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Relationship between supersaturation and calcium oxalate crystallization in normals and idiopathic calcium oxalate stone formers

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Relationship between supersaturation and calcium oxalate crystallization in normals and idiopathic calcium oxalate stone formers.

Background. In an earlier study on recurrent CaOx stone formers with no detectable abnormalities, we found that the urine of these subjects had a lower tolerance to oxalate load than controls and that the removal of urinary macromolecules with a molecular weight greater than 10,000 D improved their tolerance to oxalate.

Methods. The effects on CaOx crystallization of reduced urinary supersaturation of calcium oxalate (CaOx), induced by night water load, were studied in 12 normal males and in 15 male OxCa stone formers who were free from urinary metabolic abnormalities. The effect of the macromolecules, purified and retrieved from the natural and diluted urine, were analyzed in a metastable solution of CaOx.

Results. The water load caused an increase in urine volume (from 307 ± 111 to 572 ± 322 ml/8 hr, $P = 0.014$ in normal subjects, and from 266 ± 92 to 518 ± 208 ml/8 hr, $P = 0.001$ in the stone formers) and a concomitant reduction of the relative CaOx supersaturation (from 8.7 ± 2.5 to 5.1 ± 2.5 ml/8 hr, $P = 0.001$ in normal subjects, and from 10.4 ± 3.5 to 5.0 ± 2.7 ml/8 hr, $P = 0.001$ in the stone formers). The decrease in CaOx supersaturation was accompanied by an increase of the permissible increment in oxalate, both in normal subjects (from 43.8 ± 10.1 to 67.2 ± 30.3 mg/liter, $P = 0.018$) and in the stone formers (from 25.7 ± 9.4 to 43.7 ± 17.1 mg/liter, $P = 0.0001$), without any significant variations of the upper limit of metastability for CaOx (from 21.6 ± 5.3 to 20.5 ± 4.2 mg/liter in normal subjects, and from 18.7 ± 4.5 to 17.1 ± 3.7 mg/liter in the stone formers). The inhibitory effect of urinary macromolecules with molecular weight greater than 10,000 Daltons did not undergo any change when the latter were recovered from concentrated or diluted urine, either in normal subjects or in the stone formers.

Conclusions. Reduced CaOx supersaturation by means of water load has a protective effect with regards to CaOx crystallization in subjects who do not present any of the common urinary stone risk factors.

Key words: urinary stones, nephrolithiasis, calcium crystals, oxalate tolerance, lithogenous risk factor.

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Some idiopathic calcium oxalate (CaOx) stone formers do not present any of the metabolic alterations in their urine that are commonly found in calcium stone formers, such as hypercalciuria, hyperoxaluria, hyperuricosuria, hypomagnesiuria and hypocitraturia.

In a previous study we demonstrated that in these subjects urine has a reduced resistance to oxalate as compared to controls, due to an increased tendency to nucleation of CaOx, which would appear to depend on urinary compounds with a molecular weight (molecular wt) greater than 10,000 Daltons (D) [1].

Supersaturation and nucleation of CaOx are undoubtedly insufficient to explain the formation of stones. Yet an increased tendency to nucleation, which may be repeated in the course of one day or in certain periods of the year, may trigger subsequent events that are responsible for the formation of stones.

Urine contains powerful inhibitors of calcium crystallization and it is possible that, when nucleation occurs, the new crystals might adsorb urinary materials that normally raise the upper limit of metastability, thus creating a vicious cycle. This mechanism has recently been suggested in the formation of calcium phosphate stones in rats with hypercalciuria induced by dietary manipulation [2].

As an alternative, the formation of stones in these subjects, after the occurrence of nucleation, may depend on an altered capacity to inhibit growth and, above all, the crystalline agglomeration that is considered one of the most important lithogenous risk factors [3].

A rational program to prevent lithisiac recurrence in calcium stone formers requires identification of one or more urinary alterations (such as excess calcium, oxalate, uric acid or lack of magnesium and citrate) and the subsequent correction [4]. Naturally, this procedure is not possible in subjects with normal parameters of lithogenous risk.

On the other hand, the commonly given advice to increase water intake in order to reduce CaOx supersatu-

ration does not inevitably lead to a favorable effect in these subjects. Indeed, a reduced supersaturation due to the water load might be accompanied by a dilution of the inhibitors of calcium crystallization, with subsequent reduction of the upper limit of metastability and a reduction of their inhibitory power, thereby thwarting the effect of a reduced CaOx saturation.

In this study, the tolerance to oxalate and the upper limit of metastability were measured in normal subjects and in CaOx stone formers lacking urinary metabolic alterations, in natural urine and in diluted urine.

Furthermore, the inhibitory effect on CaOx crystallization was assessed for urinary macromolecules recovered from natural urine and from diluted urine.

METHODS

Patients and controls

The study involved 15 men with recurrent CaOx stones and 12 controls.

To participate in this study, patients had to present the following features: (*a*) recurrent, strictly idiopathic CaOx stone disease, that is, without urological anomalies, medullary sponge kidney, gastrointestinal disorders, hyperparathyroidism or other hypercalcemic disorders, sarcoidosis, Paget's disease, renal tubular acidosis, primary hyperoxaluria, gout or other hyperuricemic disorders, or drugs inducing nephrolithiasis; (*b*) absence of stone risk factors in 24-hour urine collections during free diet, such as hypercalciuria (>300 mg/24 hr), hyperoxaluria (>35 mg/24 hr), hyperuricosuria (>700 mg/24 hr), hypocitraturia (<320 mg/24 hr), hypomagnesiuria (<40 mg/24 hr), low GAG excretion (<4 mg/24 hr); (*c*) absence of renal calculi at the time of investigation; (*d*) absence of significant proteinuria, hematuria, pyuria, casts, bacteria and crystals in the overnight urine used for oxalate tolerance test; (*e*) absence of any other disease or drug consumption.

