STUDY OF THE FACTORS THAT AFFECT THE REGROWTH OF CALCIUM OXALATE DIHYDRATE FRAGMENTED CALCULI

A. Costa-Bauzá, J. Perelló, B. Isern, P. Sanchis, F. Grases

Laboratory of Renal Lithiasis Research, Institute of Health Sciences Research (IUNICS), University of Balearic Islands. 07122 Palma of Mallorca, Spain.

* Corresponding author: Prof. Dr. F. Grases
Laboratory of Renal Lithiasis Research
Faculty of Sciences
University of Balearic Islands
07122 – Palma of Mallorca
Spain
Telephone number: 034971173257
Fax number: 034971173426
E-mail address: fgrases@uib.es

Key words: Phytate
Pyrophosphate
Citrate
Renal Stone regrowth
Calcium oxalate dihydrate
Shock wave lithotripsy
ABSTRACT

**Background:** The application of extracorporeal shock wave lithotripsy (ESWL) to calcium oxalate dihydrate (COD) renal calculi gives excellent fragmentation results. Nevertheless, the retention of post-ESWL fragments within the kidney is still an important trouble. The aim of this paper is to study the regrowth of COD real spontaneously passed post-ESWL calculi fragments as a function of urinary conditions and crystallization inhibitors.

**Methods:** An “in vitro” system with synthetic urine was used to study the regrowth of post-ESWL fragments of COD calculi. Fragments growth was evaluated through their weight increase. The growth of the different fragments was uniformized by using the relative mass increase.

**Results:** In all the studied conditions the COD renal calculi fragments exhibited an important capacity to induce the growth of calcium oxalate monohydrate (COM) crystals on COD crystal faces. At urinary pH = 5.5 and normocalciuria only COM crystals were formed (growth rate = 0.22 ± 0.04 µg/mg h). At urinary pH = 5.5 and hypercalciuria COM crystals and very few new COD crystals were detected (growth rate = 0.32 ± 0.03 µg/mg h). At urinary pH = 6.5 and normocalciuria only COM and few new COD crystals were detected (growth rate = 0.35 ± 0.05 µg/mg h). At urinary pH = 6.5 and hypercalciuria large amounts of COD, COM, hydroxyapatite and brushite crystals were seen (growth rate = 3.87 ± 0.34 µg/mg h). A study of three crystallization inhibitors demonstrates that phytate attained a total inhibition of COD calculi fragments growth (2.27 µM at pH = 5.5 and 4.55 µM at pH = 6.5, both cases with hypercalciuria). 69.0 µM of pyrophosphate accomplished a reduction of 87% in the mass increase of the calculi fragments (at pH = 6.5 and hypercalciuria). Citrate (5.29 mM) did not cause any significant inhibition of COD calculi fragments mass increase at pH = 6.5 and hypercalciuria.

**Conclusion:** The growth rate of COD calculi fragments was similar in all conditions except made at pH = 6.5 and hypercalciuria, in which case the growth rate was approximately ten times superior. This demonstrates the important risk factor that could imply the presence of COD calculi residual fragments in the kidneys together with hypercalciuria and high urinary pH values. Nevertheless, crystallization inhibitors can notably slow down the calculi fragments development.
BACKGROUND

Calcium oxalate dihydrate renal calculi constitute the most prevalent and recurrent type of renal lithiasis [1, 2]. They are usually associated to hypercalciuria and in occasions to urinary pH values above 6.0 [3-7].

On the other hand, the application of Extracorporeal Shock Wave Lithotripsy (ESWL) to these renal calculi commonly gives excellent fragmentation results due to their fragility [8]. Nevertheless, the retention of post-ESWL fragments within the kidney is still an important trouble. Thus, for example, in a study with calcium stone-formers only 32% of patients were stone-free after 12 months of this treatment [9]. The data available at present seem to demonstrate that persistence and growth of fragments are quite common during history of ESWL [10-14] and consequently the interest and necessity of studies focused on knowing and minimizing such effects are obvious.

Some in vitro [15-17] and in vivo [9] studies seem to demonstrate positive effects of citrate [9, 15, 16] and phytate [17] improving the outcome of residual post-ESWL calculi fragments by reducing their growth or agglomeration. This paper belongs to a series whose purpose is to study the regrowth of residual post-ESWL calculi fragments as a function of the type of calculi, urinary conditions and presence of crystallization inhibitors. Thus, in a previous paper the regrowth of calcium oxalate monohydrate (COM) residual post-ESWL calculi fragments was studied [17]. In the present paper, the study was focused on calcium oxalate dihydrate (COD) calculi fragments.
METHODS

A collection of 48 spontaneously passed post-ESWL fragments of COD calculi collected the same day after ESWL application was chosen. The selection of the fragments was performed following the general protocol applied by our laboratory to study all renal stones. This methodology implies the appropriate combination of optical stereomicroscopy, infrared spectrometry and scanning electron microscopy (SEM) equipped with an energy dispersive X-ray analyzer (EDS) [18]. All the selected fragments had very similar morphology being this representative of that observed in the majority of spontaneously passed post-ESWL COD calculi fragments.

