Scanning Microscopy

Volume 10 | Number 2 Article 24

6-30-1996

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Scanning Microscopy, Vol. 10, No. 2, 1996 (Pages 591-603) 0891-7035/94$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

EXPERIMENTAL NEPHROLITHIASIS IN RATS: THE EFFECT OF ETHYLENE GLYCOL AND VITAMIN D_3 ON THE INDUCTION OF RENAL CALCIUM OXALATE CRYSTALS

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(Received for publication December 8, 1995, and in revised form June 30, 1996)

Abstract

Using ethylene glycol (EG) and vitamin D_3 as crystal-inducing diet (CID) in rats, we investigated the effect of the dosage of EG on the generation of chronic calcium oxalate (CaOx) nephrolithiasis. We collected weekly 24 hour urines and measured herein the amount of oxalate, calcium, glycosaminoglycans (GAG's), creatinine, protein, alkaline phosphatase (AP), γ-glutamyl transpeptidase (γ-GT), and N-acetyl-ß-glucosaminidase (NAG). The potential of these urines to inhibit crystal growth and agglomeration was also evaluated. After four weeks, the kidneys were screened by histology and radiography for the presence of CaOx crystals and the amount of kidney-associated oxalate was biochemically measured. Using 0.5 vol. % EG, only a part of the rats showed CaOx deposition in the renal cortex and/or medulla, without obvious differences between Wistar and Sprague-Dawley (SD) rats. If a dietary EG concentration of 0.75, 1.0. or 1.5 vol. % was used, the amount of kidney-associated oxalate was proportionally higher and CaOx crystal formation was consistently found in all rats. Most crystals were encountered in the cortex, whereas in the medulla and the papillary region, crystals were only occasionally detected. From these data, we conclude that in the chronic rat model, based on EG and vitamin D_3, a consistent deposition of CaOx crystals is obtained using a EG concentration of at least 0.75%.

Key Words: Calcium oxalate, urolithiasis, rat model, vitamin D, ethylene glycol, Tamm-Horsfall protein, renal pathology.

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Introduction

Hyperoxaluria is a major risk factor in calcium oxalate (CaOx) stone disease. Apart from the so-called "idiopathic" stone forming patients, in which stone formation is attributed to diurnal and seasonal variations in oxalate absorption [17], hyperoxalurias mainly occur in patients with inborn errors of metabolism [14]. Such patients have recurrent CaOx stones that lead to progressive renal failure and death at an early age.

Several animal models have been proposed to extend the understanding of the etiology of human stone disease [3, 23, 34, 41]. In rats, hyperoxaluria can easily be induced by various diets. Most of these diets are based on increasing the oxalate production by administration of oxalate precursors such as ethylene glycol (EG) [3, 23]. Moreover, in the rat kidney, the physiology and distribution of oxalate among cortex, medulla, and papilla, is reported to be similar to that in the human kidney [19].

For studies on experimental nephrolithiasis, two types of rat models are currently available [23]: acute and chronic models. In the acute models, animals are challenged with a relatively large dose of lithogen and the urinary oxalate load is increased within minutes [21, 25]. Brigman and Finlayson [1] used a single intraperitoneal injection of sodium oxalate to test the efficiency of various drugs in reducing the precipitation of intratubular CaOx salts within the renal tubules. Although in the acute models, induced hyperoxaluria is followed by crystalluria and formation of CaOx crystals within the renal tubules and the interstitium, renal stones are not formed. In the chronic models, rats are continuously challenged with smaller doses of lithogen for a longer period, up to 30 days [2, 9, 10, 11, 19]. In this model, the CaOx crystals formed are retained long enough within the tubular system to be deposited in the interstitium.

Several chronic models are available. Buck *et al.* [2] used daily injections of calcium gluconate to introduce nephrocalcinosis. In the present study, we focused our attention on the chronic vitamin D_3/EG model. This model, originally described by Okada *et al.* [32] and modified later by Reis-Santos *et al.* [35], is based on the

formation of intratubular CaOx crystals as a result of the supersaturation, generated by the vitamin D_3-induced enhancement of urinary calcium concentrations and EG-induced hyperoxaluria [33]. In order to further characterize the model, and to define the conditions consistently leading to CaOx deposition in the rat kidneys, we investigated the following parameters: (**1**) The urinary excretion and renal deposition of CaOx at successive points of time of feeding the vitamin D_3 and 0.5% EG. (**2**) At that point of time at which, with 0.5% EG, most renal crystals were encountered, the effect of increasing EG-concentrations on the renal deposition of CaOx crystals. In addition, the urinary excretion of glycosaminoglycans (GAG's) and the urinary inhibitory activities of crystal growth and agglomeration were investigated. Possible kidney damage was assessed by following the urinary excretion of creatinine and the release of protein and renal enzymes into the urine.

Materials and Methods

Thirty-three male Wistar and Sprague Dawley (SD) rats weighing 250-300 g were obtained from the Central Animal Breeding Centre (Harlan, Zeist, The Netherlands). After arrival, the rats were acclimatized for seven days. All rats had free access to 40 ml drinking water every day. Mild chronic hyperoxaluria was induced by EG and vitamin D_3 (Sigma, St. Louis, MO, USA). EG was supplemented to drinking water to a final concentration of 0.5 vol.% or higher. Vitamin D_3 was dissolved in cottonseed oil (Sigma) at a concentration of one μg/ml, of which, compulsorily, 0.5 ml was orally administrated by stomach intubation every other day. Control rats did not receive vitamin D_3 and EG had been omitted from their drinking water. All rats received a standard rat chow (Diet AM II, Hope Farms, Woerden, the Netherlands), consisting of (in weight %, wt.%): rough protein (23.3%), fat (6.4%), rough cell compound (3.2%), rough ash (4.4%), and a salt mixture, containing phosphorus (0.48%), magnesium (0.12%), sodium (0.24%), and potassium (0.8%). The calcium content varied per batch between 0.56-0.83% (mean: 0.70%).

Urines were collected at the following points in time, for each animal separately. For the first experiment, in which vitamin D_3 + 0.5% EG was used as crystal-inducing diet (CID), these urines were collected at days 0, 7, 14, 21, 28 and 42. The animals were sacrificed at the indicated points in time (Table 1). For the second experiment, in which vitamin D_3 and three increasing EG concentrations were used as CID, viz., 0.75% (group I), 1.0% (group II) and 1.5% (group III), the urines were collected at days 0, 6, 9, 14, 20 and 28. Each group consisted of three animals. The rats were

Table 1. Wistar and SD rats used for the CID experiment of vitamin D_3 + 0.5% EG. At the indicated points of time the urines were collected and the animals sacrificed. (SD = Sprague Dawley rat).

