Vitamin E therapy prevents hyperoxaluria–induced calcium oxalate crystal deposition in the kidney by improving renal tissue antioxidant status

SIVAGNANAM THAMILSELVAN and MANI MENON
Department of Urology, Vattikuti Urology Institute and Henry Ford Health Sciences Center, Detroit, Michigan, USA
Accepted for publication 14 March 2005

INTRODUCTION

Hyperoxaluria is one of the major risk factors for calcium oxalate kidney stone formation in humans [1]. Oxalate is normally excreted by the kidneys, and 60–80% of renal calculi are composed of calcium oxalate [1–3]. Oxalate present in many foods is poorly absorbed from the intestine, with only 5–15% of dietary oxalate appearing in the urine; the remaining 85% of the oxalate is produced endogenously [4]. A recent report showed an endogenous contribution closer to 50%, and the remainder being of dietary origin [5].

The prevalence of urinary tract stone disease is estimated to be 2–20 per 10 000 [3]. The recurrence rate with no treatment for calcium oxalate renal stones is ≈10% at 1 year, 33% at 5 years and 50% at 10 years [6]. Most patients with calcium oxalate renal calculi excrete large amounts of calcium and/or oxalate in their urine. Hypercalciuria can result from several causes, including increased gut absorption, reduced tubular reabsorption, and resorptive hypercalciuria characterized by increased bone demineralization [7]. Levels of urinary oxalate are increased in 15–50% of patients with idiopathic calcium oxalate urolithiasis [1,8]. The diagnosis and initial management of urolithiasis has developed considerably in recent years. Various therapies, including alkali citrate, thiazide, dietary modifications, reduction in animal protein, and foods rich in glycolate and glyoxylate, have been tried in an attempt to prevent stone recurrence [7,9]. Despite recent advances in endourological, uroteroscopic and ESWL, stone recurrence can be reduced by only half.

It is clear from our previous in vitro and in vivo studies that oxalate-induced peroxidative injury is involved in the nucleation, aggregation and development of calcium oxalate stone disease [10–13]. The cell is endowed with several antioxidant systems, including enzymatic (superoxide dismutase, SOD, catalase and glutathione peroxidase, GPX) and non-enzymatic, e.g. reduced glutathione (GSH), vitamins E, A and C, to limit the extent of lipid peroxidation. Up to a certain limit, the cells are able to control the damage with GPX, catalase, SOD or other antioxidative mechanisms. However, once a threshold of damage or rate of damage is exceeded the cellular defences are overwhelmed and a very small additional insult results in severe cellular injury. Thus, the oxidant-antioxidant balance is a critical determinant of cell sensitivity to free-radical injury. Several laboratories reported that oxalate causes renal tubular injury by increase generation of free radicals [14,15]. In the present study therefore we sought to determine whether vitamin E offers promise as a therapeutic agent for preventing kidney stone formation in an animal model of hyperoxaluria, and describe

OBJECTIVE

To determine whether vitamin E prevents hyperoxaluria–induced stone formation, using a new animal model of calcium oxalate stone disease, as our previous in vitro and in vivo studies showed that oxalate and hyperoxaluria induce free-radical generation, which results in peroxidative injury to renal tubular cells.

MATERIALS AND METHODS

Ethylene glycol (EG) was administered at 150 mg/day by gavage for 3 weeks to rats fed on diets with adequate (group 1), excess (group 2) or deficient (group 3) vitamin E. Several indicators of peroxidation, free radicals and enzymatic activity were then assessed.

RESULTS

EG treatment in group 1 lead to increased lipid peroxidation, protein thiol, excretion of urinary enzymes, oxalate and decreases in urinary calcium, antioxidant enzymes and altered glutathione redox balance. Although renal function was not altered, there was increased water intake, urine volume and lowered urinary pH in these rats. These changes were more intense, with extensive calcium-oxalate crystal deposition, in rats in group 3, and prevented in rats in group 2, except for urine renal oxalate levels, which remained high. Histopathological examination showed that there was no deposition of calcium oxalate crystals in rats in group 2.

CONCLUSION

This is the first study to demonstrate in vitro evidence that hyperoxaluria–induced peroxidative injury induces individual calcium oxalate crystal attachment in the renal tubules. In addition, excess vitamin E completely prevented calcium oxalate deposition, by preventing peroxidative injury and restoring renal tissue antioxidants and glutathione redox balance. Therefore, vitamin E therapy might provide protection against the deposition of calcium oxalate stones in the kidney of humans.

KEYWORDS
lipid peroxidation, urolithiasis, hyperoxaluria, vitamin E, antioxidants, ethylene glycol, Sprague-Dawley rats
a new model of calcium oxalate stone disease.

MATERIALS AND METHODS

Male Sprague-Dawley rats (40–45 g) were used; they were kept in a temperature-controlled room with 12-h light and 12-h dark cycles, housed individually in stainless-steel cages and given free access to diet and deionized water. The experimental protocol was reviewed and approved by the institutional animal care and use committee.

