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ROLE OF THE ORGANIC MATTER IN CALCIUM OXALATE LITHIASIS

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1. ABSTRACT

Urine contains variable amounts of organic matter derived from cell degradation. The cellular detritus is composed by membranous and cytosolic glycoproteins, etc. The aim of this paper was to study the role of organic matter in calcium oxalate crystal development and to evaluate the action of some crystallization inhibitors on this process. Crystallization studies were carried out on urine in stagnant urine as well as under flow conditions, in presence and absence of cellular debris. Low amounts of cellular debris (when batch conditions were used), exhibited some inhibitory activity on heterogeneous nucleation of calcium oxalate monohydrate crystals, probably due to glycoproteins. Increasing amounts of cellular debris, however, promoted the nucleation of calcium oxalate monohydrate crystals. When cellular debris was retained in a cavity with a urine flow, this organic matter effectively induced the development of primary aggregates of calcium oxalate monohydrate crystals (crystallization range 2.8 μg/h per mg of organic matter). Presence of crystallization inhibitors prevented or minimized crystal development. These findings show that cell debris provides the necessary elements for the development of oxalate crystals and that this process can be effectively inhibited by presence of crystallization inhibitors.

2. INTRODUCTION

Urine contains organic matter of diverse origin. Part of this material are proteins of renal source. Uromodulin (Tamm-Horsfall protein) is the most abundant protein in normal urine. It is an 85-kDa glycoprotein secreted by the thick ascending limb of the loop of Henle (1, 2), where it may act to render the nephron wall impermeable to water. Other minor proteins present in urine are osteopontin (uropontin), calprotectin and nephrocalcin (bikunin). Osteopontin is a 44-kDa secreted phosphoprotein produced by many types of epithelial cells and it is frequently associated with mineralization processes such as bone formation (3). Calprotectin is a major leukocyte protein, also present in epithelial cells. This protein shows calcium-binding properties and antimicrobial activity against several bacteria (4), and its tissue levels are markedly increased in many infections and malignant diseases. Nephrocalcin is a glycoprotein with a molecular weight 14-kDa and it is localized in the proximal tubules in kidneys (5). Urine also contains variable amounts of organic matter which originate from cell degradation. This cellular detritus is integrated by diverse materials like membranous residuous, membrane and cytosolic glycoproteins, etc.

Whereas both inhibitor (6-9) and promoter (10-13) properties of stone formation have been assigned to the

Table 1. Composition of synthetic urine

Solution A (mM)		Solution B (mM)	
$Na_2SO_4 \cdot 10H_2O$	19.34	$NaH_2PO_4 \cdot 2H_2O$	15.45
$MgSO_4 \cdot 7H_2O$	5.93	$Na_2HPO_4 \cdot 12H_2O$	15.64
NH_4Cl	86.73	NaCl	223.08
KCl	162.60	$Na_2C_2O_4$	0.91

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 2.5-3.5 10^{-3} M.

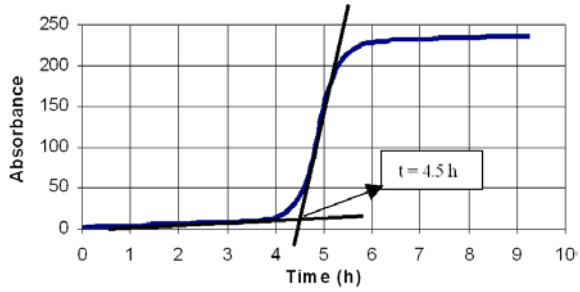

Figure 1. Absorbance-time curve obtained using the batch system, in which the induction time is assigned. Experimental conditions: $[Ca^{2+}]$ = 2.5 10^{-3} M, [Oxalate] = 4.55 10^{-4} M, pH = 5.50, T = 37 ºC.