Rat number Wistar/SD	Diet	Day of urine collection	Day of sacrifice
1 + 2	standard	0,7,14,21,28,42	42
3 + 4	CID	0, and 7	7
5 + 6	CID	14	14
7 + 8	CID	21	21
9 + 10	CID	28	28
11 + 12	CID	42	42

individually kept in metabolic cages and the urines were collected on ice without any preservative during 24 hours. The volume and the pH of each urine were measured. This pH was between 6.0 and 8.5. To one ml urine samples, 6 μl concentrated HCl was added and they were then analyzed for oxalate, calcium, magnesium, and phosphate. The remaining unacidified urine was centrifuged at 1000 x *g* for 10 minutes and the obtained supernatant was analyzed for creatinine, protein, alkaline phosphatase (AP), γ-glutamyl transpeptidase (γGT) and N-acetyl-ß-glucosaminidase (NAG). For all analyses, including the ions, Merck kits (Merck Diagnostica, Darmstadt, Germany), adapted to an Eppendorf-Merck ELAN autoanalyser, were used. These assays were performed as prescribed by the manufacturer. The oxalate assays were performed with an enzymatic kit from Sigma, which is based on the oxidation of oxalate to CO_2. The resulting H_2O_2 was measured by the formation of a blue indamine dye, with a maximum absorption at 590 nm. For each oxalate assay, 0.5 ml urine was mixed with an equal volume sample buffer (final pH 5.0-7.0). Of this mixture, 0.5 ml was pipetted in an Eppendorf tube, thoroughly mixed with 50 mg active carbon, and centrifuged at 10,000 rpm in a microcentrifuge for 2 minutes. Of the supernatant, 90 μl was used for the oxalate assay with autoanalyser. Using the oxalate standards of the test kit, the detection limit was 0.08 μmol oxalate/ml urine. Urinary GAG's were measured using 1,9-dimethylene blue as substrate [12]. Urinary inhibitory activity was assessed by measuring the potential of the collected urines to inhibit growth and aggregation of CaOx seed crystals in a metastable solution [39].

The pellets of the centrifuged unacidified urines (see above) were analyzed for the presence of CaOx crystals by Fourier-transform infrared spectroscopy (FTIR) [8]. For this purpose, the pellets were washed by resuspension in water, followed by centrifugation at 3000 rpm,

Table 2. Calcium and oxalate excretion of Wistar and SD rats fed the standard chow and rats fed a CID of vitamin D_3 and 0.5% EG for the indicated points of time. At day 0 also all CID-fed rats were fed the standard chow. All Data are expressed as μmol/24 h. The data of the Wistar rats are also shown in Figure 1 (1A: calcium; and 1B: oxalate).

Rat Type	Rat Number	Diet	Experimental day 0	7	14	21	28	42	Mean ± s.d.★
Calcium excretion									
Wistar	1	standard	17.0*	7.3*	17.8*	15.9*	29.2*	26.3*)	17.6 ± 8.0*
Wistar	2	standard	7.7*	15.8*	23.0*	12.5*	33.0*	20.5*)	
Wistar	3-7	CID	8.9*	0.6	6.4	10.6	8.9	25.3	
Wistar	6-12	CID	11.9*	9.3	11.3	4.6	4.3	11.4	
SD	1	standard	11.7∇	20.2∇	19.8∇	11.1∇	32.3∇	10.4∇)	19.3 ± 9.4∇
SD	2	standard	8.3∇	28.2∇	26.0∇	8.7∇	39.1∇	15.4∇)	
SD	3-7	CID	18.3∇	4.8	9.7	2.2	14.4	1.2	
SD	6-12	CID	20.7∇	8.0	9.5	8.9	10.0	5.8	
Oxalate excretion									
Wistar	1	standard	9.9*	10.5*	13.2*	8.2*	4.8*	9.1*)	8.8 ± 2.0*
Wistar	2	standard	6.4*	9.1*	7.4*	10.2*	10.5*	8.6*)	
Wistar	3-7	CID	8.1*	8.9	98.0	54.3	57.6	36.6	
Wistar	8-12	CID	7.8*	37.6	62.4	12.0	33.6	29.8	
SD	1	standard	3.7∇	9.3∇	9.9∇	15.6∇	8.9∇	7.8∇)	9.1 ± 3.9∇
SD	2	standard	4.3∇	8.1∇	8.9∇	16.2∇	9.0∇	14.4∇)	
SD	3-7	CID	6.5∇	41.1	18.3	85.1	81.7	12.2	
SD	8-12	CID	5.2∇	15.5	45.6	86.6	34.1	17.3	

★Pooled data of all Wistar rats (*) and SD rats (∇) fed the standard chow (for 0, 7, 14, 21, 28, and 42 days).

dried and pulverized. The crushed crystals were mixed with 200 mg KBr and analyzed with a Biorad FTS-7 (Veenendaal, The Netherlands).

At 28 days, the rats were anaesthetized with diethylether, followed by an intramuscular injection with a 12 wt.% solution of urethane (Fluka, Buchs, Switzerland) in physiological saline at a dose of 1 ml per 100 g body weight. The kidneys were perfused through the abdominal aorta with physiological saline. Small pieces of the cortex and medulla of the right kidneys were excised, fixed with 4% paraformaldehyde and embedded in paraffin. The tissue sections were stained with haematoxylin/eosin (HE) and examined by light microscopy.

The right and left kidneys were weighed and scanned by radiography for the presence of CaOx crystals. The right kidneys were homogenized as follows: the cortical/medullary- and the papillary part were digested in 6 ml, respectively, 1 ml digestion buffer, consisting of 10 mmol Tris-HCl (pH 8.0), sodium dodecyl sulphate (SDS; 1 wt.%) and proteinase K (0.14 mg/ml) (Glenny RW, personal communication, 1993). The left kidneys were entirely homogenized in 7 ml digestion buffer. The digestions were performed for 24 hours in a water bath at 45-50°C. The suspensions were centrifuged for 10 minutes at 1000 x *g*. The pellet with the crystals was dissolved in 0.3 N HNO_3 and the amount of oxalate was measured in the pellet and supernatant as described above. All data were expressed as μmol oxalate per kidney and statistically analyzed by Kruskal-Wallis 1-way ANOVA tests.