Fifteen patients with such characteristics were found out of 224 idiopathic male recurrent CaOx stone patients observed during two years in our Stone Unit (mean age 38 ± 8 years, range 25 to 59; body wt 73 ± 9 kg, range 58 to 90).

The oxalate tolerance test could be influenced by some factors such as urinary levels of calcium, oxalate, uric acid, magnesium, citrate, glycosaminoglycans and relative supersaturation of calcium oxalate (CaOx RS), calcium phosphate (CaP RS) and uric acid (Uric Acid RS) [5–10]. Therefore, the control group was formed by normal men matched for age (40 ± 11 years, range 24 to 60), body weight (73 ± 6 kg, range 62 to 85) and baseline values of the urinary stone risk parameters. Silent calculi in controls were excluded by renal echography.

In all patients and controls the renal function was normal (creatinine clearance more than 100 ml/min).

Collection and preparation of urine

Stone risk profile, evaluated in 24 hour urine during a free diet, included the following parameters: volume, creatinine (Jaffè method), sodium, potassium, calcium and magnesium (atomic absorption spectrophotometer), uric acid (uricase method), phosphate, sulfate and oxalate (ion chromatography), citrate (citrate lyase method), ammonium and chloride (colorimetric method), glycosaminoglycans (Samuell's method) and pH (pH meter). The CaOx RS, CaP RS and Uric Acid RS were obtained by Equil 2 computer program [11]. Stone risk profile was preliminarily assessed in all patients and controls on two consecutive days during free diet (data refer to the mean value of the 2 determinations).

The oxalate tolerance test and the study of the inhibitory effect of urinary macromolecules were carried out for each subject on overnight urine (8 hr, 23.30 to 7.30), the first time during free diet with no water load (natural urine) and the second time, again during free diet but with a load of 500 ml of soft mineral water (Fiuggi; Na 7 mg/liter, Ca 17 mg/liter, Mg 4 mg/liter) carried out at 23.30 hours (diluted urine).

In all cases the urine was collected at 7.30 hours from a single miction at our laboratory so as to process the urine immediately after miction to avoid any preservatives.

After the exclusion of significant proteinuria, hematuria, pyuria, casts, bacteria or crystals, by Coumbur test (Boehringer-Mannheim) and microscopic examination of the urinary sediment, overnight urine was divided into three aliquots.

The first part was stored under refrigeration to be processed later for the stone risk profile as for the 24-hour urine level, with the object of detecting possible differences between patients and controls in urinary levels of stone risk factors capable of influencing the oxalate tolerance test.

A second part was filtered through 0.22 μm Millipore filters and it was used for the oxalate tolerance test (see below).

A third part was filtered and then ultrafiltered through Amicon-Centricon 10 filters with a cut-off of 10,000 D to obtain macromolecules with molecular wt greater than 10 kDa, to be used to study their effect on CaOx crystallization (see below).

Oxalate tolerance test

A modification of the method described by Nicar, Hill and Pak [5] and already published [1] was used to determine the permissible increment in oxalate and the upper limit of metastability.

Briefly, the oxalate tolerance test was performed as follows. After pH was adjusted to 5.7 by addition of HCl or NaOH, 40 ml of filtered urine were divided into 20

test tubes and placed in a 37°C water bath. While under constant shaking, increasing amounts of sodium oxalate in a constant volume were added to obtain increments of 10 mg/liter oxalate concentration in each tube, except for the first tube that served as a control. After three hours of incubation the tubes were centrifuged at 3000 r.p.m. for 10 minutes and the calcium concentration was determined in the supernatant by atomic spectrophotometer. In this model, the measured final calcium concentrations plotted against the concentrations of oxalate added to the urine, showed a linear relation. Using this linear regression line we extrapolated the maximal amount of oxalate added at which no change in the initial calcium concentration had occurred after incubation, that is, the maximal amount of oxalate added without measurable crystallization. This amount of oxalate was called permissible increment in oxalate and it was reported as mg/liter of oxalate.

Since filtration at 0.22 μm does not produce any variation in the ionic concentrations required by the Equil program [1, 12], supersaturation was obtained at the point of precipitation, that is, the upper limit of metastability, by adding the initial measurement of oxalate concentration to the permissible increment in oxalate and entering this result in the Equil program. This method is limited by the fact that each treatment of the urine brings about a variation in the concentration of the macromolecules. We too observed that filtration with a 0.22 μm filter produces a considerable reduction of macromolecules such as glycosaminoglycans and Tamm-Horsfall mucoprotein [1]. Taken together, whole urine can contain crystals of various dimensions and compositions, cells or fragments of cells and lipid aggregates that can significantly alter the crystallization of CaOx and interfere with the measuring of the limit of metastability. Therefore, this is a methodological limitation typical for this type of investigation that cannot, for the moment, be eliminated.

Effect of macromolecules on calcium oxalate crystallization

After the urine had been passed through a 0.22 μm filter, a 12 ml sample was taken and divided up into aliquot parts of 2 ml per test tube, in 6 Centricon 10 microconcentrator test tubes (Amicon).

