STUDIES ON THE CRYSTALLIZATION PROCESS FOLLOWING ADDITION OF CALCIUM PHOSPHATE CRYSTALS TO SOLUTIONS WITH A COMPOSITION CORRESPONDING TO THAT IN THE COLLECTING DUCT

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Abstract

Crystals of calcium phosphate (CaP) were added to solutions with a composition corresponding to that at different levels of the collecting duct and with different pH. CaP crystals were rapidly dissolved at pH 5.0, 5.25 and 5.5. Only minor or no dissolution was observed at higher pH levels. Despite such an effect, CaP crystals induced nucleation or heterogeneous crystallization of CaOx up to a pH of 6.1, whereas, CaP most certainly was the type of crystalline material that precipitated at higher pH levels. Accordingly, small crystal volumes were recorded at pH 5.5 and great volumes at pH 6.7 four hours after the addition of CaP seed. Dialyzed urine appeared to counteract the dissolution of CaP and to reduce the rate of secondary crystallization. The CaP induced crystallization of CaOx was confirmed by a reduction of labelled oxalate in solution. The APCaOx required for a heterogeneous crystallization of CaOx on CaP was around 1.5 x 10⁻⁸ M² for CaP crystal growth on CaP, an APCaP (Ca²⁺ x PO₄³⁻) of approximately 50 x 10⁻¹⁴ M² appeared necessary. The CaOx crystals were microscopically found in association with the CaP material and were frequently of CaOx dihydrate type. These experimental studies give support to the hypothesis that crystallization of CaOx at lower nephron levels might be induced by CaP formed at nephron levels above the collecting duct.

Key Words: Crystallization, calcium oxalate, calcium phosphate, nephron, collecting duct, distal tubule, urine.

Introduction

Previous experimental work has shown that calcium phosphate (CaP) was the favored type of crystal precipitated at nephron levels above the collecting ducts (Hess and Kok, 1966; De Ganello et al., 1990; Asplin et al., 1991, 1996; Lupták et al., 1994; Kok and Khan, 1995). When data from urine analyses were extrapolated to a situation assumed to exist in the distal part of the distal tubule (DTd), the ion activity product of calcium oxalate (APCaOx) was very low and most certainly below the risk of crystal formation. In contrast, the ion activity products of amorphous calcium phosphate (ACP), hydroxyapatite (HAP), octacalcium phosphate (OCP) and brushite (Bru) were well above the solubility products. The risk of forming a urine supersaturated with CaP-salts in DTd was considered to be higher in stone formers than in normal subjects (Tiselius, 1996a). While the risk of CaP crystallization is negligible at low pH-levels in DTd urine, the risk increases rapidly with increasing alkalinity.

Although it is reasonable to assume that ACP is the initial crystal phase (Christoffersen et al., 1990; Lundager-Madsen and Christensson, 1991), this has been difficult to prove in our experiments, and, for sake of simplicity, we have chosen to express the supersaturation of calcium phosphate as APCaP, obtained from the product of ion activities of calcium and phosphate: APCaP = Ca²⁺ x PO₄³⁻.

Preliminary data have subsequently shown that, following titration with calcium chloride, crystals first appeared at an APCaP of 123-131 x 10⁻¹⁴ M² (Lupták et al., 1994). In solutions left at constant supersaturation levels, crystals formed spontaneously when the APCaP was in the range 225-435 x 10⁻¹⁴ M² (Tiselius, 1996a).

CaP crystals, thus, might develop in the nephron either in the loop of Henle (Hess and Kok, 1966; De Ganello et al., 1990; Asplin et al., 1991, 1996; Kok and Khan, 1995) or in the distal part of the distal tubule (Lupták et al., 1994; Tiselius, 1996a) particularly following an alkaline load together with high concentrations of calcium and phosphate.
The aim of the experiments presented below was to study the fate of CaP crystals in a urine environment roughly corresponding to that in the collecting duct. The CaP crystals used were prepared by increasing the calcium concentration in solutions, with a composition approximately corresponding to that in DTd.

