Mild Tubular Damage Induces Calcium Oxalate Crystalluria in a Model of Subtle Hyperoxaluria: Evidence that a Second Hit Is Necessary for Renal Lithogenesis

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Environment and diet have a major role in calcium nephrolithiasis by affecting urine saturation, but this is not enough to cause lithogenesis; the crystals must adhere to the tubular epithelium (TE), but it is hard to say how environment and nutrition may be involved in this step. The hypothesis that TE damage (known to enhance crystal attachment) is lithogenic in mild hyperoxaluria was tested. Mild hyperoxaluria was induced in male Wistar rats using ethylene glycol (EG; 0.5% in water) for 21 d, and TE damage was induced by intraperitoneal administration of hexachloro-1:3-butadiene (HCBD; an industrial nephrotoxin) at 10, 25, and 50 mg/kg body wt on days 7 and 14. These EG and HCBD concentrations were chosen to span from suboptimal to very low doses as far as effects on crystalluria and TE damage are concerned. Enzymuria, proteinuria, oxaluria, crystalluria, and renal pathology were investigated. All HCBD dosages induced crystalluria in mildly hyperoxaluric rats, but no intrarenal crystals were found. EG alone induced very mild hyperoxaluria but no damage to the renal tubule observable on transmission electron microscopy, and it did not cause crystalluria or intrarenal crystals. HCBD with the concomitant administration of EG caused apoptosis of the TE at the two highest dosages after the second injection. Apoptosis did not correlate with crystalluria. A TE toxin is needed for crystallogenesis to occur in borderline metabolic conditions. It may take more than just a metabolic predisposition for calcium nephrolithiasis to occur, and the second hit could come from an environmental pollutant such as HCBD.

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Idiopathic calcium nephrolithiasis (ICN) is a multifactorial disease that involves various causal factors, which may be dietary (intake of proteins, calcium, sodium, lipids, etc.) (1), environmental (ambient and occupational living conditions, lifestyle, exposure to sun, water quality, etc.) (2), and genetic (3). The case for a nutritional and environmental cause for stones is based on the following observations: (*1*) Renal stone disease was uncommon before the turn of the 20th century, but its incidence has steadily increased; (2) stone disease is more common in affluent communities (4), and a positive correlation exists between per capita income and per capita expenditure on food; and (*3*) a number of relationships between dietary/environmental factors and urinary risk factors for stones have been reported (4–6).

A body of research has investigated the role of environment and diet, concentrating mainly on their effect on urinary risk factors for stones (4–6), but the pathophysiology of renal lithogenesis makes it difficult to see these nutritional and environmental factors as being sufficient to cause a stone. Indeed, renal stone formation takes a number of predisposing events, including urine supersaturation, microcrystal formation, crystal growth, and the growth of the stone. Moreover, the retention of microcrystals by the urothelium is considered an essential, critical step in the growth of renal calculi. Considering urinary flow rates and the anatomic dimensions of the nephron, unless individual crystals can attach to a site within the nephron, they presumably would be washed away in the urine before they could grow large enough to support stone formation (7). Not all of the events that lead up to stone formation are clearly dependent on nutritional and environmental factors, and it is particularly hard to see how they can influence the crystal's attachment to the tubular lumen. The binding of crystals to kidney epithelial cells in culture has been shown to be enhanced by previous cell injury (8). Among the potential causes of such injury, hyperoxaluria has been explored and found to be capable of damaging tubular cells and increasing crystal adhesion (9,10). The level of oxalate that is needed for them to do so can be reached only in very extreme conditions of primary and secondary hyperoxaluria, however. Aminoglycosides also have been investigated (11,12) and shown to induce severe crystalluria and crystal entrapment in the kidney, but they hardly can

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have much of a role *in vivo* because they are very rarely used in healthy young adults (the most likely candidates for stone formation). Moreover, they were used in the model at very high doses and for a prolonged period of time. Other drugs are known to be toxic for the tubule (*e.g.*, acyclovir, cisplatinum), but they also are very rare in the clinical history of patients with ICN. Another group of nephrotoxic substances are the industrial toxins—cadmium, mercury chloride, organic solvents, and halogenated alkenes such as hexachloro-1:3-butadiene (HCBD)—but the role of such agents in ICN has never been considered.

