Urol Res (2007) 35:243–246
DOI 10.1007/s00240-007-0107-3

ORIGINAL PAPER

# The effect of traditional risk factors for stone disease on calcium oxalate crystal adherence in the rat bladder

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Received: 22 February 2007 / Accepted: 12 July 2007 / Published online: 1 August 2007


**Abstract** Crystal adherence in the urinary tract has been studied using the chemically injured rat bladder and cell cultures. These studies have provided evidence that mucin prevents adherence and have studied various compounds for their ability to promote or inhibit crystal adherence. Little work has been done examining the effect on crystal adherence of traditional risk factors for stone disease. The study reported here examined the effect hypercalciuria, hyperoxaluria and pH on calcium oxalate crystal adherence using the intact rat bladder model. Calcium at levels seen in hypercalciuric stone formers was associated with increased adherence. Oxalate at levels seen in stone formers had no effect on adherence. There was a tendency to increased crystal adherence at higher pH values only when phosphorus was present as the buffer. Hypercalciuria is a risk factor for stone disease by increasing the level of saturation of calcium oxalate and calcium phosphate in the urine and by decreasing inhibitor function. This study suggests that it may also play a role by increasing crystal adherence within the urinary tract.

**Keywords** Calcium oxalate · Crystal adherence · Mucin · Hypercalciuria · Hyperoxaluria

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## Introduction

Crystal adherence within the urinary tract is considered a necessary step in urolithiasis. To gain insight into the mechanism of adherence in the urinary tract, studies have been done in the bladder of intact animals [1, 2]. These studies chemically damaged the bladder and tested substances for their ability to restore resistance to crystal adherence. Although these studies provide insight into a role for mucin to prevent crystal adherence and allow examination of promoters and inhibitors of crystal adherence, they do not explain why crystals adhere in stone patients. In the study reported here, we examined the potential role of three traditional risk factors for stone disease to promote crystal adherence in the intact rat bladder.

Hypercalciuria increases the saturation of the urine for calcium oxalate and calcium phosphate and reduces the inhibitory activity of the urine for calcium oxalate precipitation [3]. Hyperoxaluria increases urinary saturation for calcium oxalate. Urine pH promotes uric acid precipitation in the acid range and calcium phosphate precipitation in the alkaline range. These abnormalities are common in stone formers but their effect on crystal adherence has received little attention.

## Materials and methods

Animal preparation was similar to a previous study examining the role of mucin to prevent crystal adherence [1]. Female Sprague–Dawley rats (225–250 g) were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg), the abdominal cavity opened and the ureters ligated to prevent urine from entering the bladder. The bladder was catherized with silicone tubing to a standard length and a

suture placed around the external urethral meatus to prevent leakage of material introduced into the bladder. The catherized bladders were irrigated twice with 0.25 ml of 0.9% NaCl and allowed to drain.

We reviewed calcium concentrations in the urine of stone formers seen in our clinic. The minimum level was 50 mg/l and the maximum was 300 mg/l. Thus we examined the effect of these and intermediate levels on crystal adherence. To isolate the effect of calcium from other urinary constituents, calcium solutions were prepared in 0.9% NaCl. A stock solution of 300 mg/l calcium solution was prepared by dissolving 120 mg $CaCl_2$ $2H_2O$ in 100 ml 0.9% NaCl. Levels of 50, 100, 200 mg/l were prepared by appropriate dilutions with 0.9% NaCl. Oxalate was examined at a concentration of 50 mg/l, as this is the upper limit of normal urine oxalate excretion in a urine volume of 1 l and levels above this have been shown to be toxic to renal tubular cells in culture [4]. The solution was prepared by dissolving 7.5 mg of $Na_2Ox$ in 100 ml 0.9% NaCl. The effect of pH at 4, 6, and 8 was examined using a TRIS buffer (10 mM TRIS in 0.9% NaCl) and a phosphate (8 g/l phosphate) buffer. Phosphate containing buffer was prepared by dissolving 3.45 gm $NaH_2PO_4$ and 3.5 gm $KH_2PO_4$ in 500 ml of 0.9% NaCl. The pH of these solutions was adjusted with HCl or NaOH as appropriate.

Test substances were introduced into mucin intact bladders for 15 min. Controls using 0.9% NaCl were run with each experiment. The bladders were allowed to drain and 0.125 ml of a suspension of radioactive calcium oxalate monohydrate crystals followed by 0.125 ml of 0.9% NaCl saturated with calcium oxalate were instilled for 15 min. In the pH experiments, the pH of the crystal suspension was adjusted to the pH studied. The bladders were allowed to drain and irrigated 12 times with 0.25 ml 0.9% NaCl to remove nonadherent crystals. Preliminary experiments had shown that this number of irrigations would yield a stable level of radioactivity in the final wash.

