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ORIGINAL PAPER

Concentrated urine and diluted urine: the effects of citrate and magnesium on the crystallization of calcium oxalate induced in vitro by an oxalate load

Angela Guerra · Tiziana Meschi · Franca Allegri · Beatrice Prati · Antonio Nouvenne · Enrico Fiaccadori · Loris Borghi

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Abstract Supplementation of certain calcium crystallization inhibitors, such as citrate and magnesium, and the dilution of urine with water are now considered consolidated practice for the prevention of calcium kidney stones. The aim of this study is to verify, using tried and true in vitro methods, whether the effect of these inhibitors can manifest itself in different ways depending on whether the urine is concentrated or diluted. Calcium oxalate crystallization was studied on 4-h urine of 20 male idiopathic calcium oxalate stone formers, first under low hydration conditions (non-diluted urine) and then under high hydration conditions (diluted urine). Both the diluted and the non-diluted urine samples were subjected to three types of load: (a) an oxalate concentration increment of 1.3 mmol/l only; (b) an oxalate concentration increment of 1.3 mmol/l with a citrate concentration increment of 1.56 mmol/l; (c) an oxalate concentration increment of 1.3 mmol/l with a magnesium concentration increment of 2.08 mmol/l. In non-diluted urine, the addition of the citrate and magnesium did not modify the crystallization parameters under study. In contrast, in the diluted urine the addition of the citrate and magnesium led to a reduction in the total quantity of crystals (equivalent to 35–45%) and their aggregates (equivalent to 30–40%); at the same time, there was an increase in the diameter of the monohydrate calcium oxalate crystals, which also underwent a morphological change. In conclusion, the inhibitory effects of citrate and magnesium on the crystallization of calcium oxalate do not manifest themselves in highly concentrated urine.

Keywords Calcium nephrolithiasis · Calcium oxalate crystallization · Urinary volume · Oxalate · Citrate · Magnesium

A. Guerra · T. Meschi · F. Allegri · B. Prati · A. Nouvenne · L. Borghi (✉)
Department of Clinical Sciences,
University of Parma, Parma, Italy
e-mail: loris.borghi@unipr.it

E. Fiaccadori
Department of Internal Medicine,
Nephrology and Health Sciences,
University of Parma, Parma, Italy

Introduction

In previous papers we demonstrated that urinary dilution, obtained through good hydration by means of soft mineral water, reduces the crystallization tendency of calcium oxalate induced in vitro by an oxalate load [1, 2].

In particular, we observed that urinary dilution leads to an increase in urinary tolerance to the oxalate load [1] and a marked reduction in crystal number and aggregation tendency [2] in normal subjects and calcium oxalate stone formers alike.

Together with the already confirmed action of urinary dilution in reducing calcium salt saturation [3], these results have contributed to clarifying the mechanisms of action that might underlie the antilithogenic effect of good hydration demonstrated in some clinical trials [4, 5].

Given the fact that supplementation with certain calcium crystallization inhibitors, such as citrate and

magnesium, is now a consolidated clinical practice for the prevention of calcium stone recurrences [6–9], we considered it useful to check whether the action of these inhibitors might manifest itself differently in concentrated urine as opposed to diluted urine.

Materials and methods

Subjects

We studied 20 male idiopathic calcium oxalate stone formers [mean age 39 years (SD 8)] selected based on the following criteria: (a) normal renal function; (b) no drug use; (c) no renal calculi in situ; (d) absence of metabolic abnormalities in 24 h urine collected on a free diet.

The study was approved by the Ethical Committee of the University of Parma and all subjects gave their consent.

Crystallization study

For each subject, the calcium oxalate crystallization study was performed on 4-h spot urine specimen collected from 4:00 to 8:00 a.m., first under low hydration conditions (undiluted urine) and then under high hydration conditions using a soft mineral water (diluted urine). The composition of the mineral water used was: Na^+ 7 mg/l, K^+ 7 mg/l, Ca^{2+} 18 mg/l, Mg^{2+} 6 mg/l, Cl^- 8 mg/l, SO_4^{2-} 3 mg/l and HCO_3^- 103 mg/l. In a previous study [1], a similar load of this type of mineral water did not change urinary calcium and oxalate excretion.

The urine was collected at 8:00 a.m. in a single micturition at our laboratory so that it could be immediately processed, avoiding the addition of preservatives. Using the methods previously described [1, 2], the first aliquot of urine was used to measure the so-called urinary stone risk profile, which includes: pH, creatinine, sodium, potassium, calcium, magnesium, chloride, uric acid, sulphate, phosphorus, oxalate, citrate, urea and ammonium. Relative saturation for calcium oxalate (CaO_x RS) and ionic strength were obtained using the Equil 2 computer program [10].