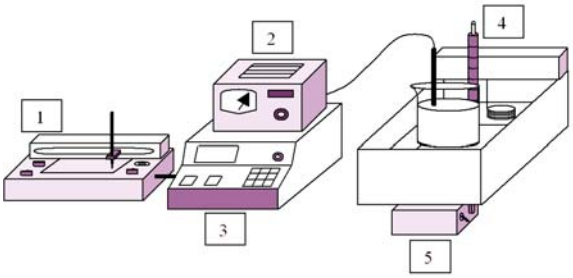

Figure 2. Diagram of the experimental device used for the batch system. 1. Recorder; 2. Altern tension stabilizer; 3. Photometer equipped with a fiber-optic light-guide measuring cell; 4. Thermostatic bath; 5. Magnetic stirrer.

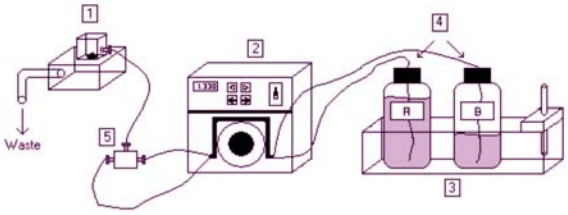

Figure 3. Diagram of the experimental device used for the flow system. 1. Cuvette; 2. Peristaltic pump; 3. Thermostatic bath; 4. A and B solutions for artificial urine; 5. Three-way T mixing chamber of A and B solutions.

mentioned urinary proteins, mainly promoter activity has been attributed to the cellular detritus (14). The aim of the present paper is to contribute to the study of the role of organic matter in calcium oxalate crystal development and also to evaluate the action of some crystallization inhibitors on such processes.

3. MATERIALS AND METHODS

3.1. Synthetic Urine

Synthetic urine supersaturated with respect to calcium oxalate ($[Ca^{2+}]$ = 2.5 – 3.5 10^{-3} M, [Oxalate] = 4.55 10^{-4} M) was prepared by mixing equal volumes of solutions A and B. Their compositions are given in Table 1. The pH of both solutions was adjusted to 5.5. The range of calcium was selected to avoid conditions that represent hypercalciuria. Conditions of mild hyperoxaluria were chosen to dispose of slight lithogenic urinary conditions. Solutions were stored for a maximum of 1 week at 4 ºC. 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.

3.2. Preparation of the cellular detritus slurry

Cellular detritus were obtained from renal tissue of Wistar rats. The kidney was lyophilized (Cryodos, Telstar, Barcelona, Spain) and pulverized to a uniform blend. 1 g of sample was homogenized in 10 ml of water using an Ultra-Turrax homogenizer (20 s at 13,500 rpm, three times).

3.3. Crystallization Studies using a batch system

To study calcium oxalate crystal formation in presence of cellular detritus, kinetic turbidimetric measurements were performed by means of a photometer (Metrohm 662) equipped with a fiber-optic light guide measuring cell with an attached light path 2 x 10 mm reflector, and using monochromatic light (550 nm). The crystallization processes were carried out in a thermostated (37 ºC) and magnetically stirred cylindrical glass flask (height 12.5 cm, diameter 9 cm). 250 ml of solution A were added to the crystallization flask, the measuring cell was immersed in the solution and the magnetic stirrer was switched on. Then 250 ml of solution B and different amounts of a cellular detritus water suspension were added to obtain a final cellular detritus concentration in the range 5-1000 mg/l. Final calcium concentration was 2.5 10^{-3} M. The chart recorder was immediately switched on in order to print the absorbance–time curve (Figure 1). A diagram of the experimental device used is shown in Figure 2.

3.4. Crystallization Studies in presence of retained cellular debris in flow conditions

A cuvette of 1 x 1 x 1 cm was placed in a temperature-controlled chamber (Figure 3). 15 mg of cellular detritus was placed at the bottom of the flask. Synthetic urine (with a calcium concentration of 3.5 10^{-3} M and pH 5.5) was introduced by a multichannel peristaltic pump, with a rate of 1.5 l/day at 3 mm from the bottom of the flask overflowing constantly. The system was kept working during a time period of 48 h. When the experiment was finished the substrate was removed from the system, rinsed with distilled water and dried at room temperature in a desicator. The amount of calcium oxalate crystallized on the substrate was evaluated dissolving the precipitate by using HCl 0.1 M and determining calcium by ICP atomic emission spectroscopy using a Perkin-Elmer P2000 spectrometer. To avoid blank errors the calcium obtained from a cellular detritus substrate, treated with identical