The urinary data are presented as mean ± standard deviation (s.d.). Since the number of data was too low to assume a normal distribution, non-parametrical tests were used for the statistical analyses: (1) for the detection, for each point in time, possible differences between the CID-fed rats and the rats fed the standard diet (day 0) Wilcoxon matched-pairs tests were used. The reason for this approach was that both populations were formed by the same rats. In these analyses, the data of group I-III were pooled for each point in time and compared with the data of the rats fed the standard diet (day 0). Significant differences ($p < 0.05$) are indicated in the various figures (*). (2) For the detection, for each point in time, of differences between the three groups

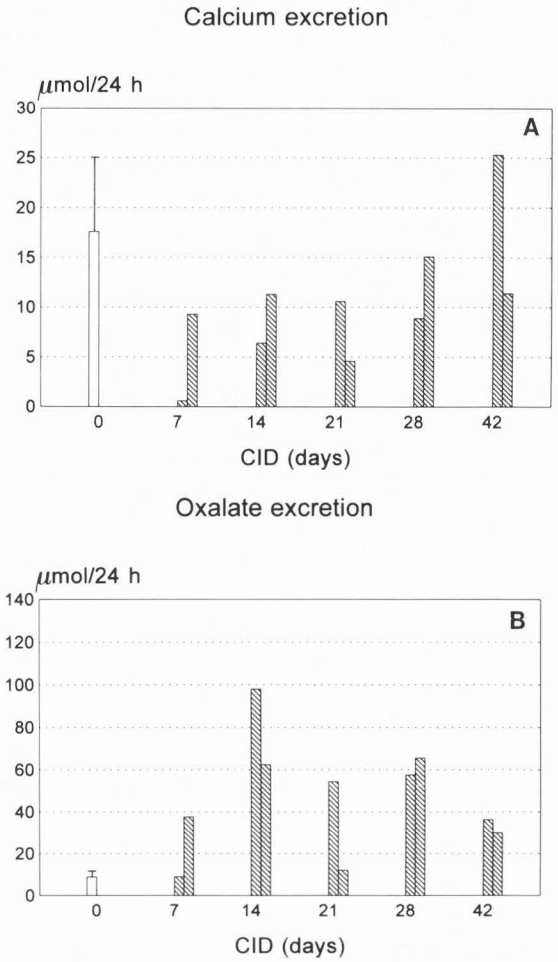

Figure 1. Urinary excretion of calcium and oxalate in control (open bars; mean ± s.d.; n = 4) and CID-fed animals at the indicated points of time (hatched bars; presented as data of individual rats). (**A**) Calcium excretion; (**B**) Oxalate excretion.

Kruskal-Wallis 1-way ANOVA tests were used. The existence of significant heterogeneity ($p < 0.05$) between these group is indicated in the various figures (***). (**3**) To detect, for each urinary parameter, significant ($p < 0.05$) time-related changes, Friedman two-way ANOVA tests were used.

Results

Time course study

In initial experiments with Wistar rats, we administered a CID of vitamin D_3 and 0.5% EG and examined the urinary excretion and renal deposition of calcium and oxalate. In addition, the composition of the excreted urinary crystals was determined. The results are presented below as data of individual rats and compared with rats fed only the standard chow (control rats).

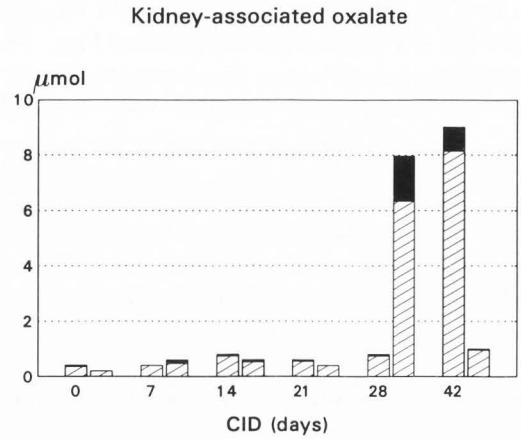

Figure 2. Oxalate content in kidneys of control and CID-fed animals. The cortical/medullary regions are indicated with the hatched areas and the papillary regions with the solid areas. The results are presented as data of individual rats.

Since it has been reported that after phosphorus- and vitamin B_6 deprivation, SD rats have a higher urinary oxalate excretion than Wistar rats [45], we performed, using the CID of vitamin D_3 and 0.5% EG, a duplicate experiment with SD rats.

Urinary excretion of calcium and oxalate: In control rats, the urinary calcium excretion varied between 10-30 µmol per 24 hours. During the first three weeks of CID, all rats showed a reduced urinary calcium excretion, although this decrease was less pronounced at 28 and 42 days (Fig. 1A, Table 2). The oxalate excretion increased considerably after CID, but also varied between the individual animals (Fig. 1B, Table 2). Maximal values were obtained at 14, 21, and 28 days. The urinary calcium/oxalate ratio was 2.2 ± 1.5 in control rats, whereas in CID-fed rats, this ratio was always lower than 0.4.

Kidney-associated oxalate: The kidneys of control rats contained approximately 0.4 µmol oxalate. During the first three weeks of CID the amount of oxalate was slightly increased. Considerably higher values were observed at 28 and 42 days, with a wide variation between the individual animals (range: 0.8-7.9 µmol, respectively, 1.0-9.0 µmol). Of the total kidney-associated oxalate ranging 0.06-1.6 µmol, maximally 0.04-0.9 µmol oxalate, respectively, was associated with the papilla at these points of time (Fig. 2).

Analyses of urinary crystals: The urinary crystals of the control rats consisted predominantly of phosphate with calcium or magnesium (apatite and struvite). CaOx monohydrate crystals (whewellite) were encountered, but