The rats received one of three diets: the first group had a diet adequate in vitamin E (Purified diet, Harlan Teklad, Madison, WI; 100 U vitamin E/kg) containing 50.0 g/kg corn oil with mineral mix #170915 and 0.2 g/kg α-tocopherol acetate (specific activity 500 U/g), with the following nutrients: vitamin-free casein, 200 mg/g; DL-methionine, 3.0 mg/g; dextrose monohydrate, 674.5 mg/g; cellulose fibre, 50 mg/g; calcium carbonate, 3.5 mg/g; choline dihydrogen citrate, 3.5 mg/g; dry vitamin A palmitate, 0.04 mg/g (specific activity 500 000 U/g); dry vitamin D3, 0.0044 mg/g (specific activity 500 000 U/g); vitamin B12, 0.05 mg/g; biotin, 0.0004 mg/g; calcium pantothenate, 0.066 mg/g; folic acid, 0.002 mg/g; inositol, 0.11 mg/g; menadione, 0.05 mg/g; niacin, 0.1 mg/g; pyridoxine HCl, 0.022 mg/g; riboflavin, 0.022 mg/g; and thiamine HCl, 0.022 mg/g. The α-tocopherol acetate content of the diet adequate in vitamin E, for 6 weeks, after which hyperoxaluria was induced in groups II, IV and VI excess vitamin E, and V and VI deficient in vitamin E, for 6 weeks, after which hyperoxaluria was induced in groups II, IV and VI by EG (by gavage) at 150 mg/rat per day for 7, 14 or 21 days. The gain in body weight was monitored every week. The rats were killed 24 h after the last dose at 7, 14 and 21 days. The kidney was removed and fixed in formaldehyde for histopathological evaluation.

For the main study, rats were divided into six experimental groups (eight per group). The rats were selected based on food intake (18.5 ± 3 g/day), water intake (24 ± 2 mL/day) and body weight (initial body weight 40 ± 5 g; body weight on the first day of EG administration 340 ± 10 g). Groups I and II received a diet adequate in vitamin E, III and IV excess vitamin E, and V and VI deficient in vitamin E, for 6 weeks, after which hyperoxaluria was induced in groups II, IV and VI by EG (by gavage) at 150 mg/rat per day for 3 weeks. Groups I, III, and V were considered as controls (no EG). Food and water intake was recorded every day, and body weights monitored weekly. Twenty-four hour urine samples were collected at 0, 7, 14 and 21 days in 50 mL centrifuge tubes kept on ice and attached to urine-collecting funnels. Water intake, urine volume, crystalluria and pH were recorded. For the enzyme determinations, urine samples were dialysed for 3 h at 4 °C against distilled water. The rats were killed 24 h after the last oral dose of EG; the animals were anaesthetized with pentobarbital (50 mg/kg body weight), and the kidneys were quickly excised and used for the following analyses. A portion of the kidney was homogenized and assayed for enzyme activities. For the histopathological analysis, kidney tissue was fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5–6 μm, and stained with haematoxylin and eosin for microscopic examination.

Markers of oxidative stress were malondialdehyde (MDA) content, representing lipid peroxidation, determined by the thiobarbituric acid reactive method [16]. Protein carbonyls were measured according to the method of Levine et al. [17]. Antioxidants comprised vitamin E, determined by the method of Arnaud et al. [18] using HPLC. SOD was measured as described by Misra and Fridovich [19] and catalase using the method of Sinha [20]. The variables in the glutathione redox system, were GSH content, analysed with a modification of the enzymatic recycling assay using 5-thio-2-nitrobenzoate to form a spectrophotometrically detectable product at 412 nm (ε = 1.36 × 10^4 mole^{-1} cm^{-1}) by the method of Tietze [21]. GPx activity was measured by the spectrophotometric method of Paglia and Valentine [22], and glutathione reductase activity in total cell homogenates with a spectrophotometric assay [23]. Glucose-6-phosphate dehydrogenase (G6PD) activity was determined according to the method of Deutsch [24].

Markers of glomerular and tubular damage were blood urea nitrogen (BUN), measured according to the method of Crockter [25]. Serum creatinine was measured by the method reported previously [26] using a spectrophotometer; 24-h urine samples were processed for the determination of the following enzymes as described in our earlier studies [27]. Lactate dehydrogenase was analysed spectrophotometrically using pyruvate as a substrate [28]. A tubular brush border marker enzyme, alanine aminopeptidase, was determined by the method of Jung and Scholz [29]. Alkaline phosphatase and γ-glutamyl transpeptidase (GGT) were assayed using a commercial kit (Trinity Biotech, MO).

Urinary oxalate and calcium were determined by an ion-exchange chromatography method with some modifications [27], with a Dionex gradient-ion chromatography system equipped with a 0.4 × 25 cm AS11 anion exchange analytical column with a AG11 guard column for oxalate or CS12A cation exchange analytical column with a CG12A guard column for calcium. Sample (25 μL) was injected using an auto-sampling injector and eluted with 40 mmol/L NaOH/ deionized water at 1.0 mL/min with a linear gradient from 10% to 75% NaOH, 90% to 25%
deionized water for oxalate or 40% of 30 mmol/l methane sulphonic acid and 60% deionized water (isocratic) for calcium, after helium degassing. The column elution was monitored using a conductivity cell and peak area measured with Peaknet chromatography automation software v6.20. Background conductivity was minimized by using an ASRS anion self-regenerating micromembrane suppressor for oxalate or CSRS cation self-regenerating micromembrane suppressor for calcium and with recycled eluent. Sample oxalate or calcium concentration was then calculated based on an oxalate or calcium standard curve.