Purification of the macromolecules with a molecular wt greater than 10,000 D was obtained by three separate centrifugations, the first of which was carried out at 4,000 × *g* for 50 minutes. At the end of the first centrifugation, the 2 ml of urine originally present in each test tube had been reduced to a volume of ≈50 μl residual. A buffer solution was added to each of the residuals (sodium veronal acetate 50 mmol/liter, NaCl 133 mmol/liter, pH 5.7) to reconstitute them to their initial volume of 2 ml. Two further centrifugations were carried out at 4,000 × *g* for 30 minutes.

At the end of this phase, the residual containing the macromolecules was easily retrieved by applying a cup, overturning the concentrator and centrifuging at 800 × *g* for three minutes. The residue left in the concentrator was then washed and mixed using a small amount of the buffer solution, and then centrifuged to avoid losing small quantities of the concentrate.

At this point, all of the concentrates were collected in a single test tube and reconstituted to a final volume of 12 ml (original volume of the urine) by adding the buffer solution, calcium and oxalate so that the macromolecules were re-suspended in a final metastable solution made of sodium veronal acetate 50 mmol/liter, NaCl 133 mmol/liter, calcium 0.5 mmol/liter, oxalate 0.44 mmol/liter, pH 5.7.

The Centricon 10 test tubes used for purifying and collecting the macromolecules consisted of a concentrator tank and a collection tank for the ultrafiltrated urine, separated one from the other by a special low-adsorption membrane that holds back macromolecular solutes with a molecular wt greater than 10,000 D and allows salts and micromolecular components to pass through, thus avoiding the long periods required for dialysis and preventing denaturation of the sample and modification of the pH.

After purification, more than 95% of the residual can be recovered in the residual cup. We ourselves were able to verify that after three centrifugations, the common urine microsolutes (sodium, potassium, calcium, magnesium, citrate, oxalate, uric acid, creatinine) had, in the residuals, reached values equivalent, or very near, to zero.

As regards the GAGs, we discovered that ≈60% had been ultrafiltered and the remaining 40% held back in the residual.

Twelve test tubes were then prepared with 1 ml of the metastable solution containing the macromolecules and increasing amounts of sodium oxalate were added to each, except the first which was used as a control, so as to increase the oxalate concentration by 10 mg/liter in each test tube while keeping the volume constant.

All the test tubes were then incubated for three hours at 37°C in a shaking water bath at 220 r.p.m., and then centrifuged at 3000 r.p.m. for 10 minutes. The supernatant was used to measure the concentration of calcium by means of an atomic spectrophotometer.

The same experiment was carried out at the same time in a CaOx metastable solution identical to the one described above but which did not contain macromolecules.

Then, as per the urine, the permissible increment in oxalate was calculated to check for differences between metastable solution with and without macromolecules, between controls and stone formers and between natural and diluted urine.

Table 1. Stone risk profile determined in two 24-hour urine collections on a free diet (mean values ± SD and ranges)

	Controls ($N = 12$)	CaOx stone formers ($N = 15$)	P
Volume *ml/24 hr*	1510 ± 450(970–2810)	1650 ± 750(850–3150)	NS
Creatinine *mg/24 hr*	1641 ± 209(1302–1908)	1679 ± 268(1249–2029)	NS
Urea *g/24hr*	24.7 ± 7.6(15–39)	26.4 ± 8.2(13–47)	NS
Sodium *mM/24 hr*	179 ± 52(83–294)	173 ± 51(100–290)	NS
Potassium *mM/24 hr*	59 ± 21(31–96)	53 ± 12(37–72)	NS
Calcium *mg/24hr*	197 ± 52(113–289)	190 ± 60(78–281)	NS
Phosphorus *mg/24 hr*	754 ± 199(479–1047)	786 ± 159(514–1018)	NS
Magnesium *mg/24 hr*	78 ± 23(52–123)	76 ± 21(42–107)	NS
Chloride *mM/24hr*	175 ± 52(78–296)	168 ± 52(97–279)	NS
Uric acid *mg/24 hr*	593 ± 81(443–695)	552 ± 74(417–690)	NS
Oxalate *mg/24 hr*	25.9 ± 5(15–33)	26.2 ± 5.8(15–34)	NS
Citrate *mg/24 hr*	603 ± 217(396–1145)	538 ± 140(374–790)	NS
Sulphate *mM/24 hr*	23 ± 4(17–31)	21 ± 4(14–30)	NS
Ammonium *mM/24 hr*	39 ± 5(27–46)	34 ± 10(22–59)	NS
GAG *mg/24 hr*	7 ± 1.4(4.7–10.5)	6.6 ± 1.9(4.4–11.7)	NS
CaOx RS	5.3 ± 1.7(2.6–8.6)	5.7 ± 3.1(1.01–14.2)	NS
CaP RS	0.67 ± 0.39(0.18–1.59)	0.74 ± 0.55(0.09–2.06)	NS
Uric acid RS	2.4 ± 1.3(0.4–4.8)	2.3 ± 1.3(0.7–4.2)	NS
Ionic strength M	0.19 ± 0.04(0.12–0.25)	0.19 ± 0.07(0.08–0.32)	NS
pH, 24 hr	5.84 ± 0.33(5.39–6.49)	5.83 ± 0.33(5.17–6.41)	NS

Abbreviations are: GAG, glycosoaminoglycans; CaOx RS, CaP RS, Uric acid RS, relative supersaturation for calcium oxalate, calcium phosphate and uric acid by EQUIL, respectively; NS, not significant.
NS when P was >0.1.