The method of Pak et al. [5] was used to prepare radioactive calcium oxalate crystals; 150 μCi of $^{14}$C-oxalic acid was added to a 10 mM solution of sodium oxalate. This solution was slowly added to a 10 mM solution of calcium chloride. Nonradioactive crystals prepared in this manner are calcium oxalate monohydrate by optical crystallography. The specific activity of the radioactive crystals from several preparations averaged $2 \times 10^5$ cpm/mg crystals. Sufficient crystals were placed in 3 ml of calcium oxalate saturated 0.9% NaCl to yield $3 \times 10^5$ cpm in 0.125 ml and homogenized with a glass piston homogenizer. The suspension of crystals was filtered through a 10-μm polypropylene filter (Gelman Sciences, Ann Arbor, Michigan, USA) yielding a suspension of crystals of 10 μm or less. Crystal suspensions were prepared daily and placed in a 25 ml Erlenmeyer flask and stirred continuously throughout the study. Preliminary studies showed that there was no significant loss of radioactivity to the solution during 8 h. The cpm instilled into the bladder was determined with each experiment by dissolving 0.125 mg of crystal suspension in 0.25 ml 1 N hydrochloric acid, followed by 3 ml NCS tissue solubilizer (Amersham International, Arlington Heights, IL, USA) and 15 ml OCS scintillation cocktail (Amersham International, Arlington Heights, IL, USA).

Bladders were removed, dried to a stable weight and placed in scintillation counting vials with 0.25 ml 1 N hydrochloric acid. After overnight digestion, 3 ml NCS tissue solubilizer was added. Bladders were heated to 50°C for several hours with periodic vortexing until dissolved and then cooled to room temperature. Fifteen milliliters of OCS scintillation cocktail was added and the specimen was counted along with the crystal suspension from that day in a LS 1801 liquid scintillation counter (Beckman-Coulter, Inc, Chaska MN).

The animal Care, Use and Research Studies Committee at Hennepin County Medical Center approved this study. Data are expressed as the percent of retained cpm in the bladder since this reflected the same changes seen when expressed as cpm/mg dry bladder weight. Data are shown as the mean ± SEM. Because of the variable sample size and relative inequality of variance between the groups, statistical significance was assessed with nonparametric procedures. The presence of significant differences between groups was confirmed using the Kruskal–Wallis *H* test. The Mann–Whitney *U* test was employed to further discern significant differences between the control and other groups. All procedures were completed using SPSS v. 13 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was assumed at $P \leq 0.05$.

## Results

Calcium concentrations studied were from 50 to 300 mg/l. Table 1 shows that compared to control, levels of 200 and 300 mg/l significantly increased crystal adherence.

The effect of oxalate was examined at a concentration of 50 mg/l, a level seen in stone formers but not toxic in renal cell cultures. Crystal adherence was 0.4 ± 0.1% ($n = 5$) in the oxalate treated animals and 0.4 ± 0.2% ($n = 5$) in control studies.

**Table 1** Effect of various calcium concentrations on crystal adherence in the mucin intact rat bladder (mean ± SEM)

| Control | 50 mg/l | 75 mg/l | 100 mg/l | 200 mg/l[a] | 300 mg/l[b] |
|---|---|---|---|---|---|
| 1.6 ± 1.1 | 1.6 ± 1.5 | 2.4 ± 2.4 | 3.4 ± 2.7 | 3.9 ± 3.4 | 4.1 ± 3.0 |

[a] $P < 0.05$

[b] $P < 0.001$

Urine pH when fixed in the acid range or the alkaline range is a risk factor for stone disease. The effect of pH was examined first using TRIS as a buffer. As seen in Table 2 there was no effect of pH on crystal adherence with this buffer. However, when phosphorus was present as the buffer, there was a tendency to increasing adherence with higher pH values although this did not reach statistical significance.

## Discussion

Calcium oxalate is the predominant component of most kidney stones. Urine is supersaturated for calcium oxalate in stone formers as well as non-stone formers [6] and the presence of calcium oxalate crystals is a common finding in both groups [7]. If these crystals pass in the urine, stone disease does not develop. If the crystals remain in the urinary tract and are exposed to urine supersaturated with calcium oxalate and/or calcium phosphate, stone disease can develop. Therefore, crystal adherence is considered an important step in the development of stone disease.

Studies of crystal adherence in the urinary tract have used two models. One model involves the bladder of intact animals [1, 2, 8]. A potential limitation of this model is that studies are done in the bladder and not in the upper urinary tract where most stones form. However, histological studies (unpublished observation) show that there is a continuous mucin layer from the terminal collecting duct and the papillae to the bladder and these tissues have a common embryological origin. This suggests that findings using this model may have application to the upper urinary tract. Studies have focused on the ability of the uroepithelium to inhibit crystal adherence and have suggested that the mucin present on the surface of the uroepithelium plays a role. Crystal adherence was significantly prevented when the mucin lining was left intact. Chemically removing the mucin layer led to an increase in adherence. Dermatan sulfate restored the ability of the chemically altered bladder to inhibit crystal adherence and sialic acid promoted adherence [1].