The working hypothesis that was explored in this study is that nephrotoxins (*i.e.*, industrial pollutants) damage the renal tubular epithelium (TE), promoting calcium oxalate (CaOx) crystal adhesion and thus favoring crystal retention and lithogenesis, and that this can coincide with extremely modest renal lesions that are induced by a very low dosage of the toxin, such as in conditions that resemble a "normal" human environmental exposure to the toxin.

A rat model was developed to investigate this hypothesis, based on the models used to study CaOx crystallogenesis (by inducing chronic hyperoxaluria by administering oral ethylene glycol [EG]) and nephrotoxicity induced by industrial toxins (by means of the intraperitoneal administration of HCBD, a typical nephrotoxin [13]). HCBD is not purely an experimental nephrotoxin, because it is widespread in the biosphere, especially in polluted industrial areas (14–18). The Wistar rat strain was chosen to develop the model instead of the more frequently used Sprague-Dawley rat because it responds to EG in the same way and with much the same sensitivity in terms of renal crystal formation (19), but we had a better knowledge of its renal susceptibility to HCBD.

Materials and Methods

Albino male Wistar rats (200 ± 10 g; Harlan, San Pietro al Natisone, UD, Italy) were placed in plastic cages, under controlled of temperature (20°C) and humidity (40 to 60%) conditions, with a dark-light cycle of 12 h; they were fed a standard rodent diet (Nuova Zoofarm, Padova, Italy) and allowed to drink tap water *ad libitum* from time −7 d to time 0 d. They were allocated to four different groups: (*1*) Group 1 ($n = 15$) received EG, (*2*) group 2 ($n = 15$) received EG and HCBD 10 mg/kg body wt, (*3*) group 3 ($n = 15$) received EG and HCBD 25 mg/kg body wt, and (*4*) group 4 ($n = 15$) received EG and HCBD 50 mg/kg body wt.

The study schedule is shown in Figure 1. Twenty-four-hour experimental periods are indicated as T followed by a number, from T7 to T21. Each period started at 1 p.m. and finished at 1 p.m. the next day. At the beginning of T7 and T14, immediately after the urine collection of the previous period, HCBD/corn oil was injected intraperitoneally.

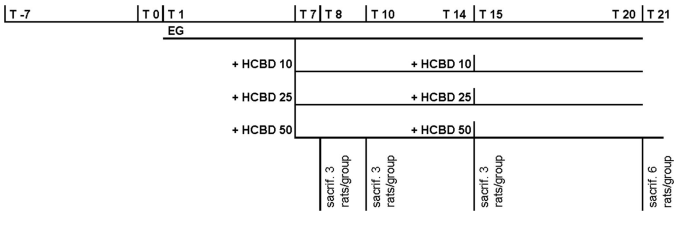

Figure 1. Experimental design (see text for details).

When rats were killed, this was done at the beginning of the period, after completion of the urine collection of the previous period. Urine was collected at the end of the periods indicated, from 8 a.m. to 1 p.m. At T0, the rats were housed in metabolic cages for urine collection. EG was administered orally in tap water (0.5% vol/vol) from T1 throughout the experiment for 3 wk; on T7 and T14, HCBD (purity > 97%; Fluka, Buchs, Switzerland) was administered intraperitoneally to the rats in groups 2, 3, and 4; the HCBD was dissolved in corn oil (5 ml/kg body wt). The rats in group 1 were given corn oil alone. The rats were killed by exsanguination under diethyl ether anesthesia on T8 (3 rats/group), T10 (3 rats/group), T15 (3 rats/group), and T21 (all remaining rats).