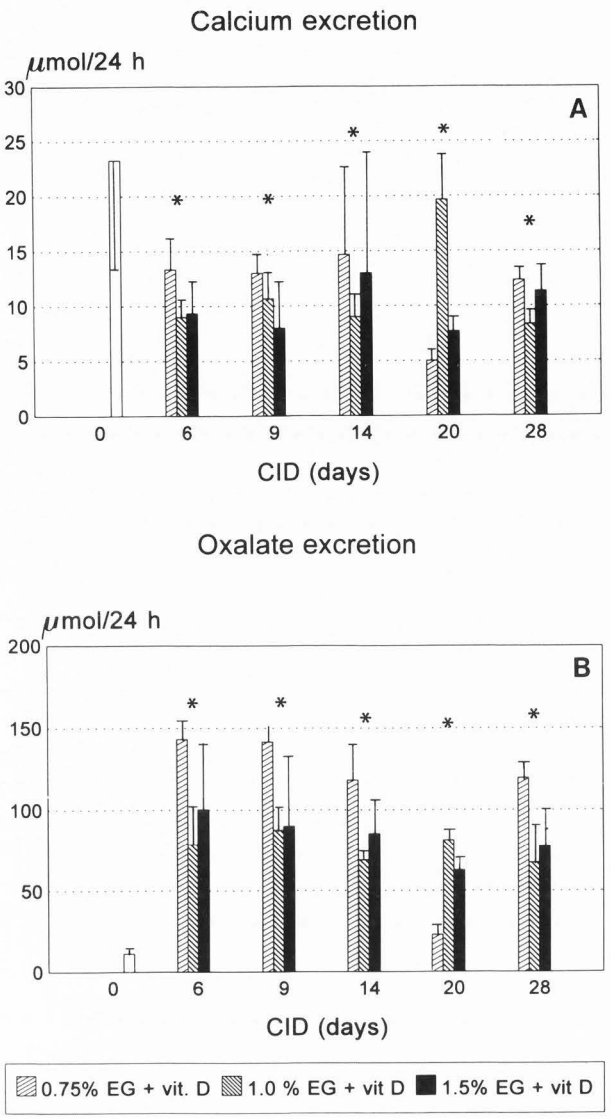

Figure 3. The effect of the concentration of dietary EG on the urinary excretion of calcium and oxalate. Each bar represents the mean (± s.d.) of nine animals (open bars; controls) or three animals (hatched bars; CID-fed animals). Significant differences with the control rats are indicated (*). The EG concentration in the drinking water is displayed in the box at the bottom of Figure 3. (**A**) Calcium excretion; (**B**) Oxalate excretion.

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only in low percentages (≤ 5%). During the first three weeks of the CID, whewellite crystals were slightly more often found (2-10%), but most frequently at both 28 and 42 days (2-50%). CaOx dihydrate crystals (weddellite) were only observed at day 42 (0-20%).

Effect of the EG concentration in the CID

In a previous study [13], we found that a CID of vitamin D_3 and 0.5% EG lead in only eight (53%) of the fifteen CID-fed animals to renal deposition of CaOx

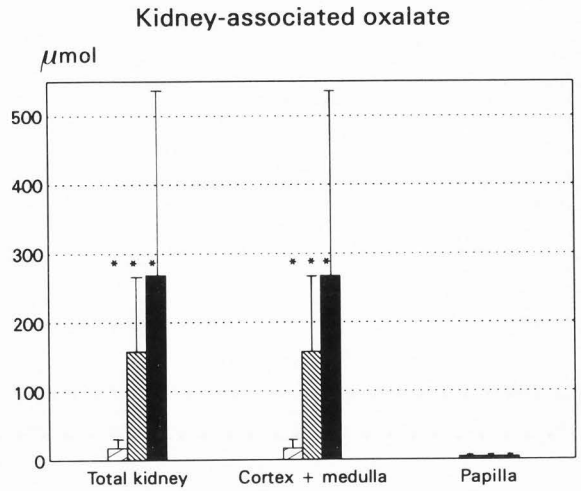

Figure 4. The effect of the concentration of dietary EG on the oxalate content of (1) the total kidney, (2) the cortex/medulla, and (3) the papilla. The animals were fed the CID of EG and vitamin D_3 for 28 days. Each bar represents the mean of three animals (± s.d.). Mutual differences between the three groups in oxalate deposition are indicated (***; $p < 0.06$). The EG concentration in the drinking water is displayed similar to that in Figure 3.

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crystals. In this study, we investigated whether increasing the EG concentration in the drinking water to 0.75% (group I), 1.0% (group II), and 1.5% (group III), respectively, achieved a consistent deposition of renal crystals. The results of this study, in which we also followed the various urinary parameters, are presented below.

Urinary excretion of calcium and oxalate: Compared to the control animals, the excretion of calcium was reduced at all points of time after CID ($p < 0.05$), without differences between the three groups (Fig. 3A). The oxalate excretion had increased considerably after CID ($p < 0.008$), without differences between the groups, and without observable changes with time (Fig. 3B). The calcium/oxalate ratio was 2.6 ± 1.6 in control animals ($n = 6$), whereas in group II and III this ratio was always lower than 0.4. No changes were observed in magnesium and phosphate excretion.

Kidney-associated oxalate: In group I, the amount of oxalate in the total kidney was 17.8 ± 12.2 µmol, of which 1.0 ± 0.6 µmol was associated with the papilla. In the cortex and medulla, the amount of oxalate was higher in group II and III, but not yet significant ($p = 0.06$). The quantity of oxalate in the papilla remained at the same low level (Fig. 4). Histological examinations and evaluation of the radiographs showed that all kidneys had small crystals. These crystals were present

Table 3. Vitamin D_3 + EG diet: the effect of EG concentration in the drinking water on the induction of renal calcium oxalate crystals ★.

EG concentration (drinking water).	0.75%			1.0%			1.5%		
Rat number	1	2	3	4	5	6	7	8	9
Radiography									
cortex/medulla/papilla	+	+	+	++++	+	+++	++++	++++	++++
small papillary stones	-	-	+	-	+	++	-	+	++
Histology	+/-	++	++	++++	+++	++++	++++	++++	++++

★+ to +++: indicates the degree of renal CaOx deposition; -: indicates the absence of renal CaOx deposition.

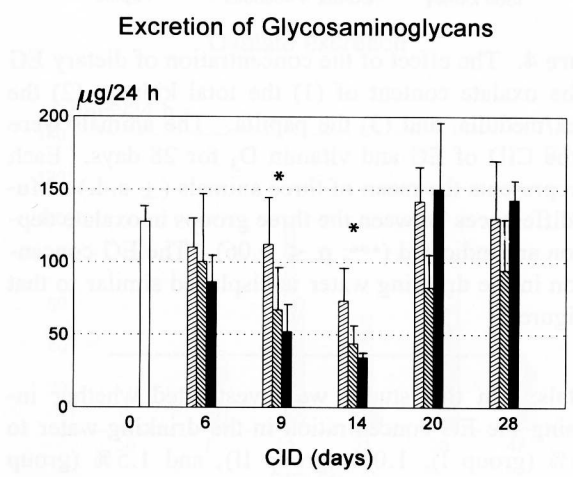

Figure 5. The effect of the concentration of dietary EG on the excretion of GAG's at various points of time of CID. Each bar represents the mean (± s.d.) of nine animals (open bars; controls) or three CID-fed animals (hatched bars). Significant differences with the control group (day 0) are indicated (*). The excretion of GAG's changes with time ($p < 0.01$; Friedman test).