Table 2. Parameters in natural and diluted urine (mean values ± SD) used for oxalate tolerance test

	Controls ($N = 12$)			CaOx stone formers ($N = 15$)		
	Natural urine	Diluted urine	P	Natural urine	Diluted urine	P
Volume *ml/8 hr*	307 ± 111	572 ± 322	0.014	266 ± 92	518 ± 208	0.0001
Creatinine *mg/8 hr*	520 ± 84	516 ± 97	NS	457 ± 71	438 ± 107	NS
Calcium *mg/liter*	214 ± 65	123 ± 78	0.009	232 ± 103	118 ± 92	0.003
Oxalate *mg/liter*	30.8 ± 11.6	19.9 ± 9.5	0.018	32 ± 10.7	16.8 ± 9.7	0.0001
Uric acid *mg/liter*	541 ± 157	350 ± 185	0.024	589 ± 198	294 ± 152	0.0001
Magnesium *mg/liter*	132 ± 47	80 ± 47	0.018	98 ± 39	59 ± 29	0.005
Citrate *mg/liter*	687 ± 360	420 ± 277	0.023	433 ± 278	258 ± 219	0.034
GAG *mg/liter*	9.5 ± 2.9	5.7 ± 2.4	0.002	9.4 ± 2.7	5.6 ± 1.8	0.002
CaOx RS	8.7 ± 2.5	5.1 ± 2.5	0.0001	10.4 ± 3.5	5.0 ± 2.7	0.0001

Overnight urine collected between 11:30 p.m. and 7:30 a.m., one time without a water load (natural urine) and one time with a water load of 500 ml at 11:30 p.m. (diluted urine). Abbreviations are: GAG, glycosoaminoglycans; CaOx RS, relative supersaturation for calcium oxalate by EQUIL.
NS when P was >0.1.

Statistical analysis

All data are expressed as mean values ± SD. The mean values were compared by Student's *t*-test for paired or unpaired data, as required. Relationships between parameters were tested by the linear regression method. A probability index $P < 0.05$ was considered significant. Statistical analysis was carried out using the Primer software program on a personal computer.

RESULTS

The essential requirement for admission to this study was the absence of any urinary stone risk abnormality.

Table 1 shows that the stone risk profile determined in two 24-hour urine collections on free diet was normal in both controls and CaOx stone formers.

Table 2 shows the parameters found in overnight urine without water loading (natural urine) and overnight urine after water loading (diluted urine). Creatinine excretion was completely superimposable both in controls and in the CaOx stone formers, which confirms the correct collection of urine in the two overnight periods examined.

As was to be expected, the concentration of all the solutes was markedly lower in the diluted urine. In particular, the most important factors of CaOx crystallization (calcium, oxalate, uric acid) and inhibitors (magnesium, citrate, glycosaminoglycans) presented a decreased concentration in diluted urine as a result of the increased urine volume. Consequently, there was also a reduced CaOx supersaturation in diluted urine (approximately half that of natural urine), without significant differences between controls and CaOx stone formers.

Figure 1 shows the results of the oxalate tolerance test.

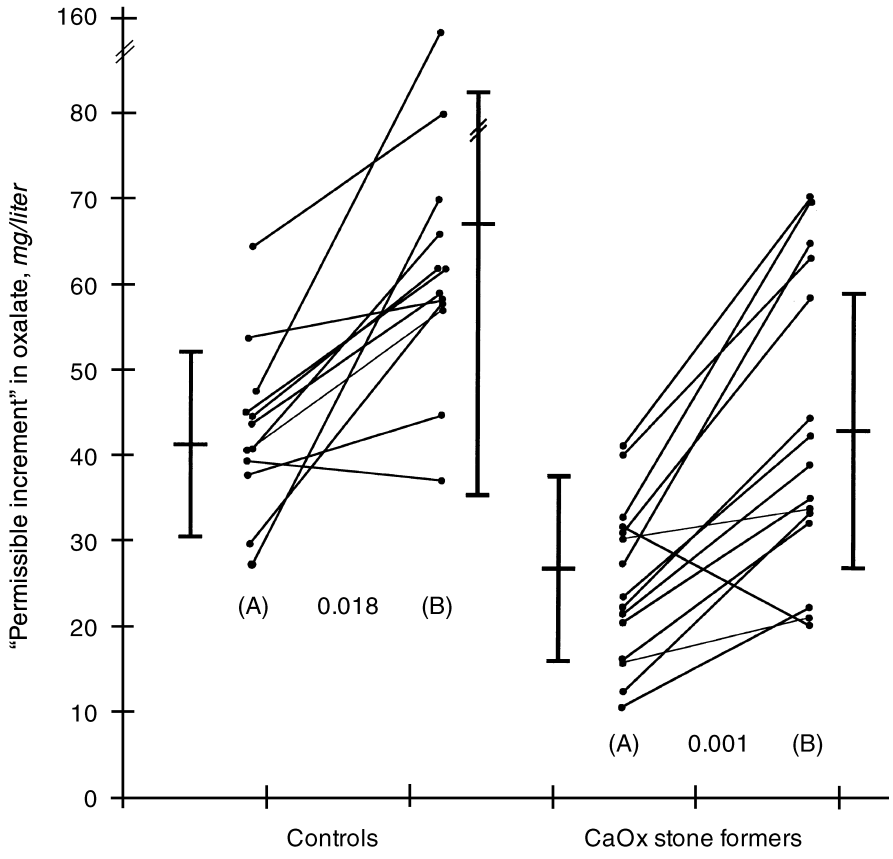

Fig. 1. Permissible increment in oxalate (mg/liter) for control subjects and calcium oxalate (CaOx) stone formers in overnight natural urine (*A*) and diluted urine (*B*). Each dot represents the value for an individual subject, while the solid bars represent the group mean ± SEM.