All experiments were repeated eight separate times in duplicate, with the results given as the mean (so). Data were analysed by three-way ANOVA with Tukey’s multiple comparison, with P < 0.05 considered to indicate significant differences. Multivariate regression analysis was used to assess the relationship between excretion of oxalate and tissue antioxidants, urinary enzymes and lipid peroxidation, and between peroxidation, tissue antioxidants and urinary enzymes.

RESULTS

Most animal models of calcium oxalate stone disease have required the generation of fairly severe hyperoxaluria. Various approaches have been used for this, including exposure to EG, oxalate infusion or feeding, and pyridoxine depletion [30]. However, the utility of these treatments is compromised by one or more limitations [31]. When we used the previously available conventional model of urolithiasis (0.75% EG through drinking water) for our experiments with rats on purified diets with adequate, excess and deficient vitamin E, we had difficulty interpreting the experiments. This widely used model for human stone disease resulted in massive crystal deposition is some rats and no or fewer crystal deposits in others. Normal Sprague-Dawley rats drink 10–35 mL of water/day. When we monitored individual rats the water intake of one rat was 10.5 (3.0) mL/day; the water intake was constant for this rat and did not differ significantly from day by day. The intake of another rat was 35.2 (4.3) mL/day, and for a third 25.4 (2.9) mL/day. Therefore, each rat has a unique, constant drinking water requirement. Rats drinking 10 mL/day of 0.75% EG had no crystal deposition in the kidney by 30 days but those drinking 25 mL/day of 0.75% EG developed calcium oxalate depositions by 15 days, and those drinking 35 mL/day of 0.75% EG did so by 7 days. Moreover, EG consumption causes polyuria or polydipsia, and therefore increased consumption of EG, from 35 to 45–50 mL/day on day 5, resulted in increased oxalate synthesis and massive blockages of renal tubules with calcium oxalate crystals in all the groups. Therefore, we developed a new calcium oxalate stone model to induce controlled endogenous oxalate synthesis.

To estimate the amount of oxalate excretion after giving EG, male Sprague-Dawley rats were divided into four groups with different amounts of EG (0, 100, 150, 200 mg) given once to each rat by gavage. Oxalate was significantly increased in urine on the first day (0–24 h) after EG in the four groups (A–D, 0–200 mg EG), at 3.52 (0.42), 32.09 (5.23), 83.45 (9.85) and 125.46 (12.6) μmol/24 h. The following day’s collection (24–48 h after EG) showed that oxalate was completely excreted within 24 h for groups B and C, whereas the oxalate was still higher in group D, with respective values of 3.31 (0.26), 2.8 (0.32), 4.50 (0.52) and 6.36 (0.45) μmol/24 h. This shows that increased excretion of oxalate was directly proportional to the amount of EG administered.

The next set of animals, treated with 0, 100, 150 or 200 mg EG each day by gavage for 7, 14 or 21 days, showed that the EG-treated rats gained substantially less body weight than the control group (data not shown). In the histopathological evaluation, each kidney was scored for crystal deposition using the scoring system shown in Table 1. From these results we estimated that EG at 150 mg/day through gavage induces a controlled amount of oxalate synthesis to produce hyperoxaluria and may prove a useful in-vivo model to study stone disease. In addition, this model may offer an appropriate option for evaluating therapeutic approaches and advantages over the uncontrolled oxalate synthesis in 0.75% EG given to rats drinking water. Although there were calcium oxalate crystals in the papillary region, a comparison between Randall’s plaque and the present observations is hampered by several difficulties. First, there is the obvious difference in species (human vs rat). Second, the approach (deductive vs inductive); and last, there is a difference between the electrolytes involved (calcium oxalate vs various calcium phosphates and carbonates) [32,33]. Khan [34] noted many similarities between the effects in experimental nephrolithiasis (including the EG protocol) induced in rats, and human kidney stone formation. It was also reported that the rat model of calcium oxalate nephrolithiasis can be used to investigate the mechanisms involved in human kidney stone formation [34].

As peroxidation was considered an important mechanism involved in many pathological conditions, we sought to determine whether vitamin E supplementation prevents peroxidation in rats treated with EG. Kidney tissue lipid peroxidation was estimated as MDA level (Table 2), and protein carbonyls were assessed as an indicator of protein peroxidation products (Table 2). Rats in group II had significant greater MDA levels and protein carbonyls than in group I. Hyperoxaluria-induced generation of MDA and protein carbonyls was significantly prevented in group IV. There was no significant change in MDA or protein carbonyl content in rats in groups I, III and V. However, rats in group VI had a dramatic increase in MDA and protein carbonyl contents. Supplementation with vitamin E therefore has a protective role against hyperoxaluria-induced oxidative injury.