The second aliquot of urine, with its pH adjusted to the value of 5.7, was used for the crystallization study. To crystallize calcium oxalate, we used an oxalate load of 1.3 mmol/l as per previous studies [1, 2], with the aim of reproducing the urinary oxalate concentration commonly found in diseases such as primary and enteric hyperoxaluria.

Calcium oxalate crystallization was studied in the following conditions:

(a) Oxalate concentration increment of 1.3 mmol/l only
(b) Oxalate concentration increment of 1.3 mmol/l with a citrate concentration increment of 1.56 mmol/l
(c) Oxalate concentration increment of 1.3 mmol/l with a magnesium concentration increment of 2.08 mmol/l

The usage 1.56 mmol/l (300 mg/l) increments of citrate and 2.08 mmol/l (50 mg/l) increments of magnesium was decided because these concentrations correspond, in a medium-to-maximum degree, to the increments that actually take place in the urine of patients under treatment with citrate and/or magnesium salts [9, 11].

In order to quantify the crystallization processes, we carried out various types of measurements using methods already reported [12]. In summary, the flat-bottomed test tubes containing the modified urines were incubated in a shaking water bath at 220 oscillation/min at 37°C for 3 h. Then the sample was centrifuged at 2,000 RPM for 10 min and the bottoms were analysed using an inverted optical Nikon microscope (Nikon Corporation, Tokyo, Japan) with a Sony CCD-IRIS camera (Sony Corporation, New York, USA), hooked up to a semi-automatic computerized image processing and analysis system (Casti Imaging program, Casti Imaging, Venice, Italy).

- To evaluate the overall quantity of crystals formed, we measured the area of the microscope field occupied by calcium oxalate dihydrate crystals (COD) and by calcium oxalate monohydrate crystals (COM), expressed as percentage of the total field area (CaO_x area/total area × 100).
- To estimate the dimension of the crystals, we evaluated the equivalent diameter expressed in micrometres (COD and COM diameter).
- To evaluate the total quantity of crystalline aggregates, we measured the area of the microscope field occupied by COD or COM crystalline aggregates, expressed as a percentage of the total field area; an aggregate was considered to be a group of two or more crystals (CaO_x agg area/total area × 100).

Statistical analysis

All data have been expressed as mean ± SD. In order to evaluate the differences between the urine samples with and without citrate and those with and without magnesium, Student's *t* test for paired data was performed. A probability index inferior to 0.05 was considered significant.

Results

Table 1 compares the urinary stone risk profile in undiluted urine (low hydration conditions) with that of the diluted urine (high hydration conditions). As expected, dilution reduced all parameters, except pH. This result is obvious, but the table is included so that the quantitative level of urinary dilution obtained through hydration might be appreciated.

Table 2 shows the values of calcium oxalate crystallization parameters in the diluted and non-diluted urine sample of 20 male idiopathic calcium oxalate stone formers, with the various experimental conditions adopted.

With regard to the non-diluted urine, it may be observed that the addition of 1.56 mmol/l of citrate and of 2.08 mmol/l of magnesium caused a significant drop in saturation for calcium oxalate, an average of 6.6 and 9.0%, respectively. Despite this drop in saturation, all the other crystallization parameters analysed did not undergo significant variations.

In the diluted urine, the behaviour encountered was different. Firstly, the addition of citrate generated a markedly greater decrease in relative saturation for calcium oxalate (mean drop of 30.2%), and the same also applied to magnesium (mean drop of 25.5%). Additionally, in the diluted urine, the addition of citrate and magnesium significantly reduced both the total quantity of the crystals (CaO_x area/total area × 100), with a drop of approx. 35–45%, and the presence of crystalline aggregates (CaO_x agg area/total area × 100), with a drop of approx. 30–40%. At the same time, there was an increase in the diameter of the crystals, especially COM crystals (mean increase of approx. 30–45%).

Finally, as shown in Fig. 1, the addition of citrate to diluted urine also brought about a morphological change in the COM crystals, which show (Fig. 1b) a more regular and more rounded polygonal shape, often hexagonal, with a reduced thickness compared to the COM crystals present in inhibitor-free urine samples (Fig. 1a).