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in the papilla or also in the tubular system and the cortical and medullary interstitium. Small papillary stones were occasionally also observed (Table 3).

Urinary excretion of GAG's and urinary inhibitory activities: Compared to the control rats (day 0), which excreted 128 ± 8.6 μg of GAG's per 24 hours, the GAG excretion was reduced at 9 and 14 days ($p < 0.03$), without prominent differences between the three groups. The Friedman test showed that the excretion of GAGs changes with time, in all probability, was caused by the relatively low excretion values at 14 days and the relatively high excretion values at 20 and 28 days (Fig. 5; $p < 0.001$).

In control animals, the inhibition of crystal growth (IG) and agglomeration (IA) were 44.0 ± 3.9% and

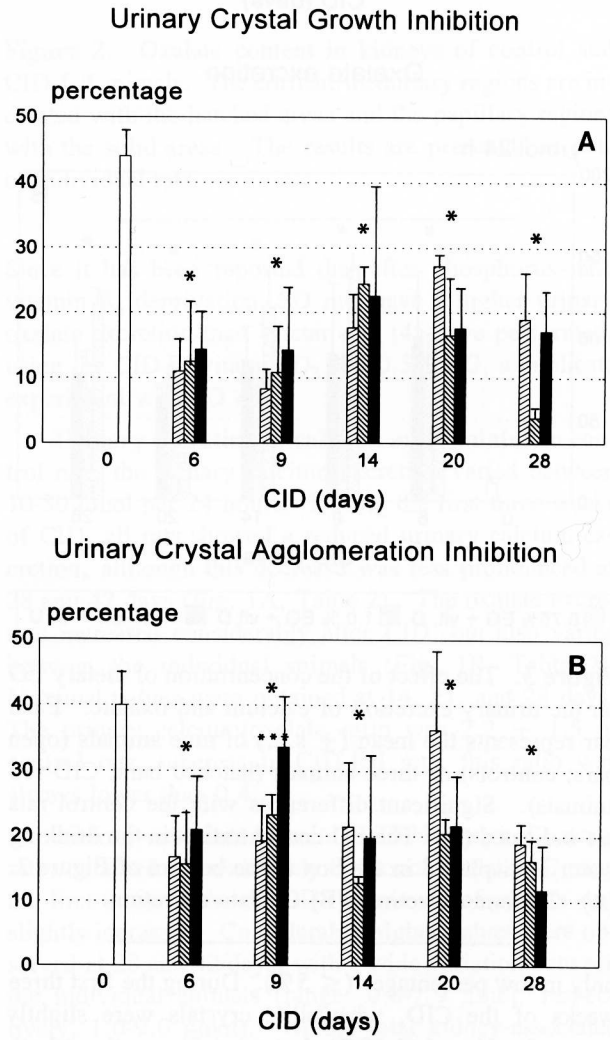

Figure 6. The effect of the concentration of dietary EG on the urinary inhibitory activities at various points of time of CID. Significant differences with the control group (*) and within the three CID groups are indicated (***). The inhibitory activity of crystal growth changes with time ($p < 0.03$). (**A**) Inhibition of urinary crystal growth; (**B**) Inhibition of urinary crystal agglomeration.

40.0 ± 5.5%, respectively. Both activities were reduced after CID ($p < 0.03$, $p < 0.02$, respectively). Only at day 9, the IA was dependent of the EG concentration (Figs. 6A and 6B; $p = 0.05$ with the Kruskal-Wallis test). A time-dependency was observed for the IG, probably caused by the relatively high values at 14, 20, and 28 days ($p < 0.03$).

Excretion of creatinine, protein and enzymes: Compared with control rats, the excretion of creatinine and NAG was roughly 20-30% reduced after CID (Figs. 7A and 7C). This decrease was not significant. Significantly lower values were found for the release of protein at day 9, 14, and 20 ($p < 0.04$; Fig. 7B) and AP at day 9, 14, and 24 ($p < 0.05$; Fig. 7D). Compared with the control rats, the activity of γ-GT was significantly reduced at day 6, 14, 20, and 28 ($p < 0.05$). The excreted amount of γ-GT was related with time ($p < 0.04$) and, at day 9, 20, and 28, also related to the amount of EG in the CID ($p < 0.05$; Fig. 7E).

Analyses of urinary crystals: The urinary crystals of the control rats consisted predominantly of phosphate with calcium or magnesium (apatite and struvite). CaOx monohydrate crystals (whewellite) were encountered, but only in low percentages (≤ 5%). During the first three weeks of the CID, whewellite crystals were found slightly more often (2-10%), but most frequently at both 28 and 42 days (2-50%). CaOx dihydrate crystals (weddellite) were only observed at day 42 (0-20%).

Discussion

For generation of CaOx crystals in the rat kidney, several diets have been described. It has been reported that after phosphorus and vitamin B_6 deprivation, SD rats show a stronger CaOx response than Wistar rats [45]. Using EG and vitamin D_3 as CID, we could not confirm this finding. Khan *et al.* [22] found that administration of 0.75% EG through drinking water for 24 days did not result in a consistent renal deposition of crystals. The fact that we found, in our experiments with this EG concentration, a consistent renal crystal retention, might be related to the additional administration of vitamin D_3.

The present results of the oxalate assays on the kidney homogenates and the collected urines confirm earlier observations [23] that for crystal formation in rat kidney, a severe hyperoxaluria is required. A prominent renal crystal retention occurred only in those animals with a daily oxalate excretion of 80-100 μmol. Assuming a production of 30 ml urine per day, this value corresponds with a urinary oxalate concentration higher than 3 μmol/ml. In stone-forming patients, the urinary oxalate concentration is much lower. In healthy adults, it is about 0.25 μmol/ml. In idiopathic stone formers, the urinary oxalate concentration lies between 0.25-0.40 μmol/ml, and in hyperoxaluric patients, between 0.4-0.8 μmol/ml [7]. *In vitro* studies have shown that in human urine, crystal formation rises if urinary oxalate concentration exceeds about 0.4 μmol/ml [17].

The rat differs from man in the low incidence of renal stones instead of crystals and the occurrence of crystals within the cortex rather than the medulla [15, 29]. In addition, vitamin D_3-induced nephrocalcinosis involves the early parts of the proximal tubule located in the superficial cortex [3]. These data agree with the results of the present study. Examination of the radiographs showed that crystal deposition in the renal papilla only increased at EG concentrations of 1.0% and higher. Our biochemical analyses showed that the increase of oxalate associated with the papilla was low, compared to the large increase in the total kidney.