Materials and Methods

Preparation of crystals

Crystals of CaP were prepared by adding 8 ml of 1.0 mol/l CaCl₂ to 200 ml of a solution with the following composition: calcium 1.04 mmol/l, magnesium 0.41 mmol/l, phosphate 4.17 mmol/l, sodium 96 mmol/l, potassium 22.5 mmol/l, citrate 0.35 mmol/l, sulfate 13.8 mmol/l, oxalate 0.04 mmol/l. The pH was adjusted to 6.75 to gel a substantial amount of crystals. The solution also contained 20% of dialyzed urine.

The urine was obtained from a normal subject and dialyzed according to principles previously described in detail (Lupták et al., 1994) and passed through a Millipore filter with a pore size of 0.8 µm (Millipore, SA Molsheim, France).

The Millipore filtered CaCl₂ solution was added drop-wise and pH maintained at 6.75 as long as the solution was macroscopically clear. When the first sign of crystal formation appeared, the remaining aliquot of CaCl₂ was added. The solution was left with magnetic stirring for 1 hour, after which the solution was poured into a separation funnel. The crystals were allowed to sediment during 40 minutes. The sediment was subsequently isolated from the remaining solution, transferred to an empty and clean separation funnel and to avoid a complete dissolution of the crystals during the experiment. The samples were subsequently placed on a shaking table and slowly moved during the following 20 hours. The crystal size distribution was assessed in a Coulter Multisizer (Coulter Electronics Ltd., Luton, U.K.) with a 50 µm tube. The number and volume of crystals were recorded.

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For determination of the formation product of CaOx crystals in the presence of a CaP precipitate, we prepared Solution M with a composition corresponding to that in the middle part of the collecting duct (CD). In the experiments, Solution M had the following ion composition: calcium 1.6 mmol/l, citrate 1.21 mmol/l, phosphate 12.1 mmol/l, magnesium 1.45 mmol/l, sodium 94 mmol/l, potassium 53 mmol/l, sulfate 7.8 mmol/l and pH 6.0. The oxalate concentration was varied in the range 0.12-0.50 mmol/l. The same solution with an oxalate concentration of 0.12 mmol/l and pH 5.00, 5.25, 5.50, 5.75, 6.00 and 6.25 was used to assess the dissolution of added CaP.

In the experiments, 0.2 ml of the CaP seed suspension was added to 100 ml of Solution M. This low concentration of seed was chosen to keep the volume of crystals as small as possible thereby avoiding a rapid saturation with calcium oxalate during CaP dissolution.

For further studies on the crystallization process, a series of solutions with different degrees of concentration and with different pH were prepared. The CD-Solution A, which was considered to correspond to an undiluted urine from the distal part of the collecting duct was given the following final ion composition: calcium 4.5 mmol/l, sodium 109 mmol/l, potassium 63.7 mmol/l, phosphate 32.3 mmol/l, sulfate 20.8 mmol/l, citrate 3.21 mmol/l and oxalate 0.32 mmol/l. Solutions B, C, D and E were obtained by diluting Solution A with water to 80, 60, 40 and 20% of its concentration, respectively. Solution A was prepared both in water and in dialyzed urine and the pH of all solutions was subsequently adjusted to 5.5, 5.8, 6.1, 6.4 and 6.7. The solutions were passed through 0.22 µm Millipore filters before use in the crystallization experiments.

The ion-concentrations and ion-activity products of different calcium salts are summarized in Table 1. In this set of samples, Solution A has a composition that corresponds to that in final urine with a 24 hour excretion of 6.75 mmol calcium, 4.6 mmol magnesium, 2.9 mmol citrate and 0.3 mmol of oxalate in a volume of 1.5 liter.

To 10 ml of the different CD-solutions was added 500 µl of the CaP suspension. This concentration of seed was considered necessary to discover a nucleation of CaOx and to avoid a complete dissolution of the crystals during the experiment. The samples were subsequently placed on a shaking table and slowly moved during the following 20 hours. Aliquots were drawn immediately after crystal addition and after 4, 8, and 20 hours. At these points of time, the crystal size distribution was assessed in a Coulter Multisizer (Coulter Electronics Ltd., Luton, U.K.) with a 50 µm tube. The number and volume of crystals were recorded.