From T6 to the end of the study, the rats were housed in individual metabolic cages. Five-hour samples of voided urine were collected on T0, T6, T7, T8, T9, T10, T11, T13, T14, T15, T16, T17, T18, and T20. Because we found an excellent correlation for both urinary calcium and oxalate, between their respective values obtained from 24-h (expressed as μmol/24 h) and 5-h morning collections (expressed as μmol/mmol creatinine; calciuria $P < 0.01$; oxaluria $P < 0.01$), we chose to perform only morning urine collections for the sake of simplicity. pH was determined immediately after collection, and samples were centrifuged at 2000 rpm for 3 min. In each sample, the supernatant urine was discarded, except for 500 μl in which the sediment was resuspended. A few drops of the suspension then were put on a slide and examined immediately under the light microscope at ×40. Ten different, randomly selected fields were observed for each sample. CaOx crystals were optically recognized and counted. The mean of 10 measurements per sample then was calculated.

Proteinuria was determined by the Pesce and Strande method (20) and expressed as a ratio to creatinine. Urine oxalate was determined enzymatically using the oxalate oxidase method (Sigma kit, Milan, Italy), and calcium was determined by atomic absorption; angiotensin-converting enzyme (ACE) and N-acetyl-β-glucosaminidase (NAG) were expressed per millimole of creatinine (Jaffe method), as described elsewhere (21).

Before the rats were killed, the right kidney was removed and cut into two parts; the first was fixed in 10% formalin in 0.2 M phosphate buffer for optical histology, and the second was cut into small tissue blocks and fixed in Karnovsky fixative for electron microscopy. For optical histology, paraffin-embedded 5-μm-thick sections were stained with hematoxylin and eosin and periodic acid-Schiff and examined using plain and polarized light to look for crystals in the kidney. Sections also were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) according to the manufacturer's recommendations (Boehringer Mannheim, Mannheim, Germany). The apoptotic index was automatically calculated as the percentage of TUNEL-positive cells of the total number of renal cells per mm^2 (morphometric analysis using the Image Pro-Plus 5.1; Media Cybernetics, Silver Spring, MD). For transmission electron microscopy (TEM), ultrathin sections were cut from Epon-embedded tissue blocks, counterstained with uranyl acetate followed by lead citrate, and then examined with a Hitachi H-700 electron microscope.

Statistical Analyses

Statistical analysis on the data involved one-way ANOVA with the Bonferroni correction for group comparisons and ANOVA for repeated measures.

Results

Studies on Urine

The oral administration of EG 0.5% induced no significant CaOx crystalluria during the 3-wk period of the study from T1

to T20 (group 1) but did raise oxaluria up to 200 μmol/mmol creatinine (Table 1). At T6, oxaluria in the four experimental arms was significantly higher than that at the baseline (T0; P = 0.001 to 0.000) and remained stable thereafter until T20 (Table 1). There was no statistically significant difference in oxaluria among the four experimental groups at any point during the study, from T6 to T20. Treatment with EG 0.5% alone (group 1) also induced no statistically significant variation in proteinuria and enzymuria throughout the study (Figure 2).

In rats that given EG, HCBD 10 mg/kg body wt induced no abnormal proteinuria or NAG enzymuria after both injections (group 1) but only a modest ACE enzymuria (Figure 2B). At the higher doses (25 and 50 mg/kg body wt, groups 3 and 4, respectively), it did cause a transient, dose-dependent rise in proteinuria after both injections (which regressed in 3 d); a transient, dose-dependent increase in ACE urinary excretion; and a transient increase in NAG excretion (at the highest dose, 50 mg/kg body wt; Figure 2).

Calciuria and urine pH were unaffected by any of the treatment and remained constant throughout the trial from T0 to T20 (Table 1). Crystalluria, mainly consisting of CaOx dihydrate, appeared in all rats in groups 2, 3, and 4 as of the first 24 h after each HCBD administration and gradually faded within the next 3 to 4 d (Tables 2 and 3).

In preliminary experiments, two HCBD injections (10, 25, and 50 mg/kg body wt) 7 d apart without any concomitant EG administration were unable to induce any crystalluria, but at the highest dosages (25 and 50 mg/kg body wt), they did, after both injections, cause much the same alterations in proteinuria and enzymuria as in group 3 and 4 rats (Figure 3). Oxaluria, calciuria, and urinary pH were unaffected.