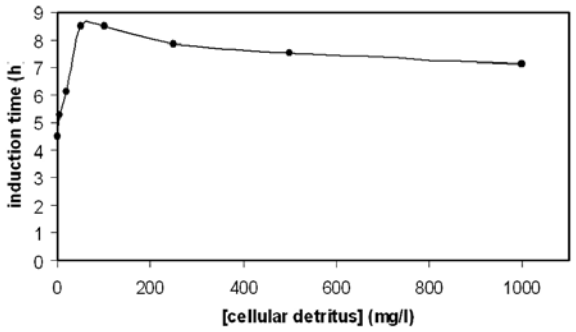

Figure 4. Effect of cellular detritus on calcium oxalate crystallization in synthetic urine using a batch system. Experimental conditions: $[Ca^{2+}]$ = 2.5 10^{-3} M, [Oxalate] = 4.55 10^{-4} M, pH = 5.50, T = 37 ºC.

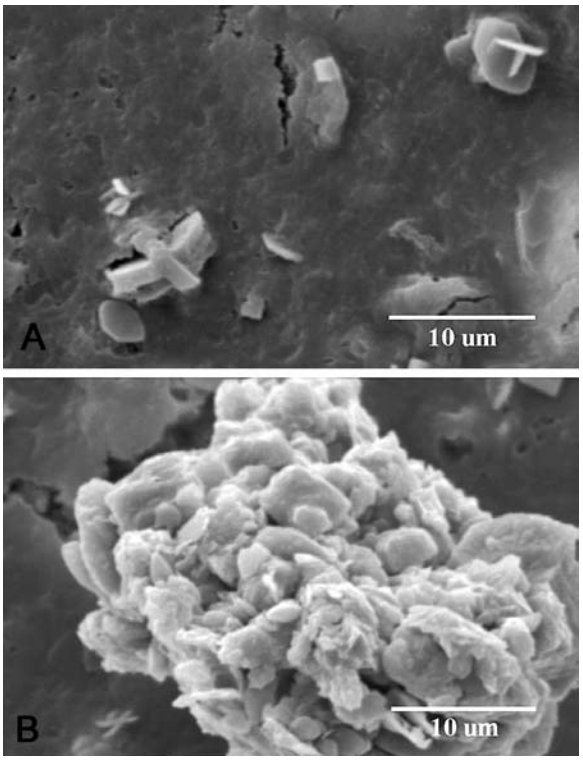

Figure 5. Images obtained by scanning electronic microscopy of the calcium oxalate monohydrate crystals (a) and primary aggregates (b) formed by heterogeneous nucleation on cellular detritus in the batch system. Experimental conditions: $[Ca^{2+}]$ = 2.5 10^{-3} M, [Oxalate] = 4.55 10^{-4} M, pH = 5.50, T = 37 ºC.

conditions but using synthetic urine without oxalate, was deducted from the former amount.

3.5. Effects of various compounds

The effects of citrate, as sodium salt, (supplied by Probus) in the concentration range 0.529-5.29 10^{-3} M, phytate, as sodium salt, (supplied by Sigma) in the concentration range 1.52-7.58 10^{-6} M, pyrophosphate, as sodium salt, (supplied by Merck) in the concentration range 5.74-40.2 10^{-6} M, and sodium pentosan polysulphate (supplied by Sigma) in the concentration range 5-40 mg/l were assayed by addition of different amounts of these substances to synthetic urine in presence of agglomerates of cellular detritus in flow conditions.

To check the reproducibility of the obtained results, each experiment was repeated three times.

3.6. Calcium-Citrate complexation

Due to the high used concentration of citrate 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 that is found in the absence of citrate. It must be considered that a decrease in the 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 selective calcium electrode (Ingold) and a potentiometer (Crison 2002). Activity of free calcium ions must be the same in citrate presence and absence, consequently when citrate was present an amount of calcium that fulfilled such conditions was added in each case. Thus, an increase of 1.5 10^{-4} M in the calcium concentration was necessary per 0.53 10^{-3} M of citrate.