In most of the controls and stone formers, the permissible increment in oxalate (that is, the minimum amount of oxalate required to produce detectable nucleation by the decrease in calcium concentration) was greater in diluted urine than in baseline urine: in controls, the mean value in natural urine was 43.8 ± 10.1 mg/liter, while in diluted urine it was 67.2 ± 30.3 mg/liter ($P = 0.018$); in CaOx stone formers the mean value of the permissible increment in natural urine was 25.7 ± 9.4 mg/liter while in diluted urine it was 43.7 ± 17.3 mg/liter ($P = 0.0001$).

These data also showed that in the CaOx stone formers the permissible increment in oxalate was significantly lower than in the controls, both in natural ($P = 0.0001$) and diluted urine ($P = 0.018$).

The study of the correlations showed that the permissible increment in oxalate was inversely correlated to the relative CaOx baseline supersaturation obtained with the Equil program (Fig. 2). A lower supersaturation at the start was accompanied by a greater permissible increment in oxalate both in controls and CaOx stone formers; however, the trend of this relationship varied in the controls and the stone formers. When baseline CaOx RS was very low, the difference in the permissible increment in oxalate between controls and stone formers was much more noticeable and, inversely, when baseline CaOx RS values were very high, this difference tended to disappear.

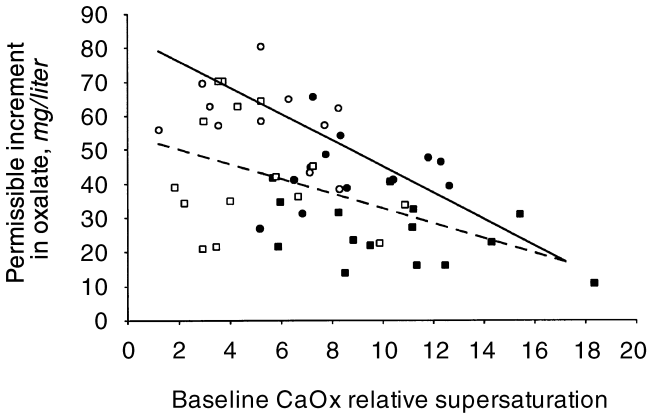

Fig. 2. Relationship between permissible increment on oxalate and baseline relative supersaturation for calcium oxalate in controls [$y = 83.3 + (-4.02 \times X)$, $r = -0.49$, $P = 0.015$] and calcium stone formers [$y = 51.4 + (-2.16 \times X)$, $r = -0.55$, $P = 0.002$]. Symbols are: (●) controls, natural urine; (○) controls, diluted urine; (■) stone formers, natural urine; (□) stone formers, diluted urine; solid line, controls; dashed line, stone formers.

Another result of the oxalate tolerance test is given in Figure 3. It has been shown [12] and confirmed by our findings [1] that urine filtration in no way alters the concentration of the various solutes required to calculate the relative supersaturation for CaOx with Equil. Thus,

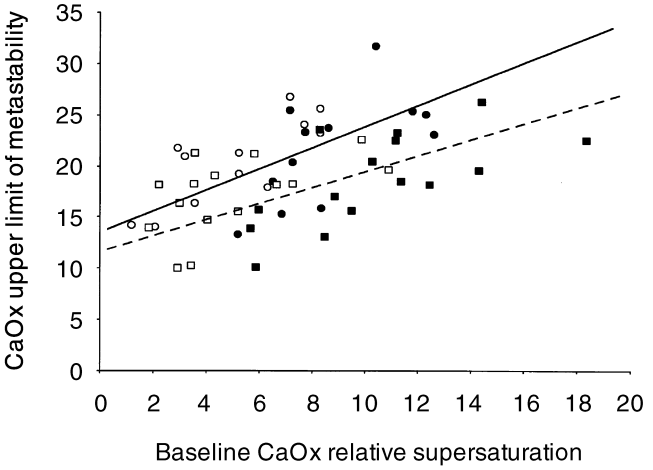

Fig. 3. Relationship between calcium oxalate (CaOx) upper limit of metastability and baseline CaOx relative supersaturation in controls [y = 14.3 + (0.98 × X), r = 0.63, *P* = 0.0001] and calcium stone formers [y = 13.4 + (0.58 × X), r = 0.58, *P* = 0.0001]. Symbols are: (●) controls, natural urine; (○) controls, diluted urine; (■) stone formers, natural urine; (□) stone formers, diluted urine; solid line, controls; dashed line, stone formers.

if one knows the minimum oxalate level at which CaOx nucleation occurs (native oxalate + added oxalate) and enters this information in Equil, it is possible to calculate the value of the upper limit of metastability for CaOx, that is, the supersaturation value at which the CaOx precipitation starts. Figure 3 shows that the upper limit of metastability was positively correlated, both in controls and in stone formers, with baseline CaOx RS.

This suggests that the reduction in the baseline supersaturation of CaOx induced by the water load could also be accompanied by a reduction in the upper limit of metastability.

The overall results of the study of the upper limit of metastability of CaOx have been summarized in Figure 4. This shows that: (*a*) In the diluted urine the upper limit of metastability for CaOx was only slightly lower than in natural urine, both in controls and in CaOx stone formers (difference not statistically significant). (*b*) In CaOx stone formers the upper limit of metastability was lower than in controls, especially in diluted urine (17.1 ± 3.7 vs. 20.5 ± 4.2, $P = 0.036$). (*c*) Nevertheless, the distance between baseline CaOx RS and the upper limit of metastability in the diluted urine of CaOx stone formers was markedly greater than in natural urine (mean difference in diluted urine was 12.2 ± 3.2, in natural urine it was 8.2 ± 3.4, $P = 0.004$).