<table>
<thead>
<tr>
<th>EG, mg/day</th>
<th>Days of EG</th>
<th>7</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-200</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 1: Scoring system for estimating crystal abundance in the kidney section of rats (six per treatment) fed a vitamin E–adequate diet followed by EG

© 2005 BJU INTERNATIONAL 119
levels of tissue vitamin E concentration; this may indicate the consumption of vitamin E by hyperoxaluria-induced free radical generation. The concentration of vitamin E in the kidney was significantly higher in the rats in group III and IV; exposure to EG was also associated with the consumption of vitamin E, as indicated by the decrease in the tissue vitamin E concentration, indicating that vitamin E positively combats hyperoxaluria-induced free radical generation. However, the tissue levels of vitamin E remained higher in group IV than in group V. Vitamin E deficiency in rats in groups I and V was validated by measuring the tissue vitamin E content. After vitamin E deprivation, there was a marked (93%) decrease in the levels of vitamin E in the kidneys. The tissue vitamin E content was significantly less in rats in group VI.

To examine the effect of vitamin E on hyperoxaluria-induced changes in kidney antioxidant and glutathione redox status, the enzymatic and non-enzymatic antioxidant levels were also assessed (Table 2). Rats in group II at 21 days had significant less SOD, catalase, GPx, glutathione reductase and G6PD activities, and GSH levels than in group I. In group IV the excess vitamin E significantly restored these enzyme activities and GSH levels towards the control levels. As expected, hyperoxaluria induced in rats in group VI produced a significant lower antioxidant enzyme and GSH concentration than in group II. There were no significant changes in the antioxidant or glutathione redox levels in rats on the three control diets. These data strongly indicate that dietary vitamin E strengthened the tissue antioxidative defense system. Vitamin E was accordingly found to reduce the hyperoxaluria-induced accumulation of reactive oxygen species and to significantly improve the tissue antioxidant status.

The indices of renal function are also summarized in Table 2. In groups II and IV, EG caused no significant increase in BUN or serum creatinine levels, whereas in group VI rats had significantly lower renal function, as indicated by the increased BUN and serum creatinine levels. As changes in renal tubular enzymes in 24-h urine are a sensitive index of renal tubular damage we studied the effect of vitamin E on EG-induced changes in the excretion of renal tubular enzymes. Rats in group II had a significant change in the activity of renal enzymes at all sample times. The cytosolic enzyme lactose dehydrogenase (LDH) had significantly greater activity in the urine of all EG-treated rats at 7, 14 and 21 days than in the control groups (Fig. 1a). Urine from rats in group IV had a significantly lower LDH activity than in group II. There were no significant differences among the control rats in any group, but the urinary excretion of LDH was significantly higher in group VI than in group II at 7 and 14 days. Brush border marker enzymes, GGT (Fig. 1b), alkaline phosphatase (Fig. 1c), and alanine amino peptidase (Fig. 1d), had significant changes in their excretion pattern in groups II, IV and VI (EG). The activity of brush border marker enzymes was significantly greater in urine at 7, 14 and 21 days in rats in group II. These enzyme excretions were further increased in group VI for up to 14 days and drastically decreased at 21 days. Rats in group IV had a significant restoration of these enzyme activities towards control levels. There were no significant changes in these urinary enzyme activities in the control rats in any of the vitamin E regimens.

Rats in groups II, IV and VI (EG-treated) had significantly greater urinary oxalate levels (Fig. 2) and lower calcium excretion (Table 2) than controls at all sample times. Rats in

### Table 2 Lipid peroxidation and protein carbonyls, vitamin E level, antioxidant and blood and urinary variables in the kidney of rats in groups I to VI after 21 days

<table>
<thead>
<tr>
<th>Mean (sd) variable</th>
<th>Group</th>
<th>I (n=8)</th>
<th>II (n=8)</th>
<th>III (n=8)</th>
<th>IV (n=8)</th>
<th>V (n=8)</th>
<th>VI (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA, nmol/mg protein</td>
<td></td>
<td>1.23 (0.22)</td>
<td>3.26 (0.18)</td>
<td>1.11 (0.16)</td>
<td>1.36 (0.24)</td>
<td>1.63 (0.21)</td>
<td>5.77 (0.48)</td>
</tr>
<tr>
<td>Protein carbonyls, nmol/mg protein</td>
<td></td>
<td>3.34 (0.42)</td>
<td>5.94 (0.39)</td>
<td>2.88 (0.27)</td>
<td>3.32 (0.35)</td>
<td>3.72 (0.41)</td>
<td>7.78 (0.37)</td>
</tr>
<tr>
<td>α-tocopherol, µg/g</td>
<td></td>
<td>37.2 (3.2)</td>
<td>22.4 (2.5)</td>
<td>70.6 (4.6)</td>
<td>60.5 (4.2)</td>
<td>2.5 (0.3)</td>
<td>1.5 (0.2)</td>
</tr>
</tbody>
</table>