Morphologic Examination

In rats that were treated with EG only (group 1), light and polarized light microscopy and TEM on kidneys that were removed at T8, T10, T15, and T21 d disclosed no significant morphologic abnormalities or crystal deposits. Moreover, the apoptotic index that was measured by TUNEL staining was irrelevant at any time points.

In rats that were treated with both EG and HCBD, light microscopy and TEM on kidneys that were removed at T8 and T10, 24 and 72 h, respectively, after the first HCBD injection and 24 h after the second HCBD dose (T15), revealed no significant morphologic abnormalities in group 2 (10 mg/kg body wt), similar to group 1, whereas light microscopy showed dysmorphic changes in the pars recta of the proximal tubule, consisting of cells with large nucleated nuclei and basophilic cytoplasm, in groups 3 and 4 (25 and 50 mg/kg body wt). No crystals were apparent under polarized light. In groups 3 and 4, TEM showed that in the S3 segment, brush border was preserved, the cytoplasm had vacuoles of variable dimensions (mainly on the basolateral side) and secondary lysosomes, and the intercellular space was enlarged. These lesions were more evident in rats in group 4. After the second HCBD injection, a few nuclei had chromatin marginal condensation, as in the initial stages of apoptosis (Figure 4). This prompted specific staining for apoptosis at all time points. A surge of apoptotic cells was observed after the second HCBD injection at T21 but only at the highest dosages (groups 3 and 4; Figure 5). TUNEL-positive cells were seen only in the TE in different nephron segments, particularly in the proximal and distal tubules (Figure 6).

In the preliminary experiments with HCBD (10, 25, and 50 mg/kg body wt), light microscopy and TEM after two HCBD injections performed 7 d apart without any concomitant EG

Table 1. Urinary pH and excretion of oxalate and calcium at T0, T6, T13, and T20[a]

	Group 1	Group 2	Group 3	Group 4
Urinary oxalate (μmol/mmol creatinine)				
baseline (T0)[b]	81.6 ± 6.1	81.6 ± 6.1	81.6 ± 6.1	81.6 ± 6.1
T6	194.7 ± 15.9	178.4 ± 26.2	198.7 ± 27.6	184.3 ± 19.2
T13	181.3 ± 26.5	189.1 ± 28.2	181.9 ± 23.6	199.8 ± 25.7
T20	201.3 ± 29.0	191.3 ± 30.9	192.7 ± 27.2	196.3 ± 31.6
Urinary calcium (μmol/mmol creatinine)				
baseline (T0)[b]	0.353 ± 0.046	0.353 ± 0.046	0.353 ± 0.046	0.353 ± 0.046
T6	0.356 ± 0.040	0.378 ± 0.051	0.366 ± 0.053	0.374 ± 0.037
T13	0.369 ± 0.053	0.342 ± 0.076	0.390 ± 0.086	0.370 ± 0.068
T20	0.380 ± 0.069	0.357 ± 0.063	0.360 ± 0.067	0.349 ± 0.078
Urinary pH				
baseline (T0)[b]	7.1 ± 0.1	7.1 ± 0.1	7.1 ± 0.1	7.1 ± 0.1
T6	7.0 ± 0.1	7.2 ± 0.3	7.1 ± 0.1	7.1 ± 0.2
T13	6.9 ± 0.3	7.2 ± 0.2	6.9 ± 0.3	6.8 ± 0.5
T20	7.2 ± 0.2	7.0 ± 0.4	7.0 ± 0.3	7.0 ± 0.4

[a]Values are mean ± SEM. At T6, oxaluria in the four experimental arms was statistically significantly higher *versus* baseline (P = 0.001 to 0.000). There was no statistically significant difference in oxaluria among the four experimental groups at any time points from T6 to T20 and in calciuria and in urinary pH from T0 to T20.

[b]Baseline values derive from the calculation on all rats.