Table 1 Urinary stone risk profile of 20 male idiopathic calcium oxalate stone formers detected under low hydration conditions (non-diluted urine) and high hydration conditions (diluted urine)

Urine parameters	Non-diluted urine	Diluted urine	*P* value
Volume (ml/4 h)	207 (66)	611 (211)	<0.001
Creatinine (mmol/l)	13.8 (4.4)	4.3 (2.1)	<0.001
Sodium (mmol/l)	108 (44)	30 (20)	<0.001
Potassium (mmol/l)	31 (13)	10 (9)	<0.001
Chloride (mmol/l)	98 (45)	32 (24)	<0.001
Calcium (mmol/l)	5.8 (3.9)	1.1 (0.6)	<0.001
Magnesium (mmol/l)	3.9 (2.3)	0.8 (0.5)	<0.001
Phosphorus (mmol/l)	28 (10)	6 (4)	<0.001
Ammonium (mmol/l)	36 (12)	11 (8)	<0.001
Sulfate (mmol/l)	20 (8)	6 (3)	<0.001
Oxalate (mmol/l)	0.28 (0.1)	0.07 (0.06)	<0.001
Citrate (mmol/l)	1.7 (1.1)	0.5 (0.4)	<0.001
Uric acid (mmol/l)	3.2 (1.1)	0.8 (0.5)	<0.001
pH	5.68 (0.28)	5.82 (0.40)	0.23
Ionic strength	0.20 (0.06)	0.06 (0.03)	<0.001
CaO_x RS	9.0 (4.1)	2.0 (1.4)	<0.001

Data are presented as mean (SD). *P* values are by Student's *t* test for paired data

CaO_x RS, relative saturation for calcium oxalate

Discussion

This study demonstrates that the addition of equal quantities of citrate and magnesium in vitro to concentrated and diluted urine produces different effects on the crystallization of calcium oxalate induced by an oxalate load.

In the concentrated urine, as already demonstrated in an earlier paper [2], an oxalate load of 1.3 mmol/l causes the formation of a large number of calcium oxalate crystals, prevalently COD crystals, with a strong aggregation tendency. In this study we found that these phenomena are not influenced by the addition of large quantities of citrate and magnesium, similar to those obtained through pharmacological treatment with potassium–magnesium citrate. The diameter of the crystals, both COD and COM, does not undergo variation in the concentrated urine. In other words, when the urine is highly concentrated, the citrate and magnesium seem to lose their known inhibitory effects [13–18].

In contrast, when the same oxalate load is added to the diluted urine of the same subjects, fewer crystals with a lower aggregation tendency are formed [2], this is also confirmed in the present study, and the addition of equal quantities of citrate and magnesium produces a further decrease in the quantity of the crystals and crystalline aggregates; this decrease is of 35–45 and 30–40%, respectively.

Therefore, the inhibitory effects of citrate and magnesium manifest themselves more readily in diluted urine.

These results are consistent with the known relationship between the concentration of inhibitors, the inhibitor/calcium or oxalate ratio, the level of supersaturation and the crystal formation [19, 20].

In particular, we know that the rate of nucleation increases exponentially with the increase of supersaturation and that, the same result, the nucleation drive will be so large that it overwhelms the most efficient inhibitors; furthermore, this result is not achieved gradually but rather suddenly.

Table 2 Crystallization parameters of calcium oxalate determined on non-diluted urine and diluted urine

Crystallization parameters	Non-diluted urine			Diluted urine		
	Oxalate[a]	Oxalate + citrate[b]	Oxalate + magnesium[c]	Oxalate[a]	Oxalate + citrate[b]	Oxalate + magnesium[c]
CaO_x RS[d]	51.2 (18.2)	47.8 (19.0)**	46.6 (16.8)**	33.1 (14.1)	23.1 (11.9)**	25.0 (10.5)**
CaO_x area/total area × 100[e]	23.9 (15.9)	22.1 (15.1)	21.9 (15.8)	8.0 (4.3)	4.5 (3.8)**	5.2 (4.3)**
COD diameter[f]	17.6 (6.6)	17.8 (7.3)	17.3 (7.4)	11.4 (2.8)	13.0 (4.9)	12.3 (4.8)
COM diameter[g]	4.5 (2.5)	5.6 (3.0)	4.7 (2.3)	8.4 (2.9)	12.1 (3.6)**	11.0 (3.7)*
CaO_x agg area/total area × 100[h]	20.8 (16.8)	19.4 (16.2)	19.3 (16.7)	2.4 (2.1)	1.4 (1.5)*	1.7 (1.7)*

[a] Oxalate concentration increment of 1.3 mmol/l only

[b] Oxalate concentration increment of 1.3 mmol/l and citrate concentration increment of 1.56 mmol/L

[c] Oxalate concentration increment of 1.3 mmol/l and magnesium concentration increment of 2.08 mmol/L

[d] Relative saturation for calcium oxalate by Equil 2

[e] Percentage of the area occupied by CaO_x crystals compared to the total field area

[f] Diameter of the calcium oxalate dihydrate crystals expressed in microns

[g] Diameter of the calcium oxalate monohydrate crystals expressed in microns

[h] Percentage of area occupied by crystalline aggregates compared to the total field area