The second model uses renal tubular epithelial cell cultures. Initial studies showed that these cells were susceptible to crystal adherence [9, 10]. However, later studies found that this susceptibility was cell type and culture condition specific [11]. MDCK-1 cells have characteristics of collecting duct cells, MDCK-2 of mixed proximal and distal tubules and LLC-$PK_1$ of proximal tubular cells. MDCK-1 cells are representative of that portion of the nephron most likely to be exposed to crystals and when cultured on permeable supports obtain a high degree of differentiation and polarization. When confluent, the cells were resistant to calcium oxalate adherence much like the intact bladder. MDCK-2 cells and LLC-$PK_1$ cells showed greater adherence. Nonconfluent cultures, as well as disruption of confluent cultures, resulted in increased adherence [12]. These studies suggest that MDCK-1 cells have the ability to inhibit the adherence of calcium oxalate crystals that is dependent on an intact layer of cells that have normal polarity.

**Table 2** The effect of pH on crystal adherence in the mucin intact rat bladder (mean ± SEM)

| Buffer | pH 4 | pH 6 | pH 8 |
|---|---|---|---|
| Phosphorus | 1.6 ± 1.1 ($n$ = 6) | 2.8 ± 1.6 ($n$ = 7) | 3.4 ± 2.1 ($n$ = 10) |
| TRIS | 0.3 ± 0.1 ($n$ = 3) | 0.5 ± 0.3 ($n$ = 3) | 1.2 ± 0.4 ($n$ = 3) |

Uroepithelial injury is listed as a risk factor for stone disease. Injuring the uroepithelium in either the intact bladder model or in the cell culture model promotes crystal adherence. Most stone formers, however, have no recognized injury to the uroepithelium that could promote crystal adherence. This led us to examine whether common risk factors for stone disease, namely hypercalciuria, hyperoxaluria and pH, could interact with the intact uroepithelium to promote crystal adherence.

The effect of calcium concentrations seen in normals to that seen in hypercalciuric stone-formers was examined. Increased adherence was present at concentrations of 200 and 300 mg/l. Similarly in MDCK-1 cell cultures, calcium concentrations up to 1,000 mg/l resulted in increased adherence [13].

The mechanism by which calcium might increase adherence has not been examined. One possibility involves negatively charged sites found on the uroepithelium. Sialic acid and annexin II are negatively charged, present on the uroepithelium and have been shown to promote crystal adherence [1, 14, 15]. These molecules could bind calcium in the urine that could then bind to available sites on calcium oxalate crystals. Another possible mechanism has to do with the role of mucin. Mucin protects cells from the environment and this protective function is related to its viscoelastic property. Calcium binds to mucin resulting in a decrease in its viscosity [16, 17]. This decrease in viscosity could potentially lead to a decrease in its ability to inhibit crystal adherence. Calcium also alters the fluidity of cell membranes and decreases in membrane fluidity have been linked to increased crystal adherence [18].

Oxalate has been suggested to be toxic and potentially injurious to the uroepithelium. Experimentally induced hyperoxaluria in intact animals leads to enzymuria and lipid peroxidation suggesting renal tubular damage [19]. Both apoptotic and necrotic cell death follow oxalate challenge in cultures of LLC-PK1 cells [20]. Most of these studies have used oxalate concentrations seen only in hyperoxaluric states such as ethylene glycol intoxication or primary

hyperoxaluria and have not shown toxicity at levels most likely present in normals and stone formers [4]. Oxalate in this study was examined at a concentration seen in patients with upper limit normal oxalate excretion but low urine volume and a level that is not toxic in cell cultures. At this level there was no effect on adherence.

Urine pH is a risk factor for stone disease if it is consistently low (uric acid stones) or consistently high (calcium stones). Studies of calcium oxalate monohydrate crystal adherence in cultures of MDCK-1 cells showed greater adherence at pH 5.1 than at pH 8.1 [21]. The buffer used in this study contained a low level of phosphorus and the primary buffer was HEPES. Similar results were found in a second study using TRIS as the buffer [13]. In the current study we found no statistically significant effect of pH on crystal adherence using TRIS as the buffer. When phosphorus was the buffer, there was a tendency to increased adherence as the urine pH increased but this did not reach statistical significance. These findings differ from those in the cell culture model. This may be due to differences in the models. It is possible that cell cultures are damaged by a low pH resulting in increased adherence. The rat bladder with its intact mucin layer may not be as susceptible to pH damage. The difference in buffers may also play a role.

In conclusion, this study supports a role for an intact layer of mucin to prevent crystal adherence. Oxalate at the concentration studied had no effect of crystal adherence. An alkaline pH in the presence of phosphorus showed a possible effect on crystal adherence although it did not reach statistical significance. Calcium at levels seen in hypercalciuric stone formers led to an increase in crystal adherence. Hypercalciuria may then promote stone disease by three mechanisms: (1) increasing the saturation of the urine for calcium oxalate and calcium phosphate leading to the precipitation of crystalline material, (2) decreasing the inhibitor capacity of the urine, and (3) promoting crystal adherence.

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