Antioxidant enzymes and glutathione-redox system components

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Group</th>
<th>I (n=8)</th>
<th>II (n=8)</th>
<th>III (n=8)</th>
<th>IV (n=8)</th>
<th>V (n=8)</th>
<th>VI (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD, U/mg protein</td>
<td></td>
<td>30.2 (2.8)</td>
<td>16.8 (1.1)</td>
<td>34.0 (1.9)</td>
<td>26.2 (2.2)</td>
<td>27.9 (3.4)</td>
<td>11.2 (1.3)</td>
</tr>
<tr>
<td>Catalase, µmol H$_2$O$_2$ consumed/min/mg protein</td>
<td></td>
<td>35.1 (2.4)</td>
<td>20.7 (1.8)</td>
<td>33.6 (2.7)</td>
<td>28.5 (2.5)</td>
<td>31.9 (1.7)</td>
<td>11.0 (1.3)</td>
</tr>
<tr>
<td>GPx, U/mg protein</td>
<td></td>
<td>160.0 (14.3)</td>
<td>82.6 (6.5)</td>
<td>166.2 (7.3)</td>
<td>131.5 (10.8)</td>
<td>155.2 (10.5)</td>
<td>48.7 (3.4)</td>
</tr>
<tr>
<td>Glutathione reductase, nmol NADPH oxidized/min/mg protein</td>
<td></td>
<td>120.5 (5.8)</td>
<td>55.2 (4.2)</td>
<td>125.2 (7.3)</td>
<td>113.4 (6.8)</td>
<td>112.0 (6.5)</td>
<td>32.6 (3.7)</td>
</tr>
<tr>
<td>G6PD, U/mg protein</td>
<td></td>
<td>17.7 (1.3)</td>
<td>9.6 (0.8)</td>
<td>18.5 (1.7)</td>
<td>14.6 (1.1)</td>
<td>16.4 (1.4)</td>
<td>5.8 (0.8)</td>
</tr>
<tr>
<td>GSH, nmol/mg protein</td>
<td></td>
<td>19.4 (1.5)</td>
<td>11.6 (0.9)</td>
<td>22.6 (1.1)</td>
<td>17.2 (1.2)</td>
<td>17.8 (1.5)</td>
<td>6.2 (0.7)</td>
</tr>
</tbody>
</table>

Blood values

<table>
<thead>
<tr>
<th>Blood values</th>
<th>Group</th>
<th>I (n=8)</th>
<th>II (n=8)</th>
<th>III (n=8)</th>
<th>IV (n=8)</th>
<th>V (n=8)</th>
<th>VI (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN, mg/L</td>
<td></td>
<td>220 (22)</td>
<td>252 (18)</td>
<td>230 (21)</td>
<td>242 (21)</td>
<td>250 (21)</td>
<td>496 (34)</td>
</tr>
<tr>
<td>Creatinine, mg/L</td>
<td></td>
<td>6.7 (0.5)</td>
<td>7.1 (0.5)</td>
<td>6.3 (0.6)</td>
<td>7.0 (0.8)</td>
<td>7.0 (0.3)</td>
<td>8.9 (0.4)</td>
</tr>
</tbody>
</table>

**Urinary variables**

<table>
<thead>
<tr>
<th>Urinary variables</th>
<th>Group</th>
<th>I (n=8)</th>
<th>II (n=8)</th>
<th>III (n=8)</th>
<th>IV (n=8)</th>
<th>V (n=8)</th>
<th>VI (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water intake, ml/24 h</td>
<td></td>
<td>22.6 (2.2)</td>
<td>44.2 (3.6)</td>
<td>23.0 (2.4)</td>
<td>31.2 (2.0)</td>
<td>22.9 (2.8)</td>
<td>55.2 (3.2)</td>
</tr>
<tr>
<td>Urine volume, ml/24 h</td>
<td></td>
<td>10.2 (1.3)</td>
<td>32.6 (3.2)</td>
<td>12.2 (1.2)</td>
<td>18.6 (1.1)</td>
<td>13.1 (1.7)</td>
<td>42.2 (3.6)</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>6.04 (0.04)</td>
<td>5.71 (0.05)</td>
<td>6.10 (0.04)</td>
<td>5.81 (0.05)</td>
<td>6.05 (0.06)</td>
<td>5.70 (0.05)</td>
</tr>
<tr>
<td>Calcium, mg/24 h</td>
<td></td>
<td>1.72 (0.32)</td>
<td>0.64 (0.08)</td>
<td>1.96 (0.22)</td>
<td>0.78 (0.10)</td>
<td>1.83 (0.17)</td>
<td>0.51 (0.09)</td>
</tr>
</tbody>
</table>

**P < 0.05, eight rats. Comparisons: a, significant vs I; b, significant vs II; c, significant vs III; d, significant vs IV; e, significant vs V.**
VITAMIN E AND HYPEROXALURIA-INDUCED CALCIUM OXALATE CRYSTAL DEPOSITION

FIG. 1. Urinary excretion of (a) LDH; (b) GGT; (c) alkaline phosphatase; and (d) alanine aminopeptidase in the rats in groups I–VI (symbols as Fig. 1), expressed as the mean (SEM). *P < 0.05.

