic changes ($p = 0.027$, $p = 0.046$). Again, papillar tubular apoptosis has been found to be significantly higher than the animals receiving verapamil and the control group animals (Fig. 4).

Sections consisting of intact papillary parenchyma of rat treated with EG showed increased number of TUNEL-positive apoptotic nuclei to total DAPI-stained nuclei ratio with increased duration of the exposure to EG (Fig. 5a, b, top panels). Vitamin E co-treatment of rats with EG did not reduce the number of TUNEL-positive apoptotic nuclei in kidney papillary sections from intermediate or late phase animals (Fig. 5a, b, middle panels). However, the number of nuclei that were stained by the TUNEL was very low in the papillary cross-sections from rats which received verapamil co-treatment with EG (Fig. 5a, b, bottom panels). Sections from the control group rats stained in the TUNEL assay did not show apparent positive staining where the nuclear DAPI staining was used as reference (Fig. 5c).

## Discussion

Both clinical as well as animal studies have clearly shown that hyperoxaluria is the main risk factor for idiopathic calcium oxalate (CaOx) crystal formation [2–5] where the interaction between renal tubular epithelial cells and CaOx crystals and/or oxalate ions plays a critical role in the

**Table 2** Evaluation of apoptotic changes during early, intermediate and late phase in different sections of the kidneys

| | Cortex | | Medulla | | Papilla | | *p* value |
|---|---|---|---|---|---|---|---|
| EG group | | | | | | | |
| Early | 0.37 ± 0.26 | 0.27 (0.18–0.66) | 0.29 ± 0.25 | 0.2 (0.1–0.58) | 0.16 ± 0.14 | 0.19 (0–0.28) | 0.670 |
| Intermediate | 1.78 ± 1.54 | 1.97 (0.16–3.22) | 2.94 ± 2.05 | 3.4 (0.69–4.72) | 21.67 ± 2.61 | 20.21 (20.11–24.68) | **0.048** |
| Late | 1.91 ± 1.27 | 2.49 (0.45–2.79) | 1.48 ± 0.7 | 1.86 (0.67–1.9) | 40.74 ± 3.6 | 39.45 (37.97–44.81) | **0.045** |
| EGV group | | | | | | | |
| Early | 1.69 ± 1.12 | 1.6 (0.62–2.86) | 1.96 ± 0.24 | 2.08 (1.68–2.11) | 0.38 ± 0.46 | 0.26 (0–0.89) | 0.099 |
| Intermediate | 0.6 ± 0.31 | 0.52 (0.34–0.94) | 2.27 ± 0.94 | 2.7 (1.2–2.91) | 4.29 ± 1.01 | 4.78 (3.12–4.96) | **0.027** |
| Late | 0.89 ± 0.97 | 0.74 (0–1.92) | 4.44 ± 5.4 | 1.71 (0.96–10.66) | 5.11 ± 1.75 | 4.32 (3.89–7.11) | 0.148 |
| EGE group | | | | | | | |
| Early | 2.18 ± 3.09 | 0.82 (0–5.72) | 2.66 ± 0.94 | 2.4 (1.88–3.71) | 1.29 ± 1.31 | 0.83 (0.26–2.76) | 0.587 |
| Intermediate | 0.39 ± 0.23 | 0.3 (0.22–0.65) | 2.63 ± 0.77 | 2.22 (2.15–3.51) | 20.99 ± 4.28 | 20.35 (17.06–25.55) | **0.027** |
| Late | 4.89 ± 5.06 | 3.21 (0.88–10.58) | 4.3 ± 2.35 | 4.89 (1.71–6.31) | 33.36 ± 7.77 | 33.33 (25.6–41.14) | **0.046** |
| Control group | | | | | | | |
| Early | 3.49 ± 3.4 | 3.89 (0–6.79) | 3.39 ± 2.49 | 3.49 (0.95–5.78) | 1.82 ± 2.19 | 0.42 (0.12–4.22) | 0.675 |
| Intermediate | 3.49 ± 3.4 | 3.89 (0–6.79) | 3.39 ± 2.49 | 3.49 (0.95–5.78) | 1.82 ± 2.19 | 0.42 (0.12–4.22) | 0.675 |
| Late | 3.49 ± 3.4 | 3.89 (0–6.79) | 3.39 ± 2.49 | 3.49 (0.95–5.78) | 1.82 ± 2.19 | 0.42 (0.12–4.22) | 0.675 |