*$P < 0.01$; **$P < 0.001$: differences compared to increment in concentrations of oxalate alone (footnote a). P values are by Student's t test for paired data

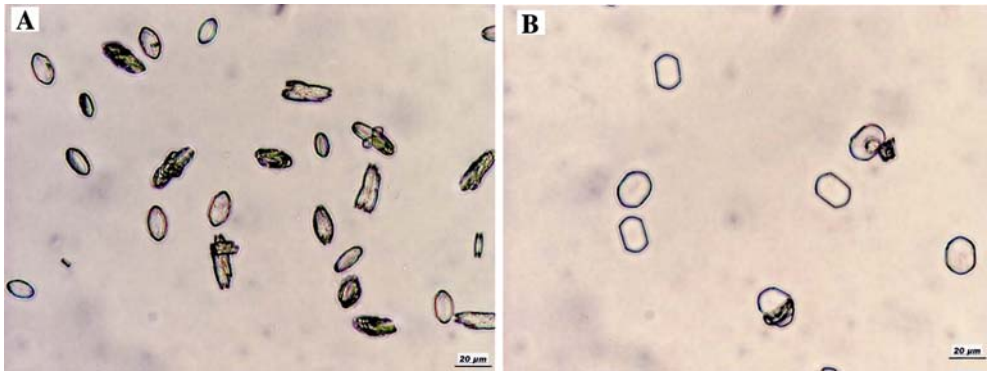

Fig. 1 COM crystals in diluted urine: **a** without increment of inhibitors; **b** with increment of citrate

In our study, the addition of citrate and magnesium to non-diluted urine shows a statistically significant decrease in supersaturation (mean reduction 6.6 and 9.0%, respectively), but not so large as to influence the crystallization process; precisely, the value of supersaturation remains at about 47–48.

On the contrary, when the same amounts of citrate and magnesium were added to diluted urine, the decrease in supersaturation was much more sizeable (mean reduction 30.2 and 25.5%, respectively), entering in a mean range of 23–25, where the inhibitory activity could carry on.

Another possible explanation could be the interactions between ionic strength and the action of the inhibitors and promoters. It is well known that the Tamm–Horsfall protein, which plays a critical role in the crystallization of calcium oxalate, a role also recently confirmed [21], can transform from inhibitor to promoter when ionic strength, its own concentration and that of calcium are high [22–24]. All these conditions were undoubtedly present in the non-diluted urine of the patients we studied and could, therefore, have led to the cancelling out of the inhibitory effect of the citrate and the magnesium. In addition, it has also been reported that, in order to inhibit aggregation effectively, the citrate should be present in the urine in equimolecular concentrations with the calcium [25], a condition which is difficult to find in non-diluted urine.

Although less evident, the action of nephrocalcin, another important inhibitory protein [26], could also be affected by different urinary ionic strengths [24].

Last but not least, we had occasion to observe that the micromolecular environment and the ionic strength have the power to strongly affect the crystalline

aggregation and, in particular, we have documented the fact that the combination of high urinary saturation for calcium oxalate associated with high ionic strength, high concentrations of citrate, magnesium and calcium, creates the ideal situation for the formation of crystalline aggregates [27].

With regard to crystal dimensions, the addition of citrate and magnesium to diluted urine leads to an increase in diameter, and this particularly applies to the COM crystals. This can be explained by assuming an inverse ratio between the number of crystalline nuclei formed and the dimensions subsequently reached by the crystals. In diluted urine, the presence of a fairly low initial saturation level and a higher inhibitor/promoter ratio, may feasibly induce the formation of a relatively low number of nuclei, the growth of which is subsequently stimulated by the availability of sufficient free energy to promote the further incorporation of crystalline material, so that the final result is a crystal with larger dimensions.

The final observation emerging from this study concerns the morphological modification of the COM crystals induced by the addition of citrate to diluted urine, as shown in Fig. 1, a more regular and more rounded polygonal shape, often hexagonal. This finding has already been reported by other authors [28] and should be considered along with the complex, simultaneous interactions between physicochemical forces, promoters and urinary inhibitors [29].

In conclusion, this study demonstrates that in highly concentrated urine, the crystalline precipitate that forms following an oxalate load cannot be modified by the addition of a further supplement of inhibitors, such as citrate and magnesium. In contrast, in diluted urine, with soft mineral water, the crystalline precipitate that forms following the same oxalate load is markedly lower and can be further reduced by a simultaneous increase in citrate and magnesium.

With all the understandable limitations of the study performed in vitro, these results suggest that it would be beneficial to advise patients under potassium–magnesium citrate treatment to maintain a high level of hydration.

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