FIG. 2. Urinary excretion of oxalate in the rats in groups I–VI (symbols as Fig. 1), expressed as the mean (SEM). *P < 0.05.

group VI had significantly greater urinary oxalate levels at 7 and 14 days, but significantly lower levels at 21 days. There were statistically significant increases in water intake and urine output, and a lower urine pH, in the rats in group II at 21 days (Table 2). These changes were drastically greater in group VI. Rats in group IV had water intake, urine output, and pH levels partly but significantly restored compared with rats in group II.

Scatterplots of the multivariate regression analysis are shown in Fig. 3; when the urinary excretion of oxalate was plotted against MDA, the correlation was much stronger (y = -33.31 + 31.88x; r = 0.99; P < 0.001). There were significant positive correlations between excretion of urinary oxalate and GGT (y = -49.06 + 17.65x; r = 0.94; P < 0.001), and significant negative correlations between urinary oxalate and catalase (y = 159.19 - 4.32x; r = -0.97; P < 0.001, plot not shown), urinary oxalate and α-tocopherol (y = 156.81 - 4.02x; r = -0.96; P < 0.001).

The kidney tissue MDA was positively correlated with the urinary excretion of GGT (y = -0.47 + 0.55x; r = 0.94; P < 0.001), and there was a significant negative correlation between MDA and GSH (y = 6.22 - 0.26x; r = -0.99; P < 0.001), and MDA and α-tocopherol (y = 5.8 - 0.12x; r = -0.92; P < 0.001).

In group II, after 7 days of EG-increased hyperoxaluria there was calcium oxalate crystalluria and enzymuria with increased MDA contents, 1.44 (0.12) in the absence of crystal deposition. Crystal deposition was completely absent even after 10 days of EG in rats in group II or IV (Fig. 4A). Occasional crystal deposits were found in rats in group VI at 10 days (data not shown). Furthermore, increased enzymuria was associated with increased MDA content of the renal tissues at 10 days (six rats); group I, 1.10 (0.09); group II, 1.79 (0.15); group IV, 1.26 (0.10); group VI, 2.6 (0.37) nmol/mg protein. After 14 days of EG in group II, individual crystals were nucleated on the renal tubular membrane surface, concurrent with increased lipid peroxidation, with an MDA content of 2.29 (0.26) nmol/mg protein, six rats), as shown in Fig. 4(B). These results strongly imply that the hyperoxaluria-induced peroxidation of renal tubular membrane is a prerequisite for nucleation of calcium oxalate crystals, and is one of the major mechanisms involved in the attachment and development of calcium oxalate kidney stones. As the renal tubules continued to be exposed to oxalate, nucleated crystals started to grow toward the centre of the tubules and resulted in complete occlusion of renal tubules, as shown in Fig. 4C; this leads to further mechanical damage to the tubular epithelium.

Kidney sections of a rat from group V had a normal epithelium under polarized light microscopy; the kidney was also of normal size, at 1.12 (0.08) g (Fig. 5A). The results were similar in groups I and III (data not shown). Calcium oxalate crystals were present in the renal parenchyma of rats in group II after 21 days but precipitation of calcium oxalate crystals (+++) showed focal involvement of the renal parenchyma, with some areas remaining free of crystal deposition. Disruption of crystal-containing tubules was also evident. Numerous crystals were present in the cortex, medulla and in the papilla. The kidney was enlarged and it was heavier than in group I (Fig. 5B). The kidney sections of rats in group IV after 21 days showed no calcium oxalate crystal deposition in any part of the nephron segment. Five of the eight rats had a normal kidney size and weight; three rats had mild enlargement of the kidney and the kidney was heavier (Fig. 5C). The pathological evaluation of kidney sections of rats in group VI showed diffuse and markedly extensive (++++) calcium oxalate deposits in the tubules of the cortex, medulla and collecting tubules (Fig. 5D). The crystal rosettes often completely occluded the tubular lumens. Intraluminal cellular debris and lymphoepithelial infiltration were sometimes found in association with crystal deposits. The decrease in urinary enzyme activities and reduced renal function, with the increase in urine volume in rats in group VI after 21 days may be caused by extensive calcium oxalate crystal deposition and significant blockage of renal tubules, which might
interfere with the filtration processes. The kidney was significantly larger than in the controls.

**DISCUSSION**

The present study provides direct evidence that vitamin E supplementation completely prevented calcium oxalate crystal deposition in the kidney, by preventing free radical-induced renal injury and by restoring antioxidant levels. Hyperoxaluria reduced vitamin E levels and increased MDA content and protein carbonyl in rats in group II, indicating increased demand for vitamin E during hyperoxaluria. This is direct evidence of increased use of vitamin E when free-radical generation is induced by hyperoxaluria, and therefore outlines the protective role of vitamin E in mitigating hyperoxaluria-induced free-radical generation. Earlier studies showed that increased lipid peroxidation reduces tocopherol levels in microsomal membranes [35].