Calcium oxalate crystals have also been experimentally induced in the kidneys of non-human primates, but the required EG dose (15 mg/kg) is much lower than that for rats [36]. In our experiments, the optimum concentration of EG in drinking water was about 0.75-1%, which corresponds to a daily dose of 750-1000 mg/kg. This is, however, far below the LD_{50} (the amount of a substance that would be required to kill, within a specified time period, 50% of the individuals in a large population of animals or organisms) for rats of 4700 mg/kg after oral administration [40]. Chronic administration of EG at concentrations higher than 1% may, however, cause toxic symptoms, such as nephrotoxicity and/or generalized metabolic acidosis and early death [20].

Although calcium and oxalate may both promote crystalluria and renal crystal formation, there are a number of indications that in humans, at calcium/oxalate ratio's larger than one, an increase in urinary oxalate concentration has a greater effect on the formation of CaOx crystals than comparable changes in urinary calcium [37, 38, 43]. It is indisputable that between healthy individuals and idiopathic stone formers, there are relatively small differences in urinary oxalate concentrations (usually less than 0.1 μmol/ml). Other evidence for an important role for oxalate comes from clinical studies. Also, particle size measurements in urines of recurrent stone formers showed that the volume of CaOx crystals is related to the urinary oxalate and not to the urinary calcium concentration [38].

In the present study, we that the excretion of calcium was lower in CID-fed than in control rats. In the control rats, the calcium/oxalate ratio was 2.6 ± 1.6, whereas, after CID of vitamin D_3 and EG, this ratio was always lower than 0.4. This indicates that in hyperoxaluric rats, the urinary calcium concentration is the rate-limiting step for the formation of CaOx crystals. The

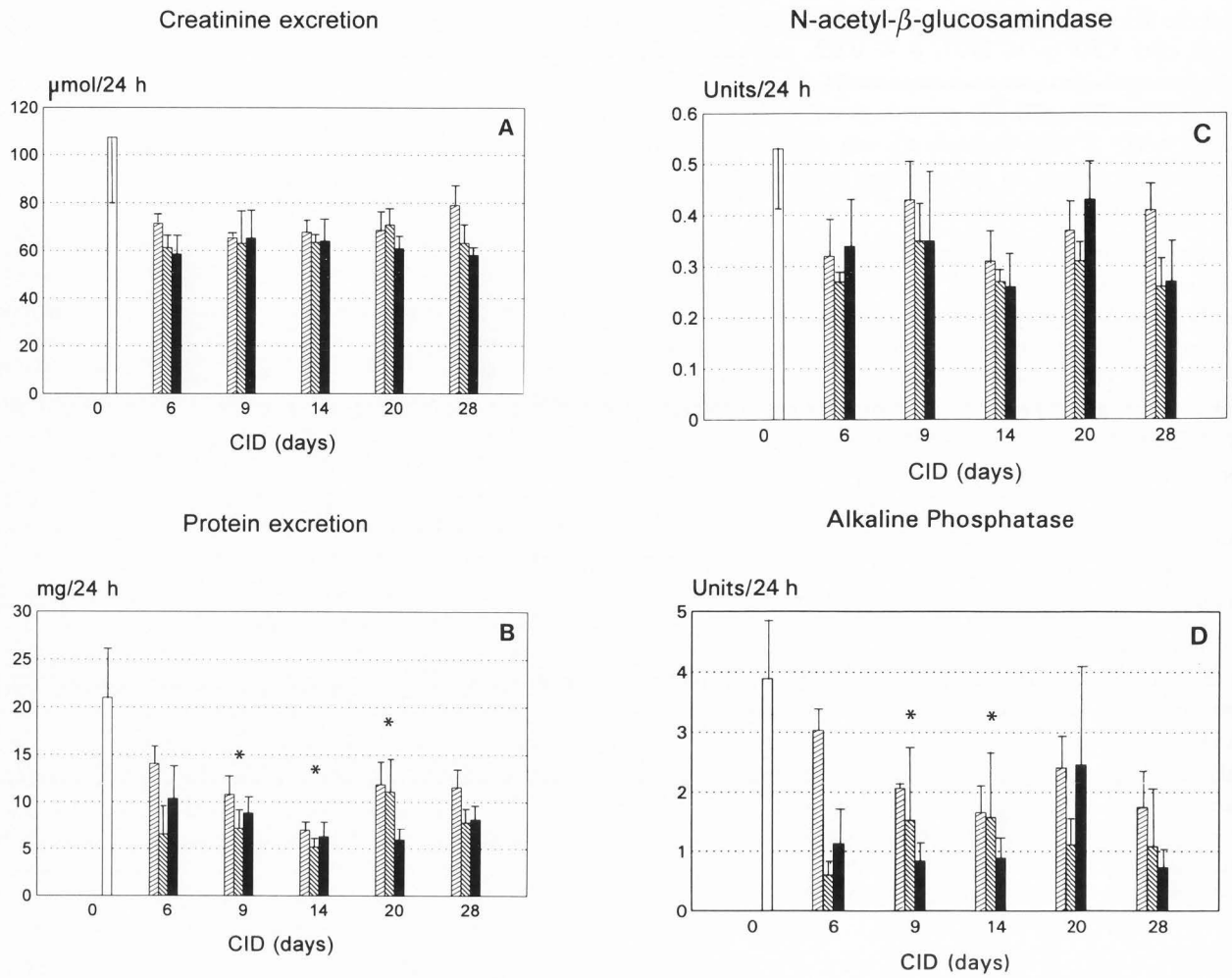

Figure 7. The effect of the concentration of dietary EG on the urinary excretion of creatinine (**A**), protein (**B**), and the renal enzymes NAG (**C**), AP (**D**), and γ-GT (**E**). Significant differences with the control (*) group and within the three CID groups (***) are indicated. The excretion of NAG and γ-GT changes with time ($p < 0.05$). The EG concentration in the drinking water is displayed conform the legend of Figure 3.

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fact that the calcium excretion in these rats was lower than in the control rats remains unexplained. This may be related to a reduced daily food consumption, since during CID diet, we observed a significant decrease in body weight in all animals, especially when the higher EG concentrations of 1.0 and 1.5% were used.