When using phytate and pyrophosphate, due to the low used levels, the decrease in the free calcium concentration was practically negligible, as it was potentiometrically observed and, consequently, in these cases it was not necessary to add a calcium supplement.

3.7. Study of the formed solid phase

Solid samples of the different experiments were studied by X-ray diffraction techniques (Siemens D-5000) and by scanning electron microscopy (Hitachi S-530), demonstrating the formation of calcium oxalate monohydrate crystals in the above-mentioned crystallization conditions.

4. RESULTS

The action of cellular debris on calcium oxalate crystal development in synthetic urine, when batch conditions were used, is shown in Figure 4. As can be observed, when cellular debris were present at low amounts (< 100 mg/l), they exhibited inhibitory activity on calcium oxalate crystal nucleation. When increasing such amounts these effects decreased and primary aggregates of calcium oxalate monohydrate crystals developed on organic matter particles (Figure 5). On the other hand, the presence of cellular debris retained in a flow system provoked the calcium oxalate monohydrate crystallization at a rate of 2.8 µg/h per mg of organic matter. The calcium oxalate monohydrate crystals formed in these conditions are shown in the Figure 6.

The effects of pyrophosphate, phytate, citrate and pentosan polysulphate (a glycosaminoglycan) on calcium oxalate monohydrate heterogeneous nucleation on retained

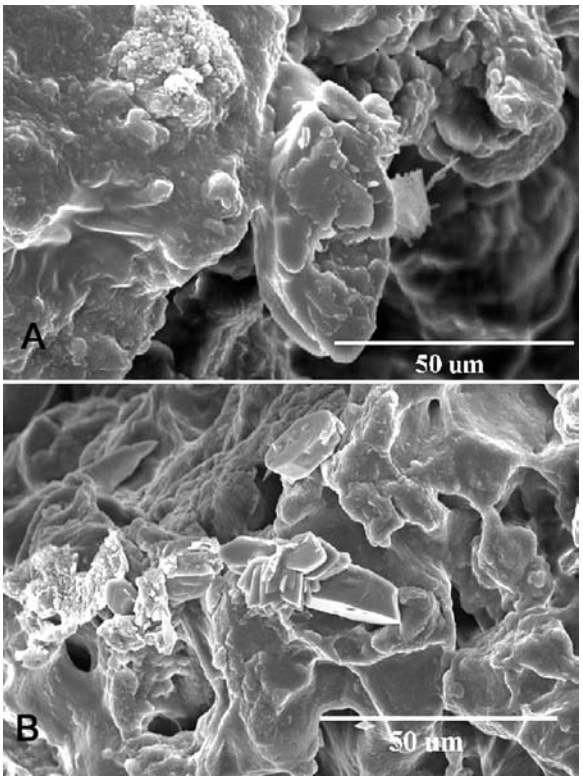

Figure 6. Images obtained by scanning electronic microscopy of the calcium oxalate monohydrate crystals (a) and primary aggregates (b) formed by heterogeneous nucleation on cellular detritus in the flow system. Experimental conditions: $[Ca^{2+}]$ = 3.5 10^{-3} M, [Oxalate] = 4.55 10^{-4} M, pH = 5.50, T = 37 ºC.

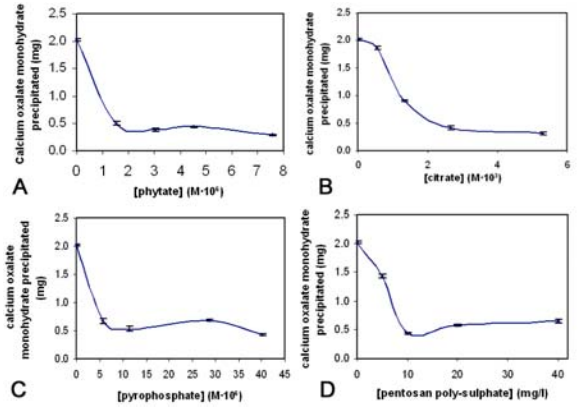

Figure 7. Inhibitory effect of phytate **(a)**, citrate **(b)**, pyrophosphate **(c)** and pentosan polysulphate **(d)** on the heterogeneus nucleation of calcium oxalate on cellular detritus, using a flow system. Experimental conditions: $[Ca^{2+}]$ = 3.5 10^{-3} M, [Oxalate] = 4.55 10^{-4} M, pH = 5.50, T = 37 ºC.