The size of the selected fragments oscillated between 2 - 4 mm. In a temperature-controlled (37ºC) chamber, four hermetic flow chambers (3 cm diameter and 4 cm high), each one containing three fragments of a COD calculus, were located. Thus, 12 fragments were used for each set of the following experiments:

1. pH = 5.5 and normocalciuria ([Ca total] = 3.75 mM).
2. pH = 5.5 and hypercalciuria ([Ca total] = 6.25 mM).
3. pH = 6.5 and normocalciuria ([Ca total] = 3.75 mM).
4. pH = 6.5 and hypercalciuria ([Ca total] = 6.25 mM).

The fragments were placed in the experimental chamber without any previous pre-treatment process. This methodology is similar to that previously described by Chow et al [16, 19]. Synthetic urine was introduced into the flow chambers, freshly prepared, by a multichannel peristaltic pump, with a rate of 750 ml/day through the bottom of the flasks (see Figure 1). The system was kept working during different periods of time that allowed the growth of new crystals on the fragments. Fragments
growth was evaluated through their weight increase using a precision balance. The growth of the different fragments of renal calculi was uniformized by using the relative mass increase. In one set of experiments, the system was kept working for 192 hours under conditions of normocalciuria / normooxaluria ([Ca total] = 3.75 mM, [Oxalate] = 0.28 mM). In the other set of experiments, the system was kept working for 48 hours under conditions of hypercalciuria / normooxaluria ([Ca total] = 6.25 mM, [Oxalate] = 0.28 mM).

The effects of crystallization inhibitors as phytate, pyrophosphate and citrate were evaluated. The assayed amounts corresponded to concentrations physiologically found in urine.

**Synthetic urine**

Synthetic urine supersaturated with respect to calcium oxalate was prepared by mixing with a three-way T mixing chamber equal volumes of solutions A and B. Their compositions are given in Table 1. The pH of both solutions was adjusted either to 5.5 or 6.5. Solutions were stored for a maximum of 1 week at 4 °C. Chemicals of reagent-grade purity were dissolved in deionized and redistilled water. All solutions were filtered through a 0.45 µm pore filter before being used.
Effects of various compounds

The effects of citrate, as sodium salt, (supplied by Probus) in the concentration range 1.32-5.29 mM, phytate, as sodium salt, (supplied by Sigma) in the concentration range 0.15-4.55 µM, and pyrophosphate, as sodium salt, (supplied by Merck) in the concentration range 11.5-69.0 µM were assayed by addition of different amounts of these substances to synthetic urine.

Calcium-Citrate complexation

Owing to the high concentration of citrate used and considering its complexing capacity of calcium ions, in experiments in which the action of citrate ions was evaluated, a supplement of calcium was added to obtain the same calcium oxalate supersaturation value as was found in the absence of citrate. It must be noted that a decrease in supersaturation would imply a decrease in the crystallization rate that could not be assigned to inhibitory effects. The amount of added calcium ions was potentiometrically calculated using a calcium-selective electrode (Ingold) and a potentiometer (Crison 2002). Calcium standards in presence and absence of citrate were prepared working with synthetic urine as matrix. The activity of free calcium ions must be the same in the presence and absence of citrate; consequently, when citrate was present, the appropriate amount of calcium was added. Thus, an increase in the calcium concentration of 0.15 mM was necessary per 0.53 mM increase in the citrate concentration.
When using phytate and pyrophosphate, due to the low levels used, the decrease in the free calcium concentration was practically negligible, as observed potentiometrically. Consequently, in these cases it was not necessary to add a calcium supplement.

**RESULTS**

In all the studied conditions the fragments of COD calculi exhibited an important capacity to induce the growth of COM crystals on COD crystal faces (Figures 2-5). New COD crystals appeared in low amount at urinary pH = 5.5 in hypercalciuric conditions (Figure 3) and at urinary pH = 6.5 in normocalciuric conditions (Figure 4). Nevertheless, large amounts were formed at pH = 6.5 in hypercalciuric conditions (Fig 5a, c). Significant amounts of hydroxyapatite (HAP) and brushite (BRU) crystals were only observed at urinary pH = 6.5 and hypercalciuria (Figure 5a, b).