The final part of the study concerned the urinary macromolecules. In all cases, both controls and stone formers, urinary macromolecules with a molecular wt greater than 10,000 D re-suspended in a metastable solution of CaOx had an inhibitory effect on CaOx crystallization that was generated by the addition of growing quantities of sodium oxalate. Figure 5 shows the typical trend of the crystallization curve, expressed as a percentage drop in the calcium of the medium, both with and without urinary macromolecules.

When we proceeded to a quantitation of the permissible increment in oxalate in the metastable solutions without macromolecules and in the metastable solutions containing the macromolecules, we noted that there were no differences between controls and stone formers (Fig. 6). The values of the permissible increment in oxalate in the natural and in the diluted urine were 33 ± 7.7 mg/liter and 32.7 ± 7.9 mg/liter for the controls and 30.1 ± 7.3 mg/liter and 34.7 ± 6.1 mg/liter for the stone formers, respectively.

Similarly, no difference was observed between natural urine and diluted urine, either in controls or in the CaOx stone formers.

DISCUSSION

Our study has shown that reduced CaOx supersaturation, obtained simply by water loading, increases the urine tolerance to an oxalate load, does not alter the upper limit of metastability for CaOx significantly, and does not produce variations in the inhibitory effect of macromolecules with molecular weights greater than 10,000 D when tested in a metastable solution of CaOx.

Some effects of CaOx supersaturation on CaOx crystallization in total urine were investigated in two previous works.

Pak et al assessed the effects of *in vivo* dilution in four patients with nephrolithiasis (all affected by hypercalciuria) and in three normal control subjects [13]. This study showed that by increasing the urine volume by ingestion of a large amount of distilled water, the urinary activity product ratio (state of saturation) of CaOx progressively declined and the formation product ratio (the limit of metastability) significantly increased. Therefore, the urine dilution required a greater degree of supersaturation in order to trigger the spontaneous CaOx nucleation.

The finding of a rise in the limit of metastability with urinary dilution was unexpected. In fact, the urine dilution must also have caused a reduction in the concentration of urine inhibitors, and therefore, if these inhibitors had been the only factor dictating the metastable limit, the minimum supersaturation required for spontaneous nucleation should have decreased following urine dilution. In conclusion, in order to explain this result, the authors hypothesized the presence in urine of substances favoring spontaneous CaOx nucleation (promoters of nucleation) whose dilution had caused an increase in the metastability limit.

Asplin et al have recently studied the relationship between supersaturation and crystal inhibition in hypercalciuric rats, CaP stone formers [2]. They found that the

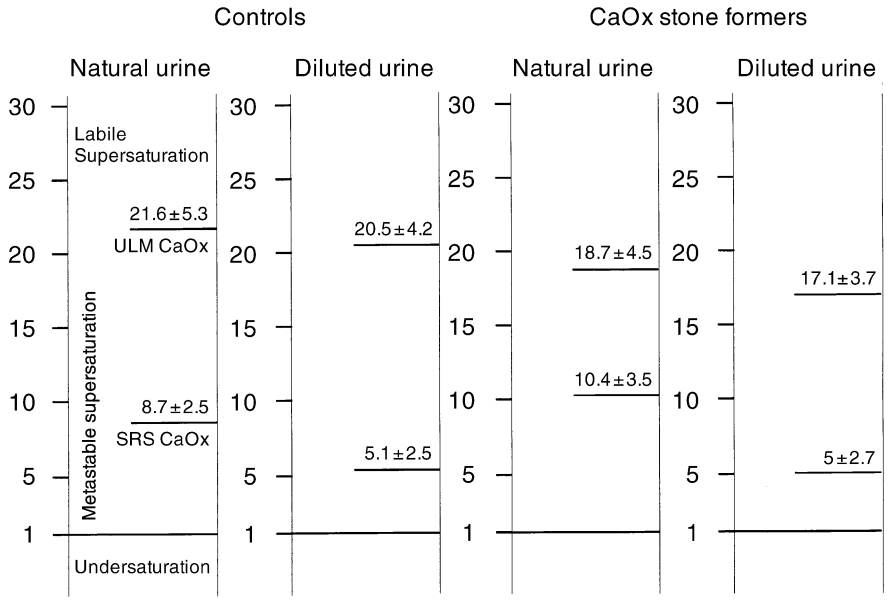

Fig. 4. Spontaneous CaOx relative supersaturation (SRS CaOx) and upper limit of metastability (ULM CaOx) for controls and CaOx stone formers in overnight natural urine and diluted urine. Details are in the Results section.

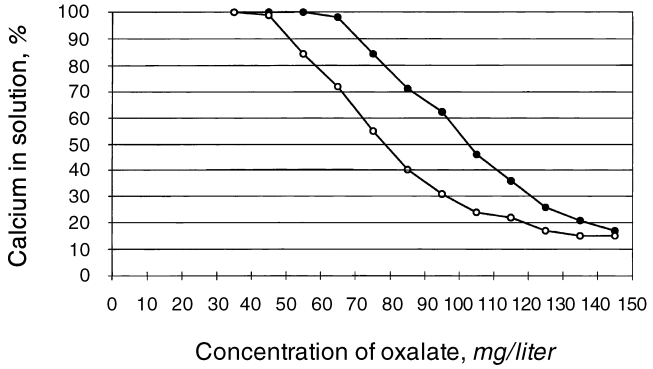

Fig. 5. Fall of calcium following the addition of sodium oxalate in a metastable CaOx with (●) or without (○) macromolecules. The inhibitory effect of macromolecules on CaOx crystallization is clearly evident.

increase in relative CaOx supersaturation was accompanied by an increase in the upper limit of metastability, as if the urine was able to bear a greater CaOx supersaturation when necessary, possibly through a change in the activity of factors affecting the CaOx nucleation.

However, the rats investigated were CaP stone formers and the pattern of CaP crystallization was different: the increase in CaP supersaturation was not accompanied by an increase in the upper limit of metastability of CaP, facilitating the theoretical risk for CaP crystallization.

For our study CaOx stone formers were chosen who were completely free from urinary metabolic alterations, and who were not distinguishable from normal control subjects with regards to urine composition. This decision was based on the need to assess whether a simple water supplement could exert favorable effects in subjects in whom it was not possible to carry out other prophylactic measures.

Both in the controls and in the stone formers the permissible increment in oxalate increased as the CaOx supersaturation decreased, demonstrating that the increased urine volume induced by the water load caused the urine to be less susceptible to CaOx crystallization even when the dilution produced a decrease in the concentration of the inhibitors magnesium, citrate and glycosaminoglycans. A difference observed between the controls and the stone formers was that in the latter the permissible increment in oxalate was lower both in natural urine and in diluted urine. In other words, in order to have an oxalate resistance equal to that of the controls, the calcium stone formers had to have a much higher urine volume, approximately double. In fact the permissible increment in oxalate in the controls had a mean value of 43.8 mg/liter with mean urine volume of 307 ml/8 hr, while in calcium stone formers it was 43.7 mg/liter with mean urine volume of 518 ml/8 hr.

The inverse correlation found between the permissible increment in oxalate and baseline CaOx supersaturation confirmed that the increase in urine volume caused an increased resistance to oxalate through a reduction of the CaOx supersaturation.

This correlation was present both in the controls and in the stone formers, but the trend was different. At the lowest saturation levels, the permissible increment in oxalate was markedly lower in the stone formers compared to the controls, while at the highest saturation levels, the two groups tended to have the same values. This suggests that, at very high saturation levels, even normal subjects can run the risk of a significant CaOx

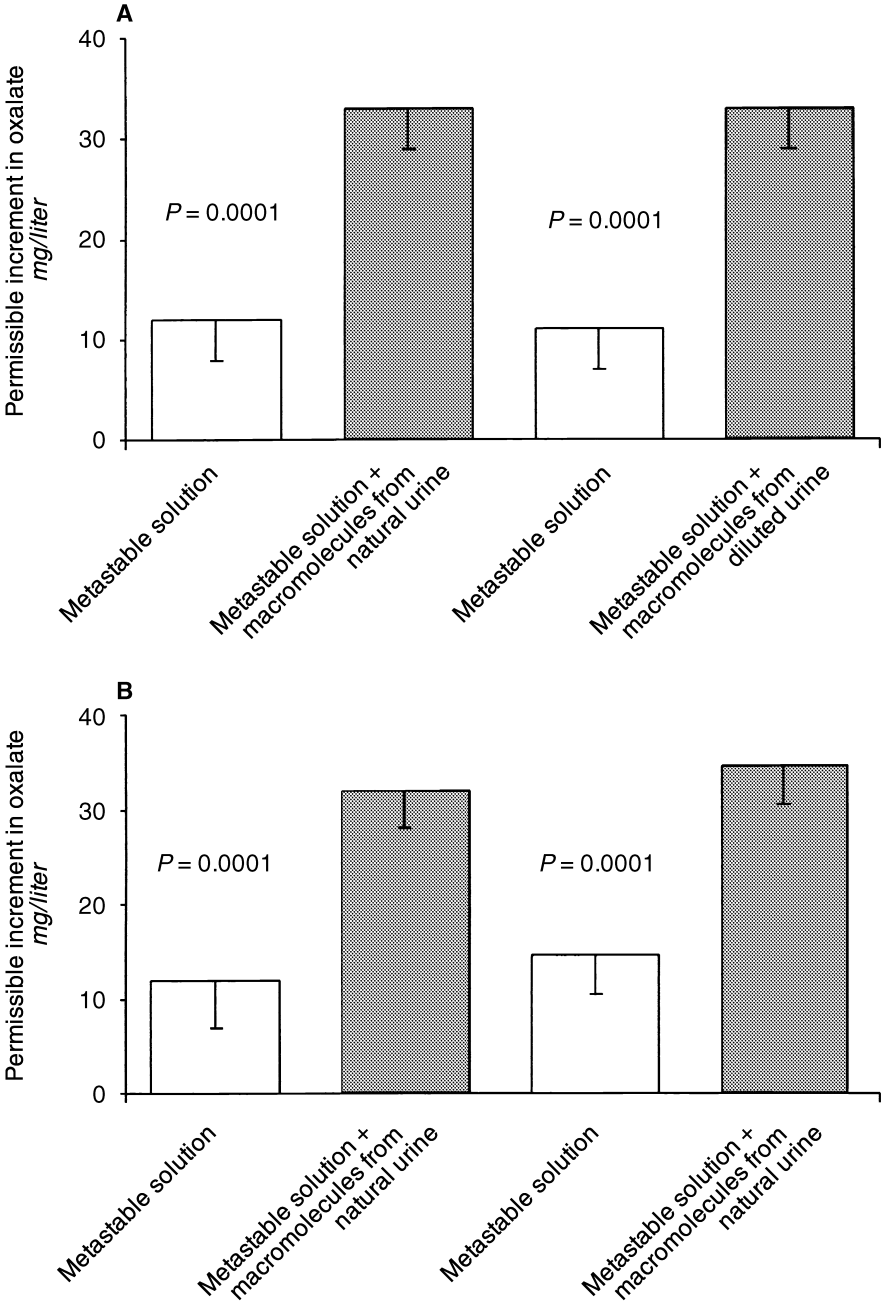

Fig. 6. Effect of macromolecules (molecular wt greater than 10,000 D) on permissible increment in oxalate in metastable solution of CaOx. No differences were found between (*A*) controls and (*B*) CaOx stone formers, and there were no differences between natural and diluted urine levels.

precipitation in the face of even slight increases in urinary oxalate.

In contrast with Pak et al [13], we found a positive correlation between the upper limit of metastability and baseline CaOx supersaturation, already described by Asplin et al [2] in experimental animals in which supersaturation had been augmented through dietary manipulation of calcium intake. This finding is consistent with the hypothesis that urinary dilution induced by water loading generates a dual effect: on the one hand, the reduction of CaOx supersaturation allows a greater tolerance to oxalate loads, and on the other, the simultaneous dilution of the inhibitors tends to lower the upper limit of metastability. It is not easy to explain the discrepancy between our data and those of Pak et al, but it is important to point out that experimental conditions were not identical.

Pak et al studied stone formers all of whom were affected by hypercalciuria, while we studied normocalciuric subjects. The fact that hypercalciuria itself can alter the activity of the natural inhibitors in the urine has been described in literature [14], and it can therefore be conjectured that the dilution of the urine might have

normalized the calcium levels in the subjects studied by Pak et al, and that this might have led to an increase in the limit of metastability.

A further difference is that Pak et al used 24-hour urine sampling, and considerably diluted it by passing from urine volumes of 1 liter/day to over 2 liter/day. We used very concentrated overnight urine, passing from volumes of 250 to 300 ml to volumes of 550 to 600 ml.

It is well known that certain macromolecular compounds, such as the Tamm-Horsfall mucoprotein, modify their molecular structure in proportion to variations in their concentration and to variations in the medium such as pH, ionic strenght, osmolarity and calcium levels. These structural modifications are also accompanied by variations in inhibitory power and, a crystallization-promoting compound can even turn into a inhibitor of the same [15].

It can therefore be conjectured that the different state of the urine could be responsible for these apparently conflicting results. In an overall evaluation of the results of our study relative to the upper limit of metastability, we found that a drop in CaOx supersaturation from 8.7 to 5.1 in the controls and from 10.4 to 5 in the stone formers only produced a slight, but not statistically significant, reduction in the upper limit of metastability. This finding suggests that when the reduction of inhibitors is accompanied by a parallel reduction of the promoters, as occurs with the water load, this does not involve a significant alteration of the point of precipitation of CaOx.

When compared to the controls the upper limit of metastability in the stone formers was observed to be slightly lower, in particular in diluted urine: mean 17.1 vs. 20.5 ($P = 0.036$). However, the distance between baseline CaOx supersaturation and the upper limit of metastability in the diluted urine of stone formers was markedly greater than in natural urine. In fact, the mean difference was 12.2 ± 3.2 in diluted urine and 8.2 ± 3.4 in natural urine ($P = 0.004$).

On the whole, these findings suggest a protective effect of a reduced CaOx supersaturation, which is only partially lessened by the reduced upper limit of metastability, presumably favored by the dilution of the inhibitors.

The final part of our study, relative to the effect of urinary macromolecules on the crystallization of CaOx, produced unexpected results.

On the basis of the results obtained from an earlier study [1], in which we had found that the removal from the urine of urinary macromolecules with a molecular wt greater than 10,000 D had provided stone formers with an improved resistance to the oxalate load, we expected that the purifying and re-suspension of these macromolecules in a metastable solution of CaOx would bring about different results in controls as opposed to stone formers. This was not the case, however, and we also noted that there was no difference in the permissible increment in oxalate between natural and diluted urine.

Since the process we used tended, as much as possible, to maintain the concentration of the macromolecules in the metastable solution of CaOx at a level equivalent to that of the original urine, it is probable that the concentration of the macromolecules retrieved from the diluted urine was lower than that retrieved from the natural urine. If this is the case, the result of an inhibitory effect of the macromolecules, similar in nature irrespective of whether it came from natural or diluted urine, suggests that the inhibitory power of the macromolecules in male subjects lies, at least within certain limits, regardless of their concentration.

Alternatively, it could be hypothesized that the concentration of the macromolecules in the undiluted urine is higher compared to normal physiological requirements.

The inhibitory effect of the urinary macromolecules has already been described by other authors [16] who have reported no difference, globally speaking, in the inhibiting potential of the macromolecules in normal male subjects as opposed to male stone formers [17]. The results of our investigation demonstrate, however, that the behavior of the macromolecules can differ greatly according to whether they are in urine or resuspended in an artificial CaOx solution. Caution must therefore be exercised when evaluating their effects, once removed from their natural milieu.

It might be possible—and this is purely a hypothetical consideration—that the ultrafiltration process eliminates crystallization promotors or substances naturally present in the urine that could interact with the macromolecules thereby modifying their behavior.

In conclusion, this study has shown that urine dilution induced by water load reduces CaOx supersaturation, increases urine resistance to an oxalate load, does not alter the limit of metastability, and maintains unaltered the inhibitory effect of urine macromolecules on CaOx crystallization when tested in a metastable solution of CaOx.

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