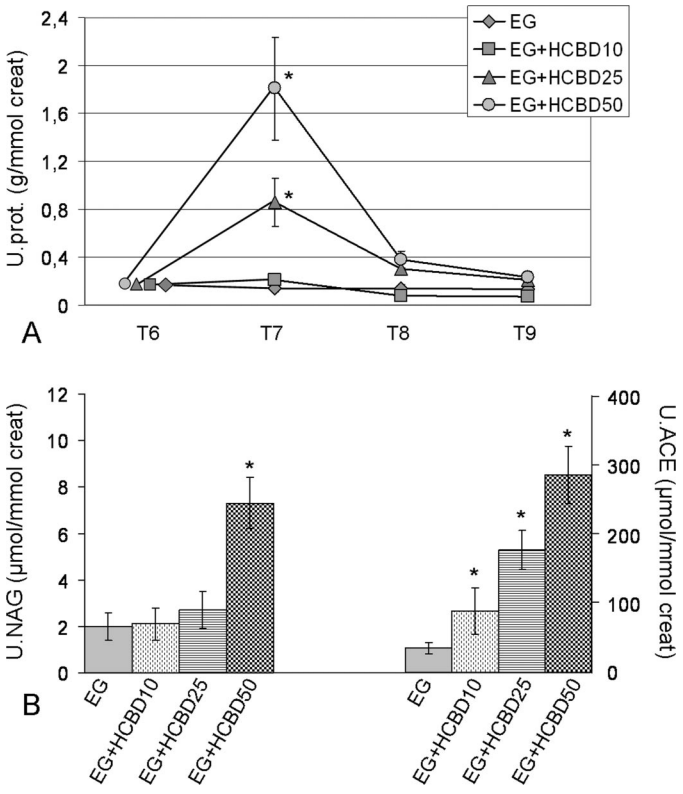

Figure 2. Urinary excretion of protein (A) and angiotensin-converting enzyme (ACE) and N-acetyl-β-glucosaminidase (NAG; B) after hexachloro-1:3-butadiene (HCBD) administration in rats that consumed ethylene glycol (EG). Urinary protein (U.prot), urinary ACE (U.ACE), and urinary NAG (U.NAG) in group 1 rats that received only EG were in the normal range. The plot in A shows data at T6, T7 (first HCBD injection), T8, and T9. The plots in B show data at T7. A similar figure in the period T13 to T16 coincided with the second HCBD administration (T14). Values are expressed as mean and SEM. *Values significantly different from the baseline.

administration disclosed very similar pictures to those described above, with the same "dose-related response," but with the noteworthy exception that no TUNEL-positive cells were observed even 7 d after the second HCBD administration at the highest dosages (25 and 50 mg/kg body wt). No crystal deposition was observed in the kidney.

Discussion

This study demonstrates that, in mild hyperoxaluric rats, crystalluria is triggered by even a very modest tubular insult caused by HCBD, as shown in the group 2 rats. That tubular damage can induce crystalluria in the presence of mild hyperoxaluria had already been demonstrated by Kumar *et al.* (10) and Hackett *et al.* (11) using repeated administrations of very high doses of gentamicin, but this caused far more severe tubular damage, characterized by proximal tubular necrosis with focal stripping of the epithelium.

The standard HCBD dosage in the experimental rat model is 100 to 200 mg/kg, which causes severe necrotic changes in the proximal TE of the rat kidney (22). We used significantly lower dosages to obtain a "mild" TE challenge, instead of causing the severe tubular damage induced by gentamicin, which is known to exacerbate crystalluria (10,11). In fact, there was no TE necrosis and no disorganization or loss of brush border, whereas there were signs of other morphologic changes that usually are observed in HCBD toxicity (vacuolization and secondary lysosomes) (13,22) at the higher HCBD dosages (25 and 50 mg/kg body wt). Moreover, the lowest dose of HCBD (10 mg/kg) alone or in combination with EG caused such a mild lesion that it was undetectable by TEM and apparent only from the increase in brush border (ACE) enzymuria (Figures 2B and 4B). The EG dosage that we used also was relatively modest and caused such a mild hyperoxaluria that the rats that were treated with EG alone had no changes in morphology or urinary biochemistry (enzymuria and proteinuria; Figure 2) and no crystalluria (Tables 2 and 3), thereby confirming the findings that were reported recently by Yamaguchi *et al.* (23) with comparable EG concentrations. Our results thus disclose a synergism in CaOx crystallization between two conditions, subtle hyperoxaluria and very mild TE damage, neither of which was able to induce crystalluria alone.