In one series of samples A to E, 100 µl of [¹³C] oxalate with a specific radioactivity of 109 µCi/µmol (Amersham, Buckinghamshire, U.K.) was added, and an aliquot filtered after 0, 4, 8, and 20 hours for assessing the isotope remaining in solution. The isotope was measured in a liquid scintillation spectrometer (model 1217 Wallac, LKB, Turku, Finland). Isotope was also added to solution M and aliquots for isotope measurements drawn from this system after 1, 2 and 4 hours.

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Microscopic examinations

The crystals were examined by light microscopy at a magnification of 400x and with a JSM 840 JEOL (JEOL Ltd., Tokyo, Japan) scanning electron microscope (SEM). For SEM examination, the solutions were filtered through polycarbonate membrane filters with a pore size of 0.2 µm (Poretics Corp., Livermore, CA, USA). The filters were rinsed with air, dried at room temperature and mounted with double stick tape on metallic stubs. The crystals were covered by a 10 nm layer of metallic platinum in a twin electron beam sputter coating unit (Model 3AM, Edwards, Crawley, Sussex, U.K.).

Results

Dissolution of CaP crystals

When CaP crystals were added to Solution M with different pH and an oxalate concentration of 0.12 mmol/l, a rapid dissolution was recorded at pH 5.0, 5.25, and 5.5. Both the number and volume of crystals were reduced already within the first minutes (Fig. 1).

Table 1. Ion composition and ion-activity products in the different experimental solutions.

<table>
<thead>
<tr>
<th>Solution</th>
<th>M</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium mmol/l</td>
<td>1.6</td>
<td>4.5</td>
<td>3.6</td>
<td>2.7</td>
<td>1.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Magnesium mmol/l</td>
<td>1.45</td>
<td>3.85</td>
<td>3.08</td>
<td>2.31</td>
<td>1.54</td>
<td>0.77</td>
</tr>
<tr>
<td>Sodium mmol/l</td>
<td>94</td>
<td>109</td>
<td>87</td>
<td>65</td>
<td>44</td>
<td>22</td>
</tr>
<tr>
<td>Potassium mmol/l</td>
<td>53.0</td>
<td>63.7</td>
<td>51.0</td>
<td>38.2</td>
<td>25.5</td>
<td>12.7</td>
</tr>
<tr>
<td>Phosphate mmol/l</td>
<td>12.1</td>
<td>32.3</td>
<td>25.8</td>
<td>19.4</td>
<td>12.9</td>
<td>6.5</td>
</tr>
<tr>
<td>Sulfate mmol/l</td>
<td>7.8</td>
<td>20.8</td>
<td>16.6</td>
<td>12.5</td>
<td>8.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Citrate mmol/l</td>
<td>1.21</td>
<td>3.21</td>
<td>2.57</td>
<td>1.93</td>
<td>1.28</td>
<td>0.64</td>
</tr>
<tr>
<td>Oxalate mmol/l</td>
<td>0.12</td>
<td>0.32</td>
<td>0.26</td>
<td>0.20</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>pH</td>
<td>5.00-6.25</td>
<td>5.5-6.7</td>
<td>5.5-6.7</td>
<td>5.5-6.7</td>
<td>5.5-6.7</td>
<td>5.5-6.7</td>
</tr>
<tr>
<td>dU%</td>
<td>0</td>
<td>0/100</td>
<td>0/80</td>
<td>0/60</td>
<td>0/40</td>
<td>0/20</td>
</tr>
<tr>
<td>AP&lt;sub&gt;CaOx&lt;/sub&gt; 10&lt;sup&gt;4&lt;/sup&gt; x M&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.54-0.47</td>
<td>1.97-1.70</td>
<td>1.58-1.36</td>
<td>1.18-1.01</td>
<td>0.78-0.66</td>
<td>0.37-0.32</td>
</tr>
<tr>
<td>AP&lt;sub&gt;CaP&lt;/sub&gt; 10&lt;sup&gt;14&lt;/sup&gt; x M&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.11-20.1</td>
<td>4.85-410</td>
<td>3.53-312</td>
<td>2.35-218</td>
<td>1.32-131</td>
<td>0.48-53.5</td>
</tr>
<tr>
<td>AP&lt;sub&gt;HAP&lt;/sub&gt; M&lt;sup&gt;9&lt;/sup&gt;</td>
<td>8.5x10&lt;sup&gt;-57&lt;/sup&gt;</td>
<td>1.05x10&lt;sup&gt;-50&lt;/sup&gt;</td>
<td>2x10&lt;sup&gt;-51&lt;/sup&gt;</td>
<td>0.02x10&lt;sup&gt;-50&lt;/sup&gt;</td>
<td>0.9x10&lt;sup&gt;-51&lt;/sup&gt;</td>
<td>0.38x10&lt;sup&gt;-55&lt;/sup&gt;</td>
</tr>
<tr>
<td>AP&lt;sub&gt;Bru&lt;/sub&gt; 10&lt;sup&gt;7&lt;/sup&gt; x M&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.99-19.6</td>
<td>2.65-14.2</td>
<td>1.93-10.8</td>
<td>1.29-7.54</td>
<td>0.72-4.54</td>
<td>0.26-1.85</td>
</tr>
<tr>
<td>AP&lt;sub&gt;OCP&lt;/sub&gt; 10&lt;sup&gt;14&lt;/sup&gt; x M&lt;sup&gt;8&lt;/sup&gt;</td>
<td>0.05-6.7</td>
<td>5.2-411</td>
<td>3.11-244</td>
<td>1.59-124</td>
<td>0.61-47.5</td>
<td>0.11-9.08</td>
</tr>
</tbody>
</table>