Crystal formation is considered to be the result of an imbalance between supersaturation and the presence of crystallization promoters on the one hand and inhibitor activity on the other [44]. In urine, a number of macromolecular inhibitors are present, among which GAG's, nephrocalcin and Tamm-Horsfall protein have been investigated most extensively [4, 5, 6, 44]. Osteopontin is, quantitatively, another macromolecular inhibitor in urine. It is present in the epithelial cells of the thin descending loop of Henle and in the papillary surface epithelium [28]. The synthesis of this glycoprotein is increased by vitamin D_3 and the control element has been found in the promoter region of the osteopontin

gene [30]. If, after a CID of vitamin D_3 and EG, the synthesis of osteopontin is increased, a rise in inhibitor activity would be expected. If osteopontin is an inhibitor of crystal agglomeration, then, this is evident on day 9 (Fig. 6B). However, the question of which inhibitors contribute to the observed reduced urinary inhibitory activity after CID deserves further attention, e.g., by measuring the urinary concentration of individual GAG's, osteopontin, Tamm-Horsfall protein, nephrocalcin and citrate.

In the present study, the CID induced evidently not only severe hyperoxaluria, but it also reduced the potential of the urines to inhibit crystal growth and agglomeration. In addition, there can be a lower production of urinary inhibitors; the observed reduction may have been due to co-precipitation of the inhibitor with CaOx crystals. During growth and agglomeration, these crystals can bind macromolecular inhibitors and the absorbed layer has been visualized by light- and electron-microscopy [13, 26]. Another explanation for the observed reduction in urinary inhibitory activity may be that, in our hyperoxaluric rat urines, less ionic calcium is available. Most inhibitors require free calcium for crystal binding [18].

It is known that hyperoxaluria with renal CaOx deposition is harmful to the tubular epithelium. Several studies have shown that oxalate ions and CaOx crystals enhance the release of the renal enzymes AP, γ-GT, and NAG [16, 24, 27]. In the present study, however, the release of protein and renal enzymes was reduced within seven days after a CID of 0.75% EG and vitamin D_3. For γ-GT, this reduction was even more pronounced, if the higher EG concentrations of 1.0 and 1.5% were used. Stonard *et al.* [42] also failed to find an increase in either NAG, AP, or protein in rats with nephrocalcinosis. Nouwen *et al.* [31], after injury of the renal epithelial cells, found a temporary loss in the expression of epidermal growth factor, Tamm-Horsfall protein, transferrin receptor and in lectin binding. From these considerations, it can be concluded that a CID of vitamin D_3 and EG in all likelihood induces renal damage. This damage may result in a decreased protein-synthesis and a lower release of proteins and renal enzymes at later points of time. In addition, it is conceivable that the urinary enzymes and other urinary glycoproteins absorb to the CaOx crystals during aggregation and agglomeration, and coprecipitate during renal deposition. Both processes can occur at the same time.

From the above considerations, we conclude that a CID of vitamin D_3 and 0.5% EG gives rise to crystalluria and a highly variable crystal deposition in the renal cortex. A higher EG concentration of 1.0, respectively, 1.5%, results in a proportionally higher crystal deposition in the cortex, whereas in the papilla, it remains at the same level and induces renal damage. We conclude that for a consistent deposition of CaOx crystal, an EG concentration of 0.75% is required.

Acknowledgements

This study received financial support from the Dutch Kidney Foundation (R. de Water and P.P.M.C. van Miert, grant number C 92.1199) and the SUWO (Stichting Urologisch Wetenschappelijk Onderzoek).

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Discussion with Reviewers

R.L. Hackett: In Figures 6A and 6B, you illustrate crystal growth and agglomeration inhibition. Although apparently not statistically different, there appears to be a trend that growth is inhibited less than agglomeration, especially in the 1.5% EG and vitamin D_3 group at earlier time periods. Could you provide an explanation for this phenomenon?

Authors: According to Ryall's group [39, 46], the Coulter Counter model has been used to characterize calcium oxalate monohydrate (COM) seeded crystal growth and agglomeration in the absence and presence of the rat urine. It is well known that crystal growth and agglomeration are different physico-chemical processes with different mechanisms, but they occur simultaneously in the test system used. The crystal growth and agglomeration can be independently evaluated based on changes in the total particle numbers and volumes measured by the Coulter Counter. This trend reflects two possibilities: (**1**) The components (inhibitors/promoters) in rat urines that may influence COM crystallization have been changed as a consequence of the CID. (**2**) The mechanisms that controls crystal growth and agglomeration might be different. Because of the increased number of particles, the possibility that the presents results are due to a second nucleation induced by the addition of the urines cannot be excluded. The advantage and weakness of the Counter model used in our laboratory have been recently discussed [47].

R.L. Hackett: What is the definition of nephro-calcinosis? Please explain the difference between it and crystallization and crystal localization.

Authors: Nephrocalcinosis is defined as crystal deposition outside the calyx and the tubular system, i.e., in the cortical and/or in the medullary parenchyma.

R.L. Hackett: In **Materials and Methods**, the text implies as though you checked crystalluria on urines that had been continuously iced throughout the collection period. If so, what do the authors conclude about the crystals that were salted out in this process?

Authors: The wish to preserve the activities of urinary enzymes and to prevent bacterial growth left us no other choice than to collect the urines on ice. Although we cannot exclude the possibility that the low temperature salted out CaOx monohydrate and dihydrate crystals, it must be realized that, in all likelihood, the urinary inhibitors of crystal growth and agglomeration are still effective at this low temperature.

R.L. Hackett: I am curious to know whether or not you provided the animals with a "mush" form of rat chow. Providing mush is important in regulating the animals dietary intake as well as limiting the contamination of the urine collectors. Also, it does not appear that the animals were "pair fed". Was the intake of EG monitored? In our experiments, some animals, if left to drink *ad libitum*, will take excessive quantities of this fluid. It also appears that there were no control animals that were intubated, stressful and invasive procedure. Would the authors enter into more detail of these points?

J.P. Kavanagh: Ideally each experimental group of rats would have a relevant control group, randomly selected from aged and weighed animals. They should be studied at the same time and housed under the same conditions. It is not clear if this was always the case. Did the different groups differ in their consumption of EG-containing water? Did the control rats receive placebo intubations?

Authors: All rats were of the same age and weight. They were housed under the same conditions and for the same period of time. The rats were fed standard chow in form of pellets. The rats were individually housed, the EG intake was not followed. We observed that all rats, fed either the standard chow or the CID of vitamin D_3 and 0.5% EG, consumed on the average 40 ml water each day. In pilot experiments, we found that the rats drank excessive amounts of EG containing water, if we increased the EG concentration in the drinking water to 0.75%, 1.0, or 1.5%. Hence, in this series of dietary

experiments, the daily ration of drinking water was limited to 40 ml. The control rats did not receive placebo intubations. As far as we could verify, the animals tolerated the intubations were very well. It appeared essential, however, that the intubations were performed by a trained biotechnician.

R.L. Hackett: In our laboratory, we have found that kidney/papilla oxalate content needs to be normalized by either protein or by weight. For one reason, since the papilla tissue is so small, alterations of its values are often masked. We find that the kidneys of these animals often become enlarged as well and that lyophilization before digestion is useful in determining true alterations in oxalate content. We have found that expressing [Ox]/kidney is not necessarily useful information. Would you like to comment on this point?

Authors: Indeed, biochemical determinations are normally related to the amount of protein. There are two reasons why we abandoned this approach. First, the oxalate determinations were performed on kidney digests, produced by proteinase K treatment. Protein determinations on such digests may not be reliable. Secondly, in the kidney crystal deposition gives rise to an enlargement of the interstitium, predominantly caused by an increase of the number of interstitial cells and by an increase of the interstitial matrix. Thus, it is likely that the amount of kidney protein is higher, if the number of interstitial crystals is increased. Both drawbacks are avoided if the oxalate determinations are related per whole kidney. We also agree that the excision of papillary tissue is inaccurate. However, one must realize that, in the papilla, most of the oxalate is present in small stones. This means that in the papilla, the amount of oxalate varies more according to the absence or presence of small stones than to the changes in the amount of protein. Thus, the distribution of oxalate over the papilla and the remainder of the kidney seems to be less dependent on the protein ratios of both compartments.

J.P. Kavanagh: It is not clear to me how many rats were used in the different experiments. It is also not clear what the control data represent in the Figures 3, 5, 6, and 7.

Authors: In total, 33 rats were used. Twenty four rats were used in the time course study, in which the calcium and oxalate response of Wistar and SD rats was compared (Table 1). For the EG concentration studies, nine rats were used, three for each group (group I-III). Since, at day 0, all rats were fed the standard chow, in this dietary study, the experimental rats of day 0 were the control group (open bars in Figs. 3, 4, 5, 6 and 7).

J.P. Kavanagh: To say that in Figure 1, during the first 3 weeks, all rats showed reduced calcium excretion does not fit with the finding in this figure that two samples during this time period showed calcium excretion within the range quoted for the controls. What was the oxalate range of the control animals? Were there any real changes in excretory patterns over time observed for the controls?

Authors: As shown in Table 2, the Wistar and SD rats, fed the standard chow, showed no prominent differences in calcium excretion. These data have a mean of 18.5 $\pm$ 8.6 μmol/24 h (range: 7.3-39.1 μmol/24 h). If in this experiment, for each point of time all data of the CID-fed Wistar and SD rats are pooled a lower calcium excretion is calculated at day 6 and 14, although not yet significant in the Wilcoxon matched-pairs test ($p < 0.07$).

E.J. Nouwen: Does the observation that the calcium excretion is reduced after CID indicate that vitamin D_3-stimulated calcium uptake in the experimental rats might be lower than the normal calcium uptake in the control rats? Also, although the authors mention that the decrease in body weight (which might better be accurately specified) was especially evident in the animals treated with the highest EG dose of 1 and 1.5%, this appears not to be correlated with the urinary calcium excretion data presented in the Figures 1A and 3A, which are similar for all experimental rats. Has a possible interference of oxalate ions with the determination of calcium been investigated methodologically?

Authors: In experimental rat nephrolithiasis, the urine is supersaturated with calcium and oxalate. Precipitation of the CaOx crystals decreases the urinary concentration of free calcium and oxalate. This decrease is most obviously noticed in the calcium concentration, since, compared with oxalate, the urinary concentration of this ion is low. We performed also a control experiment in which we investigated the effect of vitamin D_3. Compared with 0.5% EG alone, we found that vitamin D_3 + 0.5% EG does not enhance the calcium excretion (Table 4). Apparently, the administration of vitamin D_3, which has been reported to enhance the intestinal absorption of calcium, did not compensate for this decrease in urinary calcium.

H.G. Tiselius: To further emphasize the importance of the present findings, I would like to read exactly what conclusions the authors have drawn from these experiments with respect to the usefulness of this animal model. The impression I get is that, in addition to the unphysiologic concentrations of oxalate necessary for crystallization, there appears to be toxic effect on the kidney that are so pronounced that the model seems useless in studying stone formation. Do you agree?

Table 4. The effect of vitamin D_3 on the urinary excretion of calcium and oxalate in Wistar rats. In this study rats were fed a CID of 1.5% EG or a CID of vitamin D_3 + 1.5% EG . At day 0 the rats were fed the standard diet. Data are expressed as μmol/24 h (mean $\pm$ s.d.; n=3).

Experimental day	0	6	4	21	28	42
1.5% EG						
Oxalate excretion	7.7 $\pm$ 1.3	103.7 $\pm$ 24.9	34.7 $\pm$ 34.0	115.3 $\pm$ 26.7	114.7 $\pm$ 21.9	98.0 $\pm$ 36.1
Calcium excretion	26.7 $\pm$ 14.9	10.0 $\pm$ 3.3	2.3 $\pm$ 0.5	8.7 $\pm$ 0.9	8.3 $\pm$ 0.5	6.7 $\pm$ 2.4
Vitamin D_3 + 1.5% EG						
Oxalate excretion	8.7 $\pm$ 0.5	100.3 $\pm$ 42.7	0.0 $\pm$ 41.8	85.3 $\pm$ 21.0	62.7 $\pm$ 6.9	77.7 $\pm$ 11.9
Calcium excretion	23.7 $\pm$ 20.8	9.3 $\pm$ 2.9	8.0 $\pm$ 4.6	13.0 $\pm$ 11.3	7.7 $\pm$ 1.3	11.3 $\pm$ 2.1

Authors: We agree that the rat, as model for nephrolithiasis, is based upon the creation of a rather unphysiologically high concentrations of oxalate in urine. However, in an accompanying paper [13], we show that high oxalate concentration, on itself, does not lead to any kidney damage. Specifically, it was found that the retained crystals give rise to morphological and cytochemical changes, including glomerular damage, enlargement of the interstitium, tubular dilatation, increase of mitotic activity of interstitial and tubular cells, and changes in the staining pattern of Tamm-Horsfall protein. As such, this rat model is suitable to study, *in vivo*, the interaction of the retained crystals with the surrounding interstitial and tubular cells. In the renal interstitium of rats, four cell types have been described [48], and in preliminary experiments, we found that nearly all interstitial crystals are surrounded by macrophages [13]. Therefore, the present rat model is preeminently suitable to study the interaction of interstitial cells, including macrophages, lymphocytes, dendritic cells and fibroblasts, with interstitial and papillary crystals and to study the contribution of these cells to kidney damage and regeneration.

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