We previously reported that oxalate-induced lipid peroxidation in renal tubular epithelial cells in culture was associated with a greater production of superoxide and hydroxyl free radicals. The production of these free radicals is greater when the cells are exposed to oxalate and calcium oxalate monohydrate crystals. This reveals that oxalate itself is injurious to cells and that calcium oxalate crystals potentiate the toxicity [11]. The significant increase in lipid peroxidation and protein carbonyl content in rats in group VI with hyperoxaluria is in agreement with the previous reports that vitamin E and selenium deficiency enhance free radical generation in rats [36]. Therefore, dramatically increased lipid peroxidation and protein carbonyl in rats in group VI indicates that vitamin E deficiency potentiates the oxalate-induced free-radical production in the kidney. Grases et al. [37] showed that free radical-damaged cells produce a favourable environment for crystal development, and that phytic acid prevents calcium oxalate crystallization by its antioxidant properties. Recent studies show increased urinary excretion of MDA in human calcium-oxalate kidney stone formers [38]. In addition, another recent study provided more evidence indicating that oxidative stress plays a role in human calcium oxalate kidney stone formation [39]. The authors also reported increased MDA, decreased vitamin E, decreased GSH, and decreased GPx in human kidney stone formers [39], which is similar to the levels observed in our present studies with the rat model. The present results clearly show that excess dietary vitamin E significantly decreased oxalate-induced kidney lipid peroxidation, and strongly support the suggestion of our previous report that
antioxidants may have a protective effect against free-radical injury associated with oxalate treatment [10]. There is lipid peroxidation and antioxidant depletion in several pathophysiological conditions, including cigarette smoking, ischaemic stroke and congestive heart failure [40].

The decreased antioxidant enzymes (SOD, catalase, and GPx) activities in the hyperoxaluric groups were attributed to peroxidative damage to the tissue caused by increased oxalate excretion, while supplementation with vitamin E contributed to maintaining the antioxidant enzymes at an optimum level by protecting renal tubules from peroxidative injury. In the absence of a sufficient concentration of vitamin E in the diet, the kidney antioxidants are adversely affected by hyperoxaluria. These results confirm that vitamin E acts as an excellent antioxidant for the kidney, which is greatly susceptible to oxalate-induced free radical damage.

The results indicate that EG-induced hyperoxaluria significantly decreased renal GSH level. However, the greatest depletion of renal GSH, in group VI, suggests tissue antioxidant imbalance. The decrease in glutathione reductase and G6PD after hyperoxaluria appears to indicate impaired reduction of oxidized glutathione (GSSG) to GSH by depletion of reducing equivalents of NADPH, which is a cosubstrate and is required for glutathione reductase activity [41].

In the current study the treatment of rats in group II resulted in hyperoxaluria, calcium oxalate crystalluria and enzymuria. The excretion of tubular marker enzymes was further increased, indicating renal tubular damage, and appeared to correlate with the retention and deposition of crystals in the kidneys. However, renal function was unaltered. The excretion of oxalate by rats in group IV was increased and similar to that of in group II, indicating that neither oxalate synthesis nor calcium oxalate crystalluria was prevented by excess vitamin E. However, the extra vitamin E significantly reduced the levels of these enzymes in the urine of hyperoxaluria-induced rats, indicating that hyperoxaluria and formation of calcium oxalate crystals were eliminated without causing renal damage. Thus, vitamin E has a protective effect against hyperoxaluria-induced lipid peroxidative injury to the renal tubules. Earlier studies reported that increased excretion of urinary enzymes by rats occurs as a result of chronic hyperoxaluria induced by various hyperoxaluric challenges, including EG, hydroxyl-L-proline or ammonium oxalate [42]. An increase in urinary enzymes was also reported in patients with renal stones [43].

We are the first to demonstrate direct evidence in vivo that hyperoxaluria-induced peroxidation of renal tubular membrane binds individual calcium oxalate crystals and initiates kidney stone formation. The crystals are first formed in the renal proximal tubules, as calcium in calcium oxalate crystals is derived from the glomerular filtrate [44], and oxalate is readily filterable at the glomerulus and secreted by the proximal tubules [45,46]. In humans, various changes in urine chemistry, including hyperoxaluria, hypercalciuria and hypocitraturia, can lead to the development of abundant crystals within the renal tubules. Using calculations based on the concentration of ions in the renal tubules, Finlayson and Reid [47] reported that crystals are not usually retained and could not reach a size large enough to occlude the tubular lumen within the urinary transit time. In normal kidneys, it takes 3 min for urine to pass from the glomerulus to the renal pelvis; it would take several hours for crystals to become large enough to obstruct a collecting duct [47], suggesting that unless calcium oxalate crystals bind to the tubular membrane surface, stone development would not be possible. In agreement with Finlayson and Reid, we showed that hyperoxaluria-induced renal tubular peroxidative damage associated with antioxidant imbalance resulted in crystal attachment, subsequent aggregation and growth of calcium oxalate kidney stones.