Secondary crystallization in CD-solutions

As shown in Figure 2, precipitation of [14C] oxalate was observed in solutions A, B and C, but only at pH 5.5, 5.8 and pH 6.1. At pH 6.4 and 6.7, there was apparently no crystallization of CaOx during the first 20 hours following addition of CaP seed crystals. At pH 5.5, 5.8 and 6.1, crystallization of CaOx was recorded at AP<sub>CaOx</sub> level between 1.4 and 1.5 x 10<sup>-8</sup> M<sup>2</sup>. The measurements in solution M disclosed crystallization of CaOx at AP<sub>CaOx</sub> levels in the range 1.56-1.59 x 10<sup>-8</sup> M<sup>2</sup>. Although the secondary crystallization of CaOx in solutions containing CaP apparently was slow (Fig. 3), it is noteworthy that under these experimental conditions, it nevertheless occurred already at an AP<sub>CaOx</sub> around 1.5 x 10<sup>-8</sup> M<sup>2</sup>. It should also be observed that the crystallization was recorded earlier in Solution M than in Solutions A, B, and C, despite the lower CaP seed concentration in the former solution.

Four hours after the addition of CaP crystals (Fig. 4), the crystal volume was lowest at pH 5.5 remained at a plateau in the pH range 5.8 to 6.4 and increased in solutions with pH 6.7. The volume increment was most pronounced in the most supersaturated solutions. The change in crystal
volume had a similar pattern in solutions with and without dialyzed urine, albeit, the greatest volumes were recorded in the urine-free samples.

In the urine free solution, the number of crystals (Fig. 5) was low at pH 5.5 and essentially unchanged in the solutions with a pH in the interval 5.8 to 6.7. The slight reduction at pH 6.7 most certainly can be explained by difficulties in correctly counting crystals with the great size that occurred in these samples, but might also reflect an aggregation. Such an effect was accordingly most evident in Solution A and least so in Solution D. The number of observations was, however, too small for conclusions in this respect.

In contrast to the findings in solutions without urine, the crystal number was approximately the same at all pH levels in samples with urine (Fig. 5). The reduced crystal number observed at pH 5.5 in the absence of urine was not recorded in solutions containing urine.

An AP_{CaP} level of at least 50 x 10^{-14} M^2 was apparently required for growth of CaP crystals, but a more detailed analysis of the AP_{CaP} level necessary for CaP crystal growth was not carried out.

CaOx crystals were usually of dihydrate type and associated with CaP crystalline material (Fig. 6).