Our experimental design spans different conditions, from very low-grade to severe tubular toxicity. As far as histologic and enzymatic changes are concerned, EG administration had no exacerbating effect on HCBD toxicity, with the notable exception of apoptosis. Indeed, the association of hyperoxaluria with the higher HCBD dosages (25 and 50 mg/kg body wt) led to tubular cell apoptosis, but neither had any apoptotic effect on their own. *In vitro* studies have shown that oxalate concentrations at the metastability limit for CaOx cause TE cell apoptosis (9), but these concentrations probably were higher than the intraluminal oxalate concentrations that presumably were reached in our *in vivo* model. No effect of HCBD on renal cell apoptosis has been reported to date, and it disclosed no apoptotic activity in our preliminary study when administered without EG. Our data consequently suggest a synergistic effect of EG and HCBD on apoptosis. As a putative mechanism for this, the reduction of intracellular glutathione by HCBD (24) might impair the cellular buffering of reactive oxygen species that is produced by the oxalate-induced oxidative stress (25,26). Such an unbalanced availability of reactive oxygen species is a potential trigger of renal tubular cell apoptosis (27).

There is correlative evidence to suggest that apoptosis might increase crystal formation and adhesion to renal tubular cells (9,28,29). However, the profiles of apoptosis and crystalluria in our model were different, because crystalluria clearly was induced even by the very mild lesions that were reflected in an increased ACE enzymuria without any apoptosis, as in group 2. Furthermore, crystalluria was evident already after the first HCBD injection, which was unable to induce renal cell apoptosis. Therefore, apoptosis does not seem to play a part in the crystalluria that was observed in this model. Moreover, the lack of intraluminal crystals in our model seems to go against any role for renal tubular cell apoptosis in crystal adhesion. However, if apoptosis in our experiment affected cells where or when there were no crystals, then this might explain a lack of crystal binding and intrarenal crystal deposition. Further stud-

Table 2. CaOx crystalluria[a]

Time	Group 1	Group 2	Group 3	Group 4	*P*
T6	0 ± 6	1 ± 6	0 ± 5	0 ± 7	NS
T7	0 ± 6	62 ± 16	70 ± 21	60 ± 18	0.013
T8	0 ± 7	40 ± 20	65 ± 24	68 ± 16	0.034
T9	1 ± 5	20 ± 14	36 ± 16	48 ± 24	NS
T10	1 ± 5	12 ± 9	22 ± 14	28 ± 17	NS
T11	0 ± 4	4 ± 5	11 ± 10	14 ± 12	NS
T13	1 ± 5	2 ± 5	5 ± 5	3 ± 4	NS
T14	1 ± 4	56 ± 25	61 ± 15	74 ± 21	0.033
T15	0 ± 3	41 ± 20	59 ± 19	71 ± 24	0.047
T16	0 ± 5	21 ± 16	40 ± 15	52 ± 25	NS
T17	1 ± 6	15 ± 11	18 ± 18	34 ± 19	NS
T18	2 ± 6	7 ± 4	10 ± 5	22 ± 15	NS

[a]Number of crystals per ×40 power field observation by treatment group in the periods T6 to T11 and T13 to T18 around HCBD injections (at T7 and T14). *P* values refer to intergroup comparison. CaOx, calcium oxalate.

Table 3. Rats with CaOx crystalluria[a]

Time	Group 1 (%)	Group 2 (%)	Group 3 (%)	Group 4 (%)
T6	13	7	0	13
T7	7	100	100	100
T8	8	100	100	100
T9	0	67	100	100
T10	0	56	89	89
T11	22	33	56	67
T13	11	22	22	33
T14	11	100	100	100
T15	0	83	100	100
T16	17	67	100	100
T17	0	50	83	83
T18	0	50	56	83

[a]Percentage of rats by treatment group with CaOx crystalluria in the periods T6 to T11 and T13 to T18 around HCBD injections (at T7 and T14).