*EG* ethylene glycol, *EGV* ethylene glycol + verapamil, *EGE* ethylene glycol + vitamin E

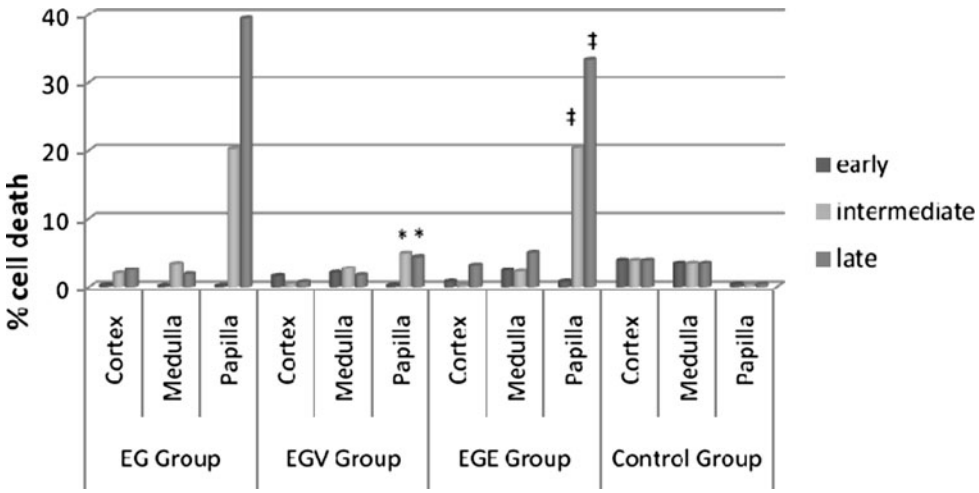


**Fig. 4** Evaluation of the tubular apoptosis rate in all groups in different sections of the kidneys. *When compared with animals receiving EG only and vitamin E, verapamil administration did decrease apoptotic changes significantly during both intermediate and late phases of evaluation ($p < 0.05$). ‡Significantly higher degree of apoptotic changes has been observed in animals receiving vitamin E than the control group and animals receiving verapamil during both intermediate and late phases ($p < 0.05$)

formation of urinary stones. Induction of hyperoxaluria has been found to be associated with cellular injury, and necrosis in these cells and the presence of the injury even in the absence of crystalluria have clearly suggested that the oxalate-induced damage was not due solely to injury produced by CaOx crystals themselves [3–7].

As a result of the possible tubular ischemia which gives rise to reactive oxygen species (ROS) production, depending on the data derived from animal studies has led the authors to propose that the interaction between oxalate ions and renal epithelial cells may initiate a programed sequence of events which can lead to cell death, in other words cell apoptosis [8, 9]. Related with this subject, studies on LLC-PK1 have clearly demonstrated that oxalate exposure may produce a variety of changes in renal epithelial cell morphology and function, including increased cellular proliferation and, at elevated concentrations, cell death [10–15]. Thus, as a distinctive form of cell death, apoptosis could also be responsible for the tubular injury induced by hyperoxaluria [8, 9]. To support this proposal, we were able to demonstrate apoptotic changes in rabbit renal tubular cells, the degree and extent of which were found to be time dependent [9].