Discussion

The possible importance of calcium phosphate dur-
The development of calcium oxalate containing stones has been emphasized by several authors (Malek and Boyce, 1977; Resnick and Boyce, 1978; Pak, 1981; Nancollas, 1983; Smith and Werness, 1983; Hering et al., 1988; Baumann et al., 1989; Achilles et al., 1994; Asplin et al., 1996). It is well recognized that calcium phosphate crystal phases, such as, HAP, OCP and Bru are capable of inducing heterogeneous growth with CaOx (Koutsoukos and Nancollas, 1981; Koutsoukos et al., 1981; Berg and Tiselius, 1989; Mandel and Mandel, 1990). Recent observations of CaP in gel systems, overgrown with calcium oxalate, gave further support for an interaction between CaP and CaOx in the stone forming process (Achilles et al., 1994). Furthermore, CaP is a common constituent in a great proportion of CaOx containing stones (Leusmann et al., 1990; Öhman et al., 1992; Tiselius and Larsson, 1993) and CaP crystals are frequently observed in urine from stone formers (Herrmann et al., 1991).

Although the details of the initiation of calcium stone formation are incompletely understood, it appears reasonable to assume that the first steps in this process take place in the nephron. At levels above the collecting duct, calcium phosphate or calcium carbonate are the most likely products of crystallization (Deganello et al., 1990; Asplin et al., 1991, 1996; Lupták et al., 1994; Kok and Khan, 1995; Hess and Kok, 1996). Irrespective of whether this process occurs in the loop of Henle or in the distal tubule,
and whether this crystallization requires a promoter or not, it is tempting to assume that CaP serves as a nucleus for the subsequent CaOx crystallization.

In the calculations published by Asplin et al. (1996), urine in the loop of Henle was supersaturated with respect to brushite and apatite and that in the collecting duct with brushite and calcium oxalate. Although, Asplin et al. (1996) found that the distal tubular urine was undersaturated with brushite, these data did not refer to urine in the late portion of the distal tubule and inasmuch as the urine in the early collecting duct commonly was supersaturated with brushite, this would probably be so also for urine in DTd.

The results in our experiments clearly showed that

Figure 5. Number of crystals in Solutions A, B, C, D, and E without (a) and with (b) dialyzed urine hours after addition of CaP seed.

Figure 6. Crystals of CaOx in association with CaP crystalline material examined by light microscopy (a) and scanning electron microscopy (b).

CaP crystals, produced by increasing the supersaturation with CaP in a DTd-like solution when added to CD-solutions, were capable of starting a CaOx crystallization. There was also microscopic evidence that the precipitated CaOx crystals were associated with the CaP material. Although the CaOx crystallization, under these circumstances, was a slow process, both in the presence and absence of dialyzed urine, it is noteworthy that CaOx crystal formation was
observed at $AP_{CaOx}$ levels as low as $1.5 \times 10^{-8}$ M$^2$. This should be compared with an $AP_{CaOx}$ in the range of $2.6-4.3 \times 10^{-8}$ M$^2$ necessary for precipitation of CaOx in similar systems without seed crystals (Tiselius, 1991) and a formation product of $2 \times 10^{-8}$ M$^2$ as reported in the literature (Robertson et al., 1968). The importance of oxalate is emphasized by the much faster CaOx crystallization recorded in Solution M which had a higher concentration of oxalate at corresponding levels of supersaturation than Solutions A, B, and C.

Half a milliliter of the crystal suspension was added to Solutions A to E. This gave a concentration of CaP seed in the experimental solution of 500-750 mg/l. For HAP, OCP and ACP, this amount corresponds to urine calcium concentrations of 5-7.5, 4.5-6.8 and 4.1-6.1 mmol/l, respectively. Precipitation of these amounts of calcium can be encountered in vivo.

It is highly interesting that CaOx crystallization only occurred in solutions with a pH between 5.5 and 6.1 and apparently despite the fact that CaP crystals at least at pH 5.5 were subject to dissolution. It is possible that the high concentration of calcium in the solution immediately surrounding the CaP crystalline material as a result of dissolution increases the risk of CaOx growth or nucleation. The constant shaking of the experimental vessels might have counteracted this process and it is likely that a less agitated solution might have resulted in a more substantial precipitation of CaOx.