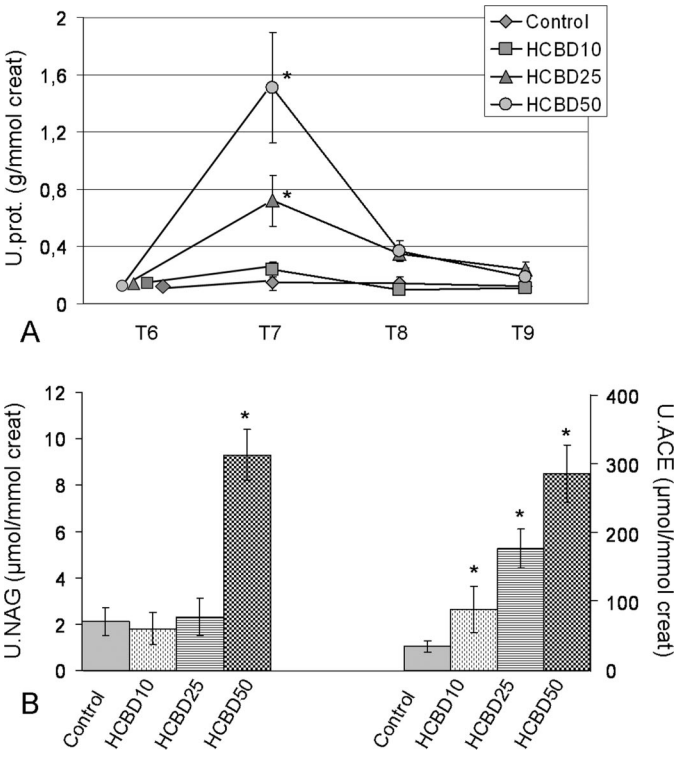

Figure 3. Urinary excretion of protein (A) and ACE and NAG (B) after HCBD administration in rats that did not consume EG. These data originated from a preliminary study that was performed to set up experimental conditions on 10 animals per group.

ies consequently are needed to confirm any *in vivo* role of TE cell apoptosis on lithogenesis, and our model may be helpful in this sense.

The four experimental groups did not differ in terms of oxaluria, calciuria, or urine pH, and there was no variation in these parameters during the trial, particularly in the periods immediately after HCBD administration. We consequently believe that the crystalluria in our model was due to the shedding of some tubular cell membrane (of which the ACE enzymuria is a marker), as suggested by Khan *et al.* (30), rather than by a change in oxaluria or urine supersaturation.

Our finding no intrarenal crystals in this model is at odds with the outcome in a similar model using gentamicin and EG (10,11). We already mentioned the differences between the two models and suggested that ours challenges the renal tubules much less severely. In fact, we induced no TE denudation, which has been claimed to have an important role in exposing the tubular basement membrane to which CaOx crystals adhere (9). However, in the light of findings reported by Evan *et al.* (31) that support the interstitial origin of Randall's plaque and its role in promoting the genesis and growth of stones in the renal pelvis, the assumption that crystals need to adhere to the TE to grow and form a cluster may not be relevant to stone formation.