Keeping the crucial role of tubular ischemia in crystal formation in renal parenchyma in mind, the ongoing controversy about the primary site of calcium oxalate stone (CaOx) formation gains more importance than ever. Anatomical studies evaluating the pathogenesis of Randall's subepithelial plaques have clearly indicated a close association between the renal vasculature and that of tubules

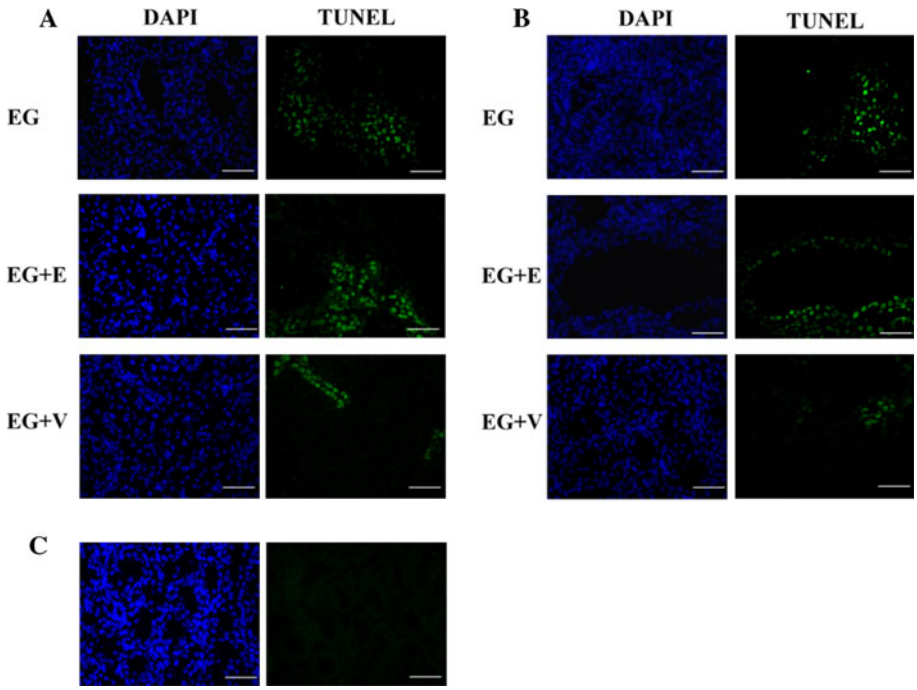


**Fig. 5** DAPI- and TUNEL-stained kidney sections after treatment with ethylene glycol (*EG*) alone, ethylene glycol plus vitamin E (*EG* + *E*), and ethylene glycol plus the drug verapamil (*EG* + *V*). **a** Rats exposed to EG and received vitamin E for 28 days had positive TUNEL staining while verapamil treatment led to a substantial decrease in TUNEL-positive nuclei. **b** Similar to group I, TUNEL-positive staining was observed in animals administered EG and EGE for 14 days unless they have received verapamil treatment (*EG* + *V*). **c** Control group showed no positive TUNEL staining in the nucleus. *Scale bar* 50 μm

[16, 17]. It has been again hypothesized that the exceptional papillary environment with low oxygen and high carbon dioxide is of interest in this context, and its impact on CaOx toxicity to tubular epithelial cells has to be evaluated [18–20]. The local low $O_2$ and high $CO_2$ pressure influence the metabolism of interstitial and tubular cells and may result in cell death promoting further stone formation. Thus, depending on the highly characteristic blood supply (as end arteries), the apoptotic changes occurring in the papillary tip of the kidneys may be of importance with respect to the crystal deposition than the other parts of the kidneys. Unfavorable low oxygen tension in this part of the kidney (due to the exceptional vascular supply of the kidney) increases the risk of toxic injury to the epithelial cells of long thin Henle's Loop located at papillary tip induced by oxalate and/or CaOx crystals being present at para-physiological concentrations [41]. Recently, Evan et al. analyzed the plaques obtained by operative biopsies of renal papillae. Based on histological examination of the tissues, they concluded that Randall's plaques begin in a unique location in the basement membrane of the thin loops of Henle and subsequently spread into the papillary interstitium. Close examination of their data also revealed that crystal deposits were always associated with the vasa recta and cells in the loops of Henle appeared normal, suggesting that an initial vascular etiology remains entirely feasible [20].

After observing the injurious effects of hyperoxaluria causing crystal deposition in renal parenchyma and apoptotic changes in renal tubular epithelium, physicians began to search for protective agents to prevent or at least minimize the extent of these pathologic alterations. Administration of antioxidants to hyperoxaluric rats has led to a decrease in renal injury; the production of lipid peroxides and CaOx crystal deposition in the kidneys indicated once again the involvement of reactive oxygen species in hyperoxaluria-induced renal injury [21, 23–25].

Among the agents used, while verapamil and nifedipine (calcium channel-blocking agents) were found to limit histologic changes and crystal deposition, possibly induced by blunt renal trauma [26–30], vitamin E has been applied to minimize free oxygen radical-induced alterations in certain tissues. As a very potent antioxidant, vitamin E appears to be an effective free radical scavenger, especially in preventing the deleterious effects of these radicals in parenchymatous organs. It has been shown that vitamin E deficiency causes damage in such organs, for example in testes or kidneys. However, studies dealing with its potential inhibitory effects on crystallization as well as subsequent stone formation could not demonstrate a well-documented positive benefit with some limited, contradictory data [37–40].

In this present animal study, following hyperoxaluria induction, we aimed to evaluate the apoptotic changes as well as crystal formation and at different regions (cortical, medullar and papillary), particularly at the papillary tip of the kidney, and to examine the possible preventive effects of verapamil and vitamin E on the presence and degree of these changes.

Our findings did reveal very limited or no crystallization in three different sections of the affected kidneys (cortical, medullar and papillary region); during early phase,

examination shows that 24 h is very early period for the initiation of crystal formation after hyperoxaluria. During intermediate phase, however, similar to control group animals, administration of verapamil was able to limit crystallization significantly in all sections evaluated when compared with the animals receiving EG only and that vitamin E. During late phase, however, none of the agents applied was able to limit crystal formation. As a result, while verapamil was effective in limiting crystal formation during intermediate phase, vitamin E application was failed to show the same effect during the whole study phases.

On the other hand, evident tubular apoptosis has been demonstrated during early phase in all sections of the affected kidneys. In other words, 24-h time period was enough for the initiation of apoptotic changes after hyperoxaluria induction. There was again no significant difference in all groups with respect to apoptotic changes in medullar and cortical regions during early, intermediate and late follow-up. But verapamil administration did significantly limit the apoptotic changes in papillar region during intermediate and late phases of evaluation. Vitamin E application, however, failed to show this effect during all phases in all sections of the affected kidneys. As a result, verapamil application was able to limit papillar apoptotic changes during intermediate and late phases of evaluation, similar to control group animals. Vitamin E was not able to show the same effect in all sections of the kidneys during all phases of evaluation.

In the light of our findings and published data in the literature, it is clear that both hyperoxaluria and CaOx crystal formation are injurious to renal epithelial cells. These findings supported the hypothesis that apoptotic changes do occur during the hyperoxaluric phase, and that these alterations may result from free radical formation causing lipid peroxidation. Both tubular cell apoptosis and crystal deposition were more prominent at papillary tip level (compared with cortical and medullar tubules) indicating the high likelihood of such specific changes due to the relatively ischemic (high $CO_2$ and low $O_2$ content) nature of this region.

Prominent apoptotic changes observed in renal papillary tubular epithelial cells damaged by hyperoxaluria-induced ischemia might result in cell degradation and could be responsible for the pathologic course of urolithiasis. Again, as demonstrated in our study, limitation of both evident crystal deposition and apoptotic changes, especially in papillary region, might well be limited by some blood flow regulating (calcium antagonists) and antioxidant agents (vitamin E). Clinical application of such agents in the prophylaxis of stone disease might limit the formation of urinary calculi, most importantly in recurrent stone formers.

## References


1. Coe FL, Evan AP, Worcester EM, Lingeman JE (2010) Three pathways for human kidney stone formation. Urol Res 38(3):147–160
2. Hackett RL, Shevock PN, Khan SR (1994) Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals. Urol Res 22:197
3. Scheid CR, Koul H, Kennington L, Hill WA, Luber-Narod J, Jonassen J, Honeyman T, Menon M (1995) Oxalate-induced damage to renal tubular cells. Scanning Microsc 9:1097
4. Thamilselvan S, Khan SR (1998) Oxalate and calcium oxalate crystals are injurious to renal epithelial cells: results of in vivo and in vitro studies. J Nephrol 11:66
5. Khan SR (1995) Calcium oxalate crystal interaction with renal tubular epithelium, mechanism of crystal adhesion and its impact on stone development. Urol Res 23:71
6. Khaskhali MH, Byer KJ, Khan SR (2009) The effect of calcium on calcium oxalate monohydrate crystal-induced renal epithelial injury. Urol Res 37(1):1–6
7. Lieske JC, Norris R, Swift H, Toback FG (1997) Adhesion, internalization and metabolism of calcium oxalate monohydrate crystals by renal epithelial cells. Kidney Int 52:1291
8. Miyazawa K, Suzuki K, Ueda Y, Katsuda S (2000) Demonstration of apoptosis and its related genes in rat tubular epithelium of calcium oxalate crystal formation. In: Urolithiasis 2000, 9th international symposium on urolithiasis, proceedings, p 253
9. Sarıca K, Yagcı F, Bakır K, Erturhan S, Ucak R (2001) Renal tubular injury induced by hyperoxaluria: evaluation of apoptotic changes. Urol Res 29:34
10. Hackett RL, Shevock PN, Khan SR (1995) Alterations in MDCK and LLC-PK1 cells exposed to oxalate and calcium oxalate monohydrate crystals. Scanning Micorosc 9:587
11. Koul H, Kennington L, Nair G, Honeyman T, Menon M, Scheid C (1994) Oxalate-induced initiation of DNA synthesis in LLC-PK1 cells, a line of renal epithelial cells. Biochem Biophys Res Commun 205:1632
12. Koul S, Fu S, Menon M, Koul H (2000) Oxalate exposure induces apoptosis in renal proximal tubular epithelial cells (LLCPK1 and HK-2 cells) in culture. In: Urolithiasis 2000, 9th international symposium on urolithiasis, proceedings, p 247
13. Scheid C, Koul H, Hill WA, Luber-Narod J, Kennington L, Honeyman T, Jonassen J, Menon M (1996) Oxalate toxicity in LLC-PK1 cells: role of free radicals. Kidney Int 49:413
14. Koul H, Kennington L, Honeyman T, Jonassen J, Menon M, Scheid C (1996) Activation of c-myc gene mediates the mitogenic effects of oxalate in LLC-PK1 cells, a line of renal epithelial cells. Kidney Int 50:1525
15. Davalos M, Konno S, Eshghi M, Choudhury M (2010) Oxidative renal cell injury induced by calcium oxalate crystal and renoprotection with antioxidants: a possible role of oxidative stress in nephrolithiasis. J Endourol 24(3):339–345
16. Randall A (1940) Papillary pathology as a precursor of primary renal calculus. J Urol 44:580
17. Vermooten V (1942) The origin and development in the renal papilla of Randall's calcium plaques. J Urol 48:27
18. Krautschick A, Esen T (1997) The role of the papilla in idiopathic calcium oxalate nephrolithiasis. World J Urol 15:213
19. Miller NL, Evan AP, Lingeman JE (2007) Pathogenesis of renal calculi. Urol Clin North Am 34:295–313
20. Evan AP, Lingeman JE, Coe FL, Parks JH, Bledsoe SB, Shao Y, Sommer AJ, Paterson RF, Kuo RL, Grynpas M (2003) Randall's plaque of patients with nephrolithiasis begins in basement membranes of thin loops of Henle. J Clin Invest 111:607–616

21. Reilly P, Schiller HJ, Bulkley GB (1992) Pharmacologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites. Am J Surg 161:488
22. Park HK, Jeong BC, Sung MK, Park MY, Choi EY, Kim BS, Kim HH, Kim JI (2008) Reduction of oxidative stress in cultured renal tubular cells and preventive effects on renal stone formation by the bioflavonoid quercetin. J Urol 179(4):1620–1626
23. Vanachayangkul P, Chow N, Khan SR, Butterweck V (2010) Prevention of renal crystal deposition by an extract of *Ammi visnaga* L. and its constituents khellin and visnagin in hyperoxaluric rats. Urol Res (in press)
24. Khan SR, Shevock PN, Hackett RL (1993) Magnesium oxide administration and prevention of calcium oxalate nephrolithiasis. J Urol 149:412
25. Zimmermann DJ, Voss S, von Unruh GE, Hesse A (2005) Importance of magnesium in absorption and excretion of oxalate. Urol Int 74(3):262–267
26. Akbay C, Sayın C, Sarıca K, Soygur T, Sabuncuoglu B (1998) Effect of verapamil on rabbit renal tissue after shock wave lithotripsy: an ultrastructural approach. Electron Microsc 4:493
27. Benyi L, Weilherg Z, Puyun L (1995) Protective effects of nifedipine and allopurinol on high energy shock wave induced acute changes of renal function. J Urol 153:596
28. Sarıca K, Bakır K, Yagcı F, Erbagcı A, Topcu O, Uysal O (2000) Unilateral testicular torsion; protective effect of verapamil on contralateral testicular histology. Urol Int 62:159
29. Sarıca K, Bakır K, Yagcı F, Topcu O, Akbay C, Sayın N, Korkmaz C (1999) Limitation of possible enhanced crystal deposition by verapamil in renal parenchyma after shock wave application in rabbit model. J Endourol 13:343
30. Strohmaier WL, Abelius A, Billes J, Grossmann T, Wilbert DM, Bichler KH (1994) Verapamil limits shock wave-induced renal tubular damage in vivo. J Endourol 8:269
31. Byer K, Khan SR (2005) Citrate provides protection against oxalate and calcium oxalate crystal induced oxidative damage to renal epithelium. J Urol 173(2):640–646
32. Fabris A, Lupo A, Bernich P, Abaterusso C, Marchionna N, Nouvenne A, Gambaro G (2010) Long-term treatment with potassium citrate and renal stones in medullary sponge kidney. Clin J Am Soc Nephrol 5(9):1663–1668
33. Tracy CR, Pearle MS (2009) Update on the medical management of stone disease. Curr Opin Urol 19(2):200–204
34. Lahme S, Feil G, Strohmaier WL, Bichler KH, Stenzl A (2004) Renal tubular alteration by crystalluria in stone disease—an experimental study by means of MDCK cells. Urol Int 72(3): 244–251
35. Vaughan WG, Horton JW, Walker PB (1992) Allopurinol prevents intestinal permeability changes after ischemia-reperfusion injury. J Pediatr Surg 27:968
36. Micali S, Grande M, Sighinolfi MC, De Carne C, De Stefani S, Bianchi G (2006) Medical therapy of urolithiasis. J Endourol 20(11):841–847
37. Biri H, Ozturk HS, Buyukkocak S, Kacmaz M (1998) Antioxidant defense potential of rabbit renal tissues after ESWL: protective effect of antioxidant vitamins. Nephron 79:181
38. Selvam R, Adhirai M (1997) Vitamin E pretreatment prevents cyclosporin *A*-induced crystal deposition in hyperoxaluric rats. Nephron 75:77
39. Parekh MH, Lobel R, O'Connor L, Leggett RE, Levin RM (2001) Protective effect of vitamin E on the response of the rabbit bladder to partial outlet obstruction. J Urol 166:341
40. Huang HS, Chen J, Chen CF, Ma MC (2006) Vitamin E attenuates crystal formation in rat kidneys: roles of renal tubular cell death and crystallization inhibitors. Kidney Int 70(4):699–710 erratum (2007) 71(7):712
41. Cao Y, Sagi S, Häcker A, Steidler A, Alken P, Knoll T (2006) Impact of hypoxia and hypercapnia on calcium oxalate toxicity in renal epithelial and interstitial cells. Urol Res 34(4):271–276