It is noteworthy that the amount of CaP seed in the experimental system with Solution M was only 20-30 mg/l. It is, thus, theoretically possible that the faster CaOx crystallization in Solution M, apart from the effect brought about by the higher oxalate concentration, also can be explained by the smaller crystal surface area available in these samples. Therefore, a localized release of calcium ions from the precipitate during dissolution might attract more oxalate per crystal surface area and, thus, result in a higher local supersaturation with CaOx than will be the case in the presence of a greater amount of seed.

The absence of CaOx crystal formation at pH 6.4 and 6.7 might be explained by a higher concentration of dissociated citrate (Berg and Tiselius, 1986; Tiselius et al. 1993) and by the fact that CaP is the favored crystal product when pH is increased (Ahlstrand et al., 1984; Tiselius, 1996b). Such an effect is also suggested by the marked increase in crystal volume at pH 6.7 particularly in Solutions A and B. It needs to be emphasized, however, that due to the crystal morphology, the Coulter measurement of precipitated CaP is less accurate than that of CaOx, particularly, when the CaP precipitate has a high concentration. Nevertheless, we believe that this method is sufficiently accurate for the conclusions we have drawn.

These preliminary results give support to a possible series of events in the formation of calcium stones: Under certain conditions, amorphous or crystalline CaP is precipitated at nephron levels above the collecting duct. This material might subsequently move down the nephron to the collecting duct, where the risk of CaOx crystal formation is much higher. The CaOx nucleation recorded in these experiments was slow, particularly, in the presence of urinary macromolecules. Its importance in stone formation, therefore, requires a retention of the CaP precipitate in the collecting duct at a level where the supersaturation with CaOx is high enough to result in the formation of a CaOx crystal phase.

At a pH below 6.4, retained CaP crystals might induce CaOx nucleation. At high pH, the CaP crystallization will dominate, whereas low pH levels might induce a CaOx crystal nucleation at least if the $AP_{CaOx}$ exceeds $1.5 \times 10^{-8}$ M$^2$. The higher the oxalate concentration, the higher is the rate of crystallization. Residual CaP might subsequently result in CaPCaOx containing stones. It is theoretically possible that after inducing CaOx crystallization, a complete dissolution of CaP might result in pure CaOx crystals, but the dissolution of CaP is apparently counteracted or retarded by urinary macromolecules. Further studies are necessary to disentangle the complexity of the events leading to calcium stone formation, but the results obtained in this limited study give further valuable support to a crucial role of calcium phosphate in calcium oxalate stone formation.

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Discussion with Reviewers

S.R. Khan: What is the role of calcium phosphate crystal associated matrix in heterogeneous nucleation of calcium oxalate crystals? Is it the crystal or the matrix that is involved in nucleation?

Authors: An exact answer to these questions is not possible to give from the experiments that have been carried out so far. In our opinion, calcium phosphate crystals that encounter an environment with a lower pH in the collecting duct will release calcium as well as phosphate by dissolution.
Although the increased concentration of calcium in the solution might increase the supersaturation with calcium oxalate, it is not likely that this causes a spontaneous nucleation of calcium oxalate if distributed freely in the solution. Therefore, a localized high supersaturation probably is one important prerequisite for the subsequent crystallization with calcium oxalate. For heterogeneous nucleation on the surface of the calcium phosphate particles, a sufficiently high supersaturation must thus be created and maintained at this level. It is reasonable to assume that macromolecules covering the calcium phosphate precipitate bind or trap calcium and, in this way, cause a localized high supersaturation with calcium oxalate. The subsequent nucleation might thus be promoted by and take place in the macromolecular gel layer surrounding the calcium phosphate. The calcium phosphate seeds, in these experiments, were prepared in the presence of dialyzed urine and the seed suspension thus contained calcium phosphate covered by a layer of macromolecules. The way in which a higher concentration of macromolecules affects the crystallization in the CD-solutions is difficult to predict but, in addition to inhibitory effects on growth and aggregation, an increased thickness of the macromolecular layer might provide more binding sites and thereby reduce the local supersaturation.

In conclusion, our hypothesis is that a heterogeneous crystallization of calcium oxalate on calcium phosphate is promoted by the macromolecular matrix when the concentration of calcium increases as a result of calcium phosphate dissolution.