In Table 2, the mean growth rates of COD calculi fragments at the studied conditions are summarized. As can be observed, the growth rates oscillate between 0.22-0.35 µg/mg·h in the majority of studied conditions, but at urinary pH = 6.5 and hypercalciuria the observed growth rate was around ten times higher (3.87 ± 0.43 µg/mg·h).

A study of the effects of three known crystallization inhibitors (phytate, pyrophosphate and citrate) was performed. The results are shown in Figures 6-7. As can be seen, phytate attained a total inhibition of the COD calculi fragments mass increase (2.27 µM at pH = 5.5 and 4.55 µM at pH = 6.5, both cases with hypercalciuria). When
low phytate amounts were present (less than 2.27 µM), COM was the mainly restricted phase. 69.0 µM of pyrophosphate accomplished a reduction of 87 % in the mass increase of the calculi fragments (at pH = 6.5 and hypercalciuria). Citrate (5.29 mM) did not cause any significant inhibition of COD calculi fragments mass increase at pH = 6.5 and hypercalciuria.

**DISCUSSION**

As it is shown in the Results section, when using normocalciuric / normooxaluric urine at pH = 5.5 only new COM crystals were developed on COD calculi fragments. At the same pH, new COD crystals were only formed using hypercalciuric urines but together with important amounts of COM crystals (Figure 3), nevertheless, in presence of low phytate amounts (less than 2.27 µM), COM was the mainly restricted phase. At pH = 6.5 COD crystals appeared in normocalciuric conditions (Figure 4), but in presence of hypercalciuria this phase was developed together with the calcium phosphates HAP and BRU. These results agree with several clinical observations. Thus, COM calculi normally have been associated to a lack of crystallization inhibitors and COD calculi to hypercalciuria and high urinary pH values [3-6]. It is important to emphasize that the growth rate of COD calculi fragments was similar in all conditions except made at pH = 6.5 and hypercalciuria, in which case the growth rate was approximately ten times superior (see Table 2). This demonstrates the important risk factor that could imply the presence of COD calculi fragments in the kidneys together with hypercalciuria and high urinary pH values (around 6.5). Thus, a
COD calculus fragment of 5 mg during only 3 months in the mentioned conditions, could become a fragment of 45 mg.

Obviously, crystallization inhibitors can notably slow down the calculi fragments development. In this way, phytate, at concentrations that can be found in real human urine [20], totally prevented the COD calculi fragments development and pyrophosphate also accomplished an important reduction of the COD calculi fragments mass increase. Nevertheless, citrate did not manifest any significant crystallization inhibitory capacity in the assayed conditions. This seems contradictory to previous results pointing out that high citrate concentrations significantly reduced the growth rate of stones by more than 50 % [16]. However, this can be explained if it is considered that the mentioned reduction in the growth rate must be assigned to citrate complexation with calcium, which resulted in an important reduction in the relative calcium oxalate supersaturation. Thus, it must be considered that in the present study a supplement of calcium was added to obtain the same calcium oxalate supersaturation value that is found in the absence of citrate. In this aspect, it is interesting to observe that whereas citrate did not manifest significative effects as crystallization inhibitor of calcium salts development on COD crystals, it exhibited an important capacity as crystallization inhibitor of calcium salt development on glycoproteins and organic matter [21, 22], this once more demonstrating the specificity of crystallization inhibitors.
CONCLUSION

The growth rate of COD calculi fragments was similar in all conditions except made at pH = 6.5 and hypercalciuria, in which case the growth rate was approximately ten times superior. This demonstrates the important risk factor that could imply the presence of COD calculi residual fragments in the kidneys together with hypercalciuria and high urinary pH values. Nevertheless, crystallization inhibitors can notably slow down the calculi fragments development.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

AC conceived of the study and participated in the search and selection of COD calculi fragments. JP carried out the crystallization studies in hypercalciuria conditions. BI carried out the crystallization studies in normocalciuria conditions and pH = 5.5. PS carried out the crystallization studies in normocalciuria conditions and pH = 6.5. FG participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.
ACKNOWLEDGEMENTS

P.S. expresses her appreciation to the Spanish Ministry of Education, Culture and Sport for a fellowship of the FPU program. Also, B.I. expresses his appreciation to the Conselleria d’Innovació i Energia del Govern de les Illes Balears for a fellowship. The financial support from Conselleria d’Innovació i Energia, Govern Balear (Grant PROIB-2002GC1-04) and from the Spanish Ministry of Science and Technology (project BQU 2003-01659) is gratefully acknowledged.