Oxalate-generated free radicals disrupt the structural integrity of the membranes in renal epithelial cells [10,14]. Wiessner et al. showed [48] that coating crystals with urinary macromolecules enhanced the attachment of the crystals to injured renal cells at a pH of <6.0. Surface exposure and redistribution of phosphatidylserine was reported to mediate stone crystal attachment to the renal tubular cell epithelium [49–51]. Studies show that crystal formation results in cell damage and cell detachment from the basement membrane, and the released degradation products can promote heterogeneous nucleation of calcium salts such as calcium oxalate and calcium phosphate. The exposed region of the tubular basement membrane could serve as a site for crystal nucleation and aggregation [52]. Smith [53] showed that dilute acid-induced damage to the rat urinary tract mucus lining promoted calcium oxalate crystal adherence and treatment with glycosaminoglycan decreased this adherence. In addition, lipid asymmetry also increased the affinity of lipid for calcium oxalate monohydrate crystal attachment [54]. Lipid peroxidation reportedly correlates with changes in membrane phospholipid asymmetry [55]. The present histopathological studies also showed a relationship between hyperoxaluria-induced
crystal deposition and peroxidative damage to the renal tubular epithelium. Even though there was EG-induced hyperoxaluria and calcium oxalate crystalluria in rats in group IV for up to 22 days, histopathological findings showed complete prevention of calcium oxalate crystal deposition.

Vitamin E is the most effective chain-breaking lipophilic antioxidant found within biological membranes and that can prevent biological damage [56]. Tocopherols lack sufficient water solubility to be excreted directly in urine and the major route of elimination of its water-soluble CEHC is through urine [57]. CEHC increased when the plasma level of RRR-\(\alpha\)-tocopherol was exceeded [58] by an excess \(\alpha\)-tocopherol supply. Recently, it was reported that \(\alpha\)-CEHC has antioxidant properties similar to those of trolox, a synthetic water-soluble vitamin E homologue [59]. Studies show that \(\alpha\)-CEHC had a protective effect against chromate- and thallium-induced nephrotoxicity in the rat model, caused by its antioxidant effect [60]. Therefore, vitamin E with its lipophilic and hydrophilic properties, could act as an effective antioxidant in vivo against hyperoxaluria-induced peroxidative damage in the kidney. In addition to the antioxidant properties of vitamin E, the absence of calcium oxalate crystal deposition in group IV might be due to interference of calcium oxalate crystals with the carboxyl group of water-soluble CEHC. The possible existence of an interaction between calcium oxalate crystals and CEHC remains, at present, a matter of speculation and worth further investigation.

In conclusion, these findings present novel and direct evidence in vivo that hyperoxaluria-induced peroxidative damage to the renal tubular membrane surface provides a favourable environment for individual calcium oxalate crystal attachment and subsequent development of kidney stones. Vitamin E treatment completely prevented calcium oxalate crystal deposition in the kidney, by preventing hyperoxaluria-induced lipid peroxidation and tissue antioxidant imbalance. From these findings, vitamin E could therefore be considered in the therapy of hyperoxaluria-induced kidney stone formation, and this could benefit individuals with recurrent kidney stone disease. However, clinical trials with estimates of the dose–response effects of vitamin E are warranted in the prevention of calcium oxalate stone deposition.

ACKNOWLEDGEMENTS

We thank Dr Raymond L. Hackett, Emeritus Professor of Pathology, Department of Pathology and Laboratory Medicine, University of Florida, Florida for valuable input and critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health RO1-DK 56249 (to S. T).

CONFLICT OF INTEREST

None declared.

REFERENCES


33 de Bruijn WC, Boeve ER, van Run PR et al. Etiology of calcium oxalate nephrolithiasis in rats. II. The role of the papilla in stone formation. Scanning Microsc 1995; 9: 115–25


38 Huang HS, Ma MC, Chen CF, Chen J. Lipid peroxidation and its correlations with urinary levels of oxalate, citric acid, and osteopontin in patients with renal calcium oxalate stones. Urology 2003; 62: 1123–8


55 Shvedova AA, Tyurina YJ, Kawai K et al. Selective peroxidation and externalization of phosphatidylserine in normal human epithelial keratinocytes during oxidative stress induced by cumene hydroperoxide. J Invest Dermatol 2002; 118: 1008–18

56 Meydani M, Vitamin E. Lancet 1995; 345: 170–5


60 Appenroth D, Karge E, Kiebling G, Wechter WJ, Winnefeld K, Fleck C. LLU-alpha, an endogenous metabolite of...
gamma-tocopherol, is more effective against metal nephrotoxicity in rats than gamma-tocopherol. *Toxicol Lett* 2001; **122**: 255–65

**Correspondence:** Sivagnanam Thamilselvan, Department of Urology, Ste 2D/34, Henry Ford Health System, One Ford Place, Detroit, MI-48202, USA.
e-mail: STHAMIL1@HFHS.ORG

**Abbreviations:** **SOD**, superoxide dismutase; **GTP**, glutathione peroxidase; **EG**, ethylene glycol; **MDA**, malondialdehyde; **GSSG**, oxidized glutathione; **GSH**, reduced glutathione; **G6PD**, glucose-6-phosphate dehydrogenase; **BUN**, blood urea nitrogen; **GGT**, γ-glutamyl transpeptidase; **LDH**, lactose dehydrogenase; **CEHC**, 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxycroman.