Scanning Microscopy Vol. 13, No. 2-3, 1999 (Pages 299-306) 0891-7035/99$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

IMPACT OF AMMONIUM CHLORIDE ADMINISTRATION ON A RAT ETHYLENE GLYCOL UROLITHIASIS MODEL

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(Received for publication March 12, 1996 and in revised form May 11, 1997)

Abstract

Ethylene glycol administration is a common method for the induction of experimental rat urolithiasis. This drug has been used alone or in combination with other drugs such as ammonium chloride for the study of kidney calcium oxalate crystal deposition. The reported crystal deposition rates with this model are controversial. The differences in crystal deposition rates may be because of varying concentrations of ethylene glycol and ammonium chloride administrated, the treatment period, and the composition of the diet. In this study, the rats were divided into four groups: 0.75% ethylene glycol alone or 0.75% ethylene glycol plus 0.5%, 0.75%, or 1.0% ammonium chloride for 1 to 4 weeks on an Agway R-M-H 3000 diet. All rats developed significant crystalluria; the extent of crystalluria was similar among the groups. Only when the ammonium chloride concentration was $\geq$ 0.75%, did all the rats develop kidney calcium oxalate crystal deposition. We conclude (1) that ethylene glycol administration is a good model for the study of crystalluria and hyperoxaluria in the rat, and (2) that at least 0.75% ammonium chloride is also necessary for kidney crystal deposition when rats are fed an Agway R-M-H 3000 diet.

Key Words: Ethylene glycol, ammonium chloride, calcium oxalate, kidney stones.

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Introduction

The most common human kidney stone is composed of calcium oxalate (CaOx) [1]. Rat experimental models of CaOx urolithiasis, induced by ethylene glycol (EG) alone, or in combination with other drugs such as ammonium chloride (AC), are often used to study the pathogenesis of kidney crystal deposition [2-4, 6, 9-13]. Ethylene glycol is a metabolic precursor of oxalate. Administration of EG to rats results in hyperoxaluria, CaOx crystalluria, and occasional deposition of CaOx crystals in the kidney. However, various crystal deposition rates have been reported with EG. Boevé's group found that none of the rats developed renal crystal deposition when treated with 0.8% EG for up to 24 days [2]. Khan reported crystal deposition rates of 16.6% and 50% when rats were treated with 0.5% and 0.75% EG, respectively, for 24 days [7]. Lee *et al.* found a 71.4% kidney crystal deposition rate when rats were treated with 0.5% of EG for four weeks [11]. Ammonium chloride ingestion, which induces metabolic acidosis, has been used in conjunction with EG ingestion to promote the deposition of CaOx crystals in rat kidneys. At a dose of 1% [2] or 2% [8] AC in combination with EG, all rats form kidney CaOx depositions within 4 to 7 days.

The purpose of the current study was to systematically investigate the doses and effects of AC ingestion on urinary chemistry, CaOx crystalluria, and kidney CaOx crystal deposition, in the rat EG urolithiasis model.

Materials and Methods

Male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), weighing 225 to 275 g at the start of the experiments, were used for the study. The rats were divided into four groups depending on the amount of AC ingestion: **Group 1**: 0.75% EG alone for 28 days; **Group 2**: 0.75% EG plus 0.5% AC for 28 days; **Group 3**: 0.75% EG plus 0.75% AC for 7 days; and **Group 4**: 0.75% EG plus 1.0% AC for 7 days. There were 6 rats each in Groups 1, 3 and 4, and 3 rats in Group 2. Our preliminary studies showed that with either 0.75% or 1% AC administration, 6 out of 9 rats (67%) died within two weeks, and all had extensive kidney

crystal depositions. Therefore, only one week of treatment was selected for rats receiving these higher doses of AC (Groups 3 and 4). Both EG and AC were given through the drinking water (tap water) and the access to water was *ad libitum*. An Agway R-M-H 3000 diet (Agway Inc., Syracuse, NY) was used for the entire length of the study. This diet contained 22.5% protein, 0.97% calcium, 0.85% phosphorus, 0.21% magnesium, 0.44% sodium, 0.95% potassium, Vitamin A 20,229 I.U., and Vitamin D_3 1,045 I.U. The rats were weighed weekly. Water consumption was determined before treatment and twice a week after treatment had begun. All rats were sacrificed at the end of the treatment period for histological studies.

Urine collection and analysis

Urine samples were collected from each rat one day before the treatment was begun and one day before sacrifice, using a metabolic cage. A 3-hour fresh urine sample was first collected in the morning without any preservatives for crystalluria analysis. For microscopy, 1 ml of the fresh urine sample was centrifuged at 3,000 rpm (revolutions per minute) for 10 minutes, and then 950 μl of the supernatant was discarded. Ten microliters of the vortex-mixed sediment was then transferred to a hemocytometer. The type and number of the crystals were identified and counted using a Olympus CH-2 light microscope (Olympus America, Inc., Lake Success, NY). The severity of crystalluria was graded as follows: "-" no crystals; "+" 1 to 5 crystals per high power field (x400); "++" 6 to 20 crystals per high power field; and "+++" > 20 crystals per high power field. Additionally, a filter technique and chemical analysis was also used in four rats, two each from Groups 1 and 3, to quantitatively identify crystalline material in the sediments. Briefly, 1 ml of urine sample was filtered through a 0.45 μm Millipore filter (Millipore Corporation, Bedford, MA), and the filter was then rinsed with 2 ml of distilled water. The filtration was assisted by vacuum suction. The crystals obtained by the filter were dissolved in 2 ml of 0.05 M HCl solution at 4°C overnight. Calcium (Ca), oxalate (Ox), and phosphate (PO_4) concentrations in the HCl solution were then determined by methods described below for urinary chemistries.

After the 3-hour fresh urine sample collection, a 24-hour urine sample was also collected. The 24-hour urine sample was collected at room temperature with thymol (Sigma Chemical Co., St. Louis, MO) as preservative. Twenty-four hour urine pH was measured by an Accumet 1003 pH meter (Fisher Scientific, Pittsburgh, PA) immediately after the collection. The urine was filtered with Whatman #1 filter (Whatman International Ltd., Maidstone, Kent, UK; distributed by Fisher Scientific) and stored at -20°C until further analysis. A portion of the urine was acidified with concentrated HCl to pH < 3.0 for the Ox assay. Urinary Ca, PO_4, uric acid (Ua), magnesium (Mg), sodium (Na), potassium (K), and creatinine (Cr) were measured in the Hospital Laboratory using a Hitachi 747 autoanalyzer (Boehringer Mannheim Corp., Indianapolis, IN); citrate (Cit) and Ox were measured in our Urolithiasis Research Laboratory using enzymatic methods (Sigma). The urinary CaOx relative free energy (ΔG) and ion-activity product (AP) were calculated using EQUIL II computer software [15] based on the 9 urinary parameters measured. AP was presented as $-\log_{10}$ of the ion-activity product. Our unpublished data indicates (n = 7) that rat urinary oxalate excretion after one week of 0.75% EG administration is similar to values obtained after 4 weeks of EG administration.

Kidney harvest and measurements

The rats were sacrificed in a 100% CO_2 chamber, and both kidneys were removed and placed in crushed ice immediately. After the kidneys were weighed, the right kidney was fixed with 10% buffered formalin, embedded in paraffin, cut to 5 μm section for slides, and stained with hematoxylin and eosin. The slides were examined with a polarizing light microscope. The number of crystal deposits were counted over 6 low power fields (x100), 2 each from the cortex, medulla and papilla; crystal deposits were graded (using the mean value) as: "-" no crystal deposition; "+" < 5 crystals per low power field (x100); "++" 6-10 crystals per low power field; and "+++" > 10 crystals per low power field.

The left kidney was used for kidney tissue Ca, Ox and PO_4 measurements. The kidney tissue was homogenized by a Sonifier II Cell Disrupter (Branson Ultrasonics Co., Danbury, CT) and dissolved in 1 M HCl overnight. After centrifugation, the supernatant was removed and neutralized with 1 M NaOH. Calcium, oxalate, and phosphate were determined by the methods described above for urinary chemistries. To further verify the composition of the crystals, X-ray diffraction was done by a Siemens D-5005 Diffraction System (Siemens Analytic X-ray Systems, Inc., Madison, WI) fitted with parallel-beam optics and a silicon X-ray detector in two of the experimental rat kidneys with crystal deposits. Briefly, fresh kidney tissue was cut into several 0.5 mm thick slices, placed on a microscope glass slide, and stored at -20°C until use. The kidney tissue sample was transferred onto a quartz plate and the diffraction pattern determined using Cu Kα radiation at a fixed incidence angle of 5° (step interval 0.02°) and a step time of 2 seconds per step by scanning the detector over an angular range of 5° to 65° (measured from the incident beam direction). A Siemens/Socabim Diffrac-AT software package was then used to compare the diffraction pattern obtained to that from standard diffraction patterns of known crystals.

Table 1. Rat body weight (g) before and after treatment with ethylene glycol and ammonium chloride (see text for description of various groups). SE = standard error.

	Group 1 n = 6	Group 2 n = 3	Group 3 n = 6	Group 4 n = 6
Pre-treatment; mean ± SE	241.2 ± 6.8	245.3 ± 3.8	258.7 ± 3.5	250.7 ± 2.3
After-treatment; mean ± SE	353.0 ± 10.7	360.0 ± 26.6	232.2 ± 10.1[1]	229.7 ± 14.6[1]
Total body weight gain; range	68 to 171	85 to 160	2 to -53[1]	20 to -76[1]
Daily body weight gain; mean ± SE	3.16 ± 0.28	5.73 ± 1.15	-3.76 ± 1.09[1]	-3.02 ± 2.02

[1]$p < 0.05$ versus Group 1.

Table 2. Twenty-four hour (24h) urinary data (see text for description of various groups).

	Pre-treatment n = 16	Group 1 n = 6	Group 2 n = 3	Group 3 n = 6	Group 4 n = 6
Volume; ml/24h	7.55 ± 0.85	15.90 ± 4.97	25.33 ± 12.42	19.67 ± 1.64[1]	16.60 ± 3.55
pH	7.28 ± 0.22	7.17 ± 0.26	6.41 ± 0.34	6.18 ± 0.28	5.75 ± 0.05[1,2]
Oxalate; mmol/24h	3.42 ± 0.36	60.60 ± 8.64[1]	21.13 ± 10.41	30.48 ± 3.62[1]	9.98 ± 4.00[2]
Calcium; mmol/24h	36.59 ± 5.51	7.85 ± 1.16[1]	17.80 ± 7.09	4.88 ± 1.15[1]	25.85 ± 11.16
Phosphate; mmol/24h	166.0 ± 41.5	420.6 ± 47.4	432.6 ± 54.6	576.5 ± 26.1[1]	622.6 ± 77.5[1]
Uric Acid; mmol/24h	7.18 ± 0.78	11.28 ± 2.54	7.63 ± 0.35	4.45 ± 0.59	5.80 ± 0.68
Citrate; mmol/24h	37.09 ± 5.18	31.18 ± 2.83	3.77 ± 1.13	1.82 ± 0.90[1,2]	1.08 ± 0.65[1,2]
Magnesium; mmol/24h	91.0 ± 18.4	128.5 ± 41.4	143.5 ± 63.7	89.6 ± 18.4	168.7 ± 34.5
EQUIL ΔG_{CaOx}	3.15 ± 0.24	3.17 ± 0.34	1.75 ± 0.59	1.60 ± 0.36[1]	1.45 ± 0.14[1]
EQUIL AP_{CaOx} *	4.15 ± 0.08	4.15 ± 0.11	4.62 ± 0.20	4.67 ± 0.12[1]	4.72 ± 0.05[1]

[1]$p < 0.05$ versus pre-treatment [2]$p < 0.05$ versus Group 1 *AP = $-\log_{10}$(ion-activity product)

Statistical analyses

The statistical analyses were done using a StatView 4.01 computer software (Abacus Concepts, Inc., Berkeley, CA); Krustal-Wallis and Mann-Whitney methods (part of StatView software) were used for the multigroup and two group comparisons, respectively.

Results

General information

In Groups 1 and 2, the rats were active and healthy throughout the study period. Their body weight increased from 68 to 171 g after four weeks of treatment. The daily water consumption was similar for both groups: 25 to 35 ml/day, which was the same as before treatment. Most of the rats in Groups 3 and 4 lost body weight after one week of treatment. Their daily water consumption was only 10 to 20 ml, and the rats generally became lethargic 4 to 7 days after treatment had begun (Table 1). No rats died during the course of the study.

Urinary chemistry

The 24-hour urinary pH decreased as the AC ingestion increased (Group 1 to Group 4); a significant decrease in pH occurred in Group 4 (Table 2). Urinary citrate also correspondingly decreased in all AC treated rats; in some Group 3 and Group 4 rats, urinary citrate was undetectable. There was a significant increase in phosphate excretion in all groups. Urinary calcium excretion decreased after treatment, but there was no change in urinary magnesium or uric acid excretion. Oxalate excretion increased 3 to 18 times after EG ingestion; the highest increase occurred in the pure EG treated rats (Group 1). The highest urinary ΔG_{CaOx} and AP_{CaOx} also occurred in Group 1 (Table 2).

Crystalluria

All rats had extensive microscopic CaOx crystalluria (+++) after treatment, except one rat in Group 1 who had only struvite crystals. The most common crystals were calcium oxalate dihydrate (COD). There were no differences between the groups. No calcium phosphate crystals were

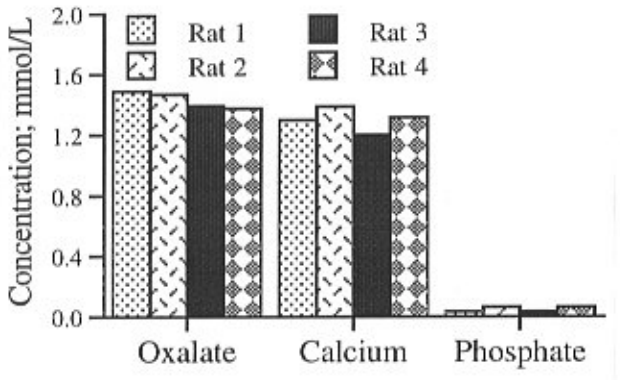

Figure 1. Chemical analysis results of the sediment Ox, Ca, and PO_4 using the filter technique. Rat 1 and 2 were treated with 0.75% EG for 4 weeks. Rat 3 and 4 were treated with 0.75% EG and 0.75% AC for 1 week.

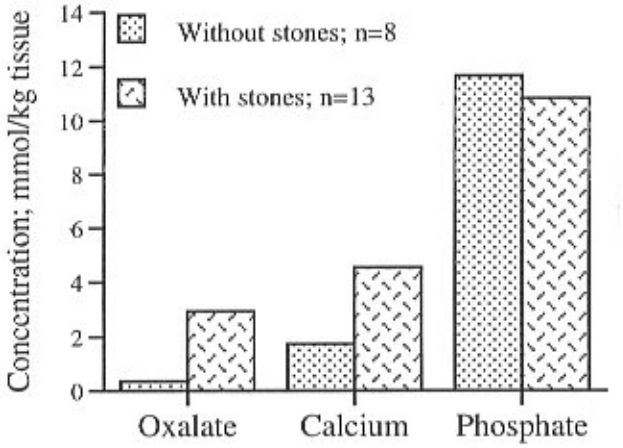

Figure 2. Wet tissue Ox, Ca, and PO_4 concentrations in rat kidneys with and without crystal deposits ($p < 0.05$ for Ox and Ca).

found in any of the groups. Figure 1 shows the results of crystalluria analysis by the filter technique in 4 rats. All these rats had "+++" microscopic crystalluria. Two rats from Group 1 (Rat 1 and Rat 2) had no kidney crystal deposits, and the other two from Group 3 (Rat 3 and Rat 4) had "+++" crystal deposition in the kidney. However, both sediment Ca and Ox were similar among the rats (Fig. 1).

Kidney tissue analysis (Table 3)

Kidney tissue pathological examination showed that all rats in Groups 3 and 4 had crystal deposits in the kidney. The crystal density in Group 4 was slightly higher than that of Group 3. Only one rat in Group 1 (none in Group 2) developed kidney crystal deposits. The CaOx crystals seemed to have a uniform distribution throughout the kidney. The relative kidney weight (% of the body weight) was significantly higher in kidneys with crystal deposits. Wet kidney tissue Ca and Ox concentrations in Groups 3 and 4 rats were 2 to 3 times higher than that of Groups 1 and 2, but the phosphate concentrations were similar in all groups. There was a good correlation between wet tissue Ca and Ox concentrations and the presence of kidney crystal deposits (Fig. 2). When compared to kidneys without CaOx crystals, the wet tissue Ca and Ox concentrations of kidney with CaOx crystals were significantly higher ($p < 0.05$). The X-ray diffraction spectrum from two crystal deposits showed strong CaOx crystal peaks, 85% of them being CaOx monohydrate. No calcium phosphate crystalline peaks were identified.

Discussion

Ethylene glycol ingestion has been widely used as an experimental model for the study of nephrolithiasis. However, when EG is used alone, kidney crystal deposition can be quite variable [2, 13]. To achieve a uniformly high rate of kidney crystal deposition, other drugs such as ammonium chloride [2, 3, 8], vitamin D_3 [6], and gentamicin [5], or a magnesium deficient diet have been used in conjunction with EG. Among these drugs, AC has been used most widely.

Ammonium chloride ingestion induces urinary acidification. The reported kidney CaOx crystal deposition rate in male rats with this animal model are controversial. Table 4 lists some of the reported crystal deposition rates. When rats were treated with EG alone with similar doses and treatment period, kidney crystal deposition occurred anywhere from 0% to 75%. These differences may be explained, in part, by the composition of the rat diet. For example, the AIN-76 rat diet which has a much lower magnesium content compared to the other commonly used rat diets, may itself cause calcium phosphate kidney crystal deposition [8, 14]. On the other hand, when rats are treated in conjunction with 1% or 2% AC, almost all rats demonstrate deposition of the CaOx crystals in the kidney within a week. At doses of $\geq$ 1% of AC, however, the rats become sick, stop eating, and appear lethargic after one week of treatment [8]; 61% of the rats die within 4 weeks [13]. Our preliminary experiments also demonstrated a 67% mortality within 2 weeks when rats were treated with 0.75% or 1% AC. Rats treated with AC alone have been reported to be metabolically indistinguishable from untreated control rats [2].

In the present study, we examined the effect of various AC doses in combination with 0.75% EG ingestion on urinary chemistry, crystalluria, and the deposition of CaOx crystals within the kidney. The rat chow used in our study was Agway R-M-H 3000. As shown in Table 1, when the dose of AC was $\leq$ 0.5% (Groups 1 and 2), the rats remained

Table 3. Kidney tissue data (see text for description of various groups).

	Group 1 n = 6	Group 2 n = 3	Group 3 n = 6	Group 4 n = 6
Kidney weights				
Right kidney; g	1.24 ± 0.07	1.40 ± 0.12	1.45 ± 0.05	1.26 ± 0.06
Left kidney; g	1.24 ± 0.09	1.29 ± 0.11	1.46 ± 0.04	1.23 ± 0.05[2]
Total; g	2.48 ± 0.15	2.69 ± 0.22	2.91 ± 0.07	2.49 ± 0.10[2]
% of body weight	0.70 ± 0.03	0.75 ± 0.01	1.26 ± 0.05[1]	1.10 ± 0.06[1]
Crystal deposits				
None	5	3	0	0
Crystals: +	0	0	1	1
++	0	0	4	2
+++	1	0	1	3
Wet tissue concentrations				
Oxalate; mmol/kg tissue	0.324 ± 0.051	0.415 ± 0.073	1.181 ± 0.126[1]	0.957 ± 0.191[1]
Calcium; mmol/kg tissue	1.523 ± 0.227	2.062 ± 0.079	3.711 ± 0.242[1]	4.708 ± 0.680[1]
Phosphate; mmol/kg tissue	11.91 ± 0.60	11.77 ± 0.44	9.28 ± 0.13[1]	12.19 ± 1.18

[1]$p < 0.05$ versus Group 1 [2]$p < 0.05$ versus Group 3

Table 4. Literature review of experimental ethylene glycol and ammonium chloride urolithiasis models in male rats.

Literature	Rat strain	Diet	Treatment	Period	Renal crystals*
Boevé *et al*. [2]	Wistar		0.8% EG; n = 2	24 days	0%
			1% AC; n = 2	24 days	0%
			0.8% EG + 1% AC; n = ?	4 days	100%
Khan *et al*. [7, 8, 10]	SD**	Purina	0.5% EG; n = 6	24 days	16.7%
			0.75% EG; n = 6	24 days	50.0%
			1% EG; n = 8	15, 29 days	75% and 50%
			0.5% EG + 2% AC; n = 4	7 days	100%
			0.75% EG + 2% AC; n = 4	7 days	100%
Lee *et al*. [11]	SD**	AIN-76	0.5% EG; n = 7	28 days	71.4%
Li *et al*. [12]	Wistar		1% EG; n = 12	28 days	62.5%
Lyon *et al*. [13]	SD**	Purina	1% EG; n = 13	28 days	23.1%
			1% EG + 1% AC; n = 6	28 days	83.3%

*Renal CaOx crystal deposition rate **Sprague-Dawley rats

active and gained weight. However, when the AC dose was $\geq$ 0.75%, the rats became sick, drank less water, and lost body weight after 4 to 5 days of treatment.

Ingestion of AC resulted in higher urinary acidification and a corresponding decrease in urinary citrate excretion. Urinary oxalate excretion increased 3- to 17-fold after EG treatment; both oxalate excretion and urinary CaOx supersaturation were the highest in the pure EG treated rats (Group 1). Urinary calcium excretion decreased in all treated rats with a seemingly negative correlation between urinary calcium and urinary oxalate excretion. To determine if urinary calcium excretion may be lowered in treated rats by retention of calcium salts or by calcium binding to other urinary macromolecules by the Whatman #1 filter, we measured the retained calcium by the filter in additional 16 rats (unpublished data). These studies showed that the Whatman #1 filter may retain up to 40% (mean = 26%) of the total urinary calcium and only 8% of the total urinary oxalate in rats treated with 0.75% EG for 2 to 4 weeks. The total calcium excretion (soluble + sediment) after treatment was only slightly lower than that of pre-treatment values.

The magnitude of crystalluria was similar in all four treatment groups. This data indicates that the severity of the CaOx crystalluria is primarily dependent upon the amount of EG ingestion. However, increased urine acidification by the addition of AC decreases urinary citrate excretion, which in turn may be responsible for increased deposition of CaOx crystals in the kidneys.

A semiquantitative assay (microscopic scoring system) and a quantitative assay (chemical analysis of wet kidney tissue) of the crystal deposits were used in this study to compare the severity of kidney CaOx crystal deposition among the various study groups. When AC concentration was $\geq$ 0.75% (Groups 3 and 4), all rats developed significant CaOx crystal deposition in the kidneys (83% of the rats had greater than 6 deposits per low power field). The severity of microscopic kidney crystal deposition correlated well with wet tissue Ca and Ox concentrations. As the tissue Ca and Ox concentrations correspondingly increased 2- to 3-fold, while the phosphate concentration remained the same, these crystal deposits were likely to be CaOx. Our X-ray diffraction studies confirmed this finding.

We conclude (1) ethylene glycol administration is a good model for the study of crystalluria and hyperoxaluria, and (2) that at least 0.75% ammonium chloride is also necessary for kidney crystal deposition when rats are fed an Agway R-M-H 3000 diet.

Acknowledgement

This work was supported in part by NIH grant No. 1 R29 DK45009-01A1.

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Discussion with Reviewers

S.R. Khan: The authors confirm results of earlier investigations. However, it is suggested that results of earlier studies are "controversial", which is not the case. When different protocols are used, or different diets are used, results are going to be different. Even when experiments are carried out in the same laboratory using similar protocols, results may sometimes come out differently. The authors have indicated that ethylene-glycol-induced CaOx crystal deposition in the kidney is not a model of CaOx kidney stone formation. Such a statement should not be made without providing reasons. The authors should provide a criterion for a kidney stone model. Would the authors consider a model where animals are on the verge of death as is the case when both ethylene glycol and ammonium chloride are administered, as a good model of stone disease? Would it be possible for any experimental or theoretical model to meet all the conditions?
Authors: As suggested by the reviewer, we agree that kidney CaOx crystal deposition and/or stone formation rate with the rat EG urolithiasis model are variable among the different research groups even when the EG doses and treatment period are similar. To develop a good animal model, the degree of crystalluria and/or crystal deposits in the kidney should be reproducible under similar experimental conditions. We agree with the reviewer that the animal diet is an additional variable that may play a important role in kidney stone formation. Because of the variability of lithogenicity obtained in experimental models with different protocols, this study is an attempt to systematically address the consequences of one additional variable, i.e., ammonium chloride. We also agree with the reviewer that EG plus AC is not a good model of stone disease. However, AC seems to be necessary to induce adequate renal crystal deposits. The optimal experimental model for the study of human idiopathic CaOx stone disease has still not been developed.

Y. Ogawa: Ethylene glycol administration is a common method for the experimental induction of urolithiasis in rats. However, vitamin B6 deficiency, magnesium deficiency, chloride depletion, and oxalate, glycolate, or glyoxylate administration, have so far also been tested for this purpose. The authors should review the historical background and explain why they chose this particular model. I do not agree with the use of this kind of unphysiological model (also vitamin B6 or magnesium deficiency) which leads to weight loss (body consumptive state) without adequate nutritional state. Various factors may be involved in the pathogenesis of urolithiasis in the model and the disease (ethylene glycol toxicity) itself does not seem to be a pure condition (isolated hyperoxaluria). A critical point lies in the fact that all rats lost body weight which may be associated with a deficiency of vitamin B6 or magnesium. The authors may state that the treatment time is too short to cause vitamin B6 deficiency, and that magnesium depletion is only related to apatite. However, this model can hardly be extrapolated to clinical urolithiasis. Do the authors want to use the model just for studying the morphology of crystalluria and the pathogenesis of hyperoxaluria? Or are they interested in treating crystalluria and hyperoxaluria using this model?
Authors: We agree with the reviewer that many rat CaOx urolithiasis models have been reported in the literature. To our knowledge, the EG model is the one that is used most widely. It is not our intention to compare the various models of urolithiasis. The purpose of this study was to only examine the effect of AC on the rat EG model. To our knowledge, there is no rat (or other animal) urolithiasis model that mimics human idiopathic CaOx stone formation. Our results indicate that rats treated with EG alone were active and gained body weight, and we did not find signs of EG toxicity within 4 weeks. With EG alone (Group 1) under the present experimental conditions, we believe this model can be used for the study of crystalluria and/or hyperoxaluria.

H.-G. Tiselius: Regarding the fact that the stone formation was better correlated with tissue concentrations of calcium and oxalate than urine concentrations, it would be interesting to know the authors' opinion on the mechanism of stone formation during EG+AC treatment.
Authors: The kidney tissue calcium and oxalate concentrations are similar in kidneys that do not develop crystal deposits compared to that of normal untreated rats (unpublished data). However, once CaOx crystal deposition occurs in the kidney, tissue calcium and oxalate concentrations increase. The tissue concentrations of calcium and oxalate thus serve as a quantitative estimation of renal CaOx crystal deposition. We think that urinary acidification induced by AC changes other urinary micro and/or macro molecular components quantitatively and/or qualitatively, such as decreased citrate excretion resulting in renal stone formation or crystal deposition.

W.C. de Bruijn: A confusing aspect is the total amount of AC ingested over the induction periods by the various groups of rats. My rough calculation indicates that the total ingested AC amount at the end of each experiment is higher in groups 1 and 2 as compared to groups 3 and 4 (28*35*0.5 versus 7*20*0.65 or 1.0, respectively). Could the authors please comment?
Authors: Ingested ammonium chloride will be either absorbed and metabolized and/or excreted in the feces by the rat. We have used AC as that traditionally used in experimental models (Group 1 rats were not given any ammonium chloride). While it is true that the total amount

of AC ingested in Group 2 is higher than in Groups 3 and 4, the impact on stone formation is determined on daily solute excretion and urinary ionic concentrations. The ingestion of AC is not expected to have a cumulative effect, rather a chronic metabolic acidosis that is accommodated by increased urinary acidification.

W.C. de Bruijn: Your (semi)quantification of the crystalluria is rather vague. According to the description in **Materials and Methods**, it allows in the hemocytometer, data to be acquired as crystal number/μm^3 urine/h. In your measurements in the kidney sections, measured in Table 3, you can acquire crystal data as number/μm^2 tissue, provided that one, in an additional step, converts the field of view into μm^2. Could you please add this information? Moreover, in the past, crystal localization has been differentiated into cortex, medulla or papilla, whereas you are taking the whole kidney as a reference. Was the aliquot that you measured, representative for the whole kidney? Was the COD identification made based on crystal shape?
Authors: The kidney crystal deposition scoring system used in this paper is widely accepted, based on the number of crystals per defined magnification. Unless an image analysis system is used, which gives more accurate information such as crystal number, mean crystal size and size range, the mean crystal volume per unit kidney tissue, to convert the renal crystal data to crystal number per μm^2 will give no additional information than that provided by the scoring system because the crystals are not evenly distributed and crystal size may vary from 2 µm (the detection limit for light microscope used in this study) up to 50 µm or more. The same argument applies to crystal counting methods in the urine. For renal and urinary crystal quantitative analysis, a new chemical analysis approach has been used. As we did not find any favoured site for crystal deposition, i.e., crystal deposits were similar in the cortex, medulla, and papilla, we chose to use the while kidney as the reference. COD crystal identification was based on crystal shape by polarized microscopy.

W.C. de Bruijn: In the left kidneys, the chemical parameters were obtained after tissue digestion in 1 M HCl. Is this digestion complete (this was not always the case in our hands)? Are the crystals completely dissolved after that treatment (this was also not always the case in our hands). Therefore, is it possible that the values that you measured were influenced by the duration of the "digestion"?
Authors: The kidney tissue Ca, Ox and PO_4 concentrations in kidneys without crystal deposits (Groups 1 and 2 in this study) were similar to normal rats (unpublished data). For kidneys with crystal deposits, the tissue calcium and oxalate concentrations correlated directly with the amount of the crystal deposits. Therefore, we believe that the "digestion" was almost complete. This is the reason for comparing the kidney tissue calcium, oxalate and phosphate concentrations with and without crystal deposits (Fig. 2).