REFERENCES


FIGURE LEGENDS

Figure 1. Diagram of the experimental flow system device used for crystallization studies with COD calculi. 1. Temperature-controlled chamber; 2. Flask containing the post-ESWL calculi fragments; 3. Three-way T mixing chamber of A and B solutions; 4. A and B solutions for artificial urine; 5. Peristaltic pump.

Figure 2. COM crystals formed on a post-ESWL fragment of a COD renal calculus when working with normocalciuric (3.75 mM) and normooxaluric (0.28 mM of oxalate) synthetic urine (pH = 5.5), using the in vitro system after 192 h.

Figure 3. COM and COD crystals formed on post-ESWL fragments of COD renal calculi when working with hypercalciuric (6.25 mM) and normooxaluric (0.28 mM of oxalate) synthetic urine (pH = 5.5), using the in vitro system after 192 h.

Figure 4. COM, COD and HAP crystals formed on post-ESWL fragments of COD renal calculi when working with normocalciuric (3.75 mM) and normooxaluric (0.28 mM of oxalate) synthetic urine (pH = 6.5), using the in vitro system after 192 h.

Figure 5. a) HAP and BRU crystals, b) COM and HAP crystals and c) COD and HAP crystals formed on post-ESWL fragments of COD renal calculi when working with hypercalciuric (6.25 mM) and normooxaluric (0.28 mM of oxalate) synthetic urine (pH = 6.5), using the in vitro system after 48 h.

Figure 6. Increase of relative weight of post-ESWL fragments of COD renal calculi maintained in normooxaluric ([oxalate] = 0.28 mM) synthetic urine at pH = 5.5 during 192 hours in absence and presence of phytate. Values are mean of 12 fragments ± SE.
a. Normocalciuric urine ([Ca total] = 3.75 mM).

b. Hypercalciuric urine ([Ca total] = 6.25 mM).

**Figure 7.** Increase of relative weight of post-ESWL fragments of COD renal calculi maintained in normooxaluric ([oxalate] = 0.28 mM) synthetic urine at pH = 6.5. Values are mean of 12 fragments ± SE.

a. Normocalciuric urine ([Ca total] = 3.75 mM). System kept working for 192 hours in absence and presence of phytate.

b. Hypercalciuric urine ([Ca total] = 6.25 mM). System kept working for 48 hours in absence and presence of phytate.

c. Hypercalciuric urine ([Ca total] = 6.25 mM). System kept working for 48 hours in absence and presence of pyrophosphate.

d. Hypercalciuric urine ([Ca total] = 6.25 mM). System kept working for 48 hours in absence and presence of citrate.
Table 1. Composition of synthetic urine

<table>
<thead>
<tr>
<th></th>
<th>Solution A (mM)</th>
<th>Solution B (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂SO₄ · 10H₂O</td>
<td>19.34</td>
<td>NaH₂PO₄ · 2H₂O</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>5.93</td>
<td>Na₂HPO₄ · 12H₂O</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>86.73</td>
<td>NaCl</td>
</tr>
<tr>
<td>KCl</td>
<td>162.60</td>
<td>Na₂C₂O₄</td>
</tr>
</tbody>
</table>

Different volumes of a 1 M calcium solution (prepared by dissolving calcium carbonate with hydrochloric acid) were added to solution A to obtain a final calcium concentration in the range of 3.75-6.25 mM.
**Table 2.** Mean growth rate (relative mass increase) of post-ESWL fragments of COD renal calculi working with the *in vitro* system under different experimental conditions and with an oxalate concentration of 0.28 mM. Results are expressed as mean (µg/mg·h) ± SE (n = 12).

<table>
<thead>
<tr>
<th>pH = 5.5</th>
<th>pH = 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ca total] = 3.75 mM</td>
<td>[Ca total] = 6.25 mM</td>
</tr>
<tr>
<td>[Ca total] = 3.75 mM</td>
<td>[Ca total] = 6.25 mM</td>
</tr>
<tr>
<td>0.22 ± 0.04</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>0.35 ± 0.05</td>
<td>3.87 ± 0.43</td>
</tr>
</tbody>
</table>
Figure 1
Figure 6

(a) Graph showing the relationship between phytate concentration and Δm/m (%). The x-axis represents the concentration of phytate (μM), and the y-axis represents the change in Δm/m (%). The graph displays bars with error bars indicating the variability at each concentration level.

(b) Another graph similar to (a), showing the relationship between phytate concentration and Δm/m (%). The x-axis represents the concentration of phytate (μM), and the y-axis represents the change in Δm/m (%). The graph displays bars with error bars indicating the variability at each concentration level.