cellular debris have been studied in flow conditions. The results are shown in Figure 7. As can be seen, 5.74 μM (≈ 1 mg/l) of pyrophosphate caused a decrease of 67 % of the calcium oxalate crystallized on organic matter and no superior decrease was obtained when pyrophosphate increased over this concentration. 2.27 μM (≈1.5 mg/l) of phytate caused a decrease of 83 % of the calcium oxalate crystallized and also no further decrease was obtained when increasing this phytate concentration. The maximum inhibitory effects by citrate were obtained around 3.17 mM (≈ 600 mg/l) of this molecule with a decrease of 82 % of the calcium oxalate crystallized. Pentosan polysulphate (a semisynthetic glycosaminoglycan) also inhibited the calcium oxalate heterogeneous nucleation on cellular debris and maximum effects were observed in the presence of 10 mg/l of the macromolecule (78 % of decrease of the calcium oxalate crystallized). When increasing this glycosaminoglycan amount, the inhibitory effects slightly decreased.

5. DISCUSSION

From the presented results it can be observed how the cellular debris suspensions, at low amounts, exhibited some inhibitory capacity of the heterogeneous nucleation of calcium oxalate monohydrate crystals. This probably should be attributed to their glycoprotein content. Nevertheless, when increasing the amounts of cellular detritus the manifestation of promoter properties also increased, due to the formation of suspended organic matter agglomerates that act as heterogeneous nucleants of calcium oxalate monohydrate, allowing development of typical primary aggregates of these crystals (see Figure 4). This apparently contradictory behaviour as an inhibitor and promoter of these renal cellular debris can be easily explained if the nature of the crystallization inhibitory processes is considered. Thus, the crystallization inhibitory processes should be assigned to the interaction between inhibitors, probably glycoproteins in the present case, and calcium oxalate crystals. This interaction must be attributed to the affinity between these macromolecules and calcium ions. As a consequence of these interactions, disturbances in the nucleation and growth of crystals are produced and crystallization inhibitory effects are detected. Nevertheless, also due to the capacity to interact with calcium ions, if these macromolecules are integrated in bigger solid particles, then they can also behave as active heterogeneous nucleants, enhancing the formation of calcium oxalate crystals. In fact, this behaviour allows to justify the controversy of inhibitory or promoter properties of urinary proteins as Tamm-Horsfall glycoprotein (6, 7, 10, 11) or osteopontin (15). Hence, the Tamm-Horsfall protein in its monomeric form inhibits (16), whereas in its aggregated polymeric form, this property is reduced or changed into a promoter capacity (13). Osteopontin is a urinary protein, which inhibits calcium oxalate crystal growth in the seed crystal methodology (8, 9), but when osteopontin was immobilized on collagen granules, it promoted aggregation and adhesion of calcium oxalate crystals (15). In the present study it was clearly observed that when cellular detritus was retained in a cavity where urine flows, this organic matter effectively induced calcium oxalate crystal development (see Figure 5). Hence, the existence of renal cavities of very low urodinamic efficacy could be an important stone formation risk factor when urine contains important amounts of cellular debris that consequently can be accumulated in such cavities. Obviously, the action of

crystallization inhibitors that can avoid or minimize calcium oxalate crystal development then would be very important. According to the results obtained in the present paper, pyrophosphate, phytate, citrate and pentosan polysulphate effectively reduced calcium oxalate crystal nucleation on cellular detritus. Thus, 11.49 μM of pyrophosphate, 1.52 μM of phytate, 2.65 mM of citrate and 10 mg/l of pentosan polysulphate provoked a 75-80 % of decrease on the calcium oxalate crystallized. It is important to emphasize that the concentrations of these substances with important inhibitory capacity of calcium oxalate crystal development were of the same order as the concentrations normally found in urine, demonstrating the important role that the crystallization inhibitors can play in urolith development.

6. ACKNOWLEDGEMENTS

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