According to our data, a two-step (or two-hit) model can be

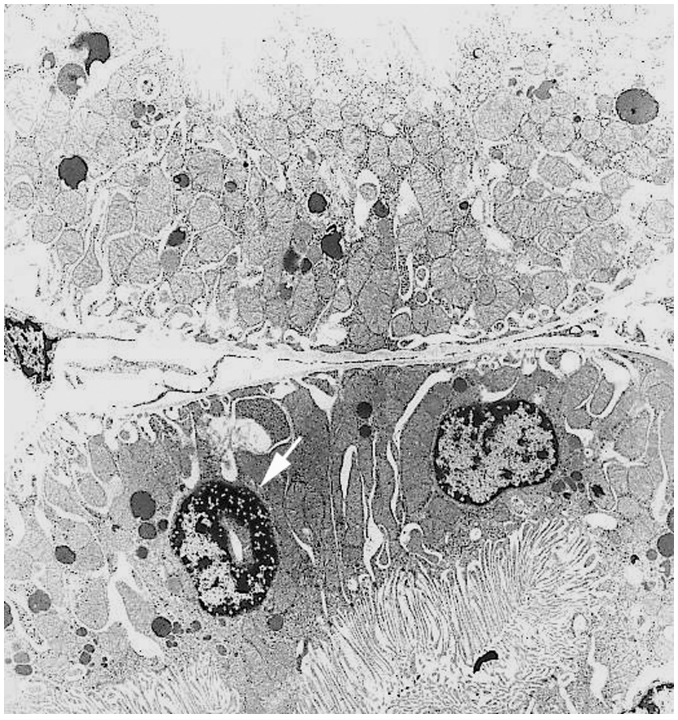

Figure 4. Nuclei with chromatin condensation in proximal tubule epithelial cells. The arrow points to one of these nuclei (group 3, T21 d, transmission electron microscopy). Magnification, ×5000.

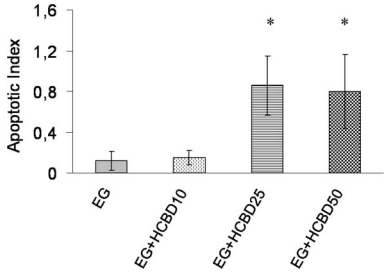

Figure 5. Apoptotic index in the four experimental groups at T21. *Statistically significant higher than group 1.

proposed: In urinary metabolic risk conditions (the first step, *i.e.*, subtle hyperoxaluria below a certain threshold) that are necessary but not sufficient for the onset of nephrolithiasis, there is a triggering event (second step) that causes stone formation. We suggest that this applies to a condition that is similar to human ICN. Actually, Evans *et al.* (31) did not show any CaOx crystals on renal biopsies from patients who had ICN, who may present mild (marginal) chronic hyperoxaluria among the many risk factors for renal stones. The scenario is different in the rat that is challenged with much higher EG intakes, in which oxaluria is high enough to cause spontaneous crystallization in the kidney. Such a circumstance seems to be more akin to the one seen in patients with primary hyperoxaluria.

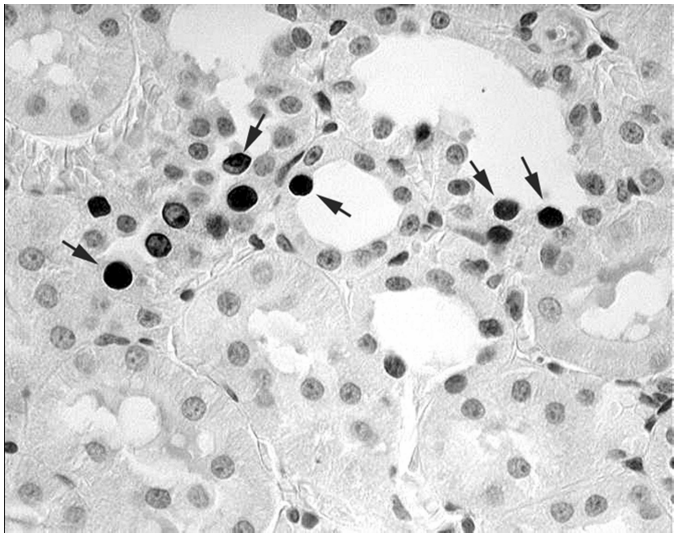

Figure 6. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling–positive epithelial cells in proximal and distal tubules. Arrows indicate apoptotic cells (group 3, T21 d). Magnification, ×50.

Conclusion

We believe that to explain the pathogenesis of stones in ICN, we need to fill the gap between the stone-predisposing conditions (*e.g.*, hypercalciuria, hyperoxaluria, hypocitraturia, a lower crystallization and aggregation inhibitory activity), which usually are chronic, and the stone-forming episode that is sporadic. Presumably something happens to throw a delicate urinary equilibrium off balance, thereby leading to the renal stone episode. Such a putative event could be exposure to an event that is capable of damaging the TE and thereby facilitating crystal adhesion or inducing acute crystalluria. Whether industrial/environmental nephrotoxins such as HCBD actually play a part is still highly speculative and needs specific studies.

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