Lessons from a Stone Farm

John P. Kavanagh and P. Nagaraj Rao

Department of Minimally Invasive Urology and Stone Management, South Manchester University Hospitals Foundation Trust, Manchester, M23 9LT, UK

Abstract. The stone farm is a system for measuring macroscopic stone growth of 12 calcium stones simultaneously. It is based on mixed suspension, mixed product removal continuous crystallization principles and the stones are grown continuously for about 500 hours or more. The growth of the stones follows a surface area dependent pattern and the growth rate constants are very similar irrespective of whether the starting materials are fragments of human stone or pieces of marble chip. Increasing citrate from 2mM to 6mM caused a significant growth inhibition which persisted in the presence of urinary macromolecules. Phytate was a very effective inhibitor (about 50% at sub-μM concentrations) but the effective concentration was increased by an order of magnitude in the presence of urinary macromolecules. The effective concentration for inhibition in a crystallization assay was a further two orders of magnitude higher. Urinary macromolecules or almost whole urine were also strongly inhibitory although neither human serum albumin nor bovine mucin had any great effect. The relationship between the size distribution of crystals in suspension and the stone enlargement rate suggests that the primary enlargement mechanism for these in vitro stones is through aggregation. The stone farm is a powerful tool with which to study crystallization inhibitors in a new light. Some differences between inhibition of crystallization and inhibition of stone growth have emerged and we have obtained quantitative evidence on the mechanism of stone enlargement in vitro. Our findings suggest that the interface between crystals in suspension and the stone surface is the key to controlling stone enlargement.

Keywords: Urolithiasis, calcium oxalate, crystallization, calculi
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INTRODUCTION

Crystallization studies have been the mainstay of in vitro urolithiasis research with the underlying assumption that what is learnt from these investigations is applicable to stone formation and growth in vivo. With a view to bridging the gap between these two extremes, we have developed a system for growing multiple calcium stones in vitro. This is a continuous flow system, based on mixed suspension mixed product removal (MSMPR) principles. Fresh feed solutions are provided on a daily basis while the stones are growing and we refer to the system as a stone farm.

Our goal was to achieve macroscopic stone growth in an urine like environment which might be analogous to a residual fragment enlarging in the renal spaces. We found that this could be achieved by placing a small stone fragment in the small scale MSMPR crystallization chamber which we have previously developed to study
crystallization kinetics in nearly whole urine [1]. In our pilot studies we grew individual stones [2] and while this proved the possibility of achieving our goal, the system was not suitable for making quantitative comparisons between different growth conditions. We therefore scaled the system up to grow 12 stones at once [3] and here we review our experience and findings with the stone farm.

MATERIALS AND METHODS

The Stone Farm

The details of the construction and operation of the stone farm have been given elsewhere [3]. Here we describe the system with the focus on incidental details. Twelve stone fragments (~100mg) are separately suspended in twelve identical crystallization chambers, each with a working volume of 20ml, maintained at 37°C by external water circulation and stirred by PTFE coated magnetic fleas rotating at 400rpm. These chambers are organised as six pairs, one pair of which is shown in Fig. 1. The surfaces in contact with the feed solutions are glass, stainless steel or PTFE. It was not practicable to maintain a completely closed system throughout the experimental period so the following precautions against infection were used. Freshly purified water was filtered (0.22μm) before being used to make up the salt solutions. Once fully dissolved and made up to the correct volume these were dispensed into sterile bottles, again through 0.22μm filters and in a sterile atmosphere. The artificial urine (AU) solutions were stored at 4°C for up to 5 days. In use, the air inlets to the feed solution bottles were protected by 0.22μm filters. The likelihood of contamination is greatest in the crystallization chambers which are opened daily. We therefore include 0.04% NaN₃ in one of the feed solutions. We never saw any evidence of infection such as cloudiness of stock solutions or unexpected changes in pH of the mixed suspension in the crystallization chambers. Details of the composition of the feed solutions making up the AU urine are described elsewhere [3] but our standard conditions give rise to 6mM Ca and 1.2mM oxalate, pH 6.0 in the crystallization chamber (before any precipitation takes place). The flow rate through each pair of crystallizers was approximately 1 litre per day. Because of the large volumes being prepared, we measured the added water to the salts gravimetrically, taking into account the specific volume of the included salts. Where additions to the feed solutions for half the crystallization chambers were made, we did this by dividing batches of feed solutions in two and then making the addition, rather than preparing separate feed stocks. Feed solutions were replenished each day. At this point the stones and their supports were removed and the tubing and chambers were cleaned by flushing with 0.1M HCl followed by copious purified water. In-line syringes are fitted to enable purging of the lines and priming of the crystallization chambers with fresh feed solutions. Stone masses were measured every two or three days; they were carefully placed and rolled on absorbent paper and allowed to air dry for 15 minutes before being weighed. Between different experiments all tubing was replaced and the crystallization chambers and stone supports were cleaned with chromic acid.
In initial experiments the stone fragments used as the core of the growing stones were derived from human stone fragments. In later experiments we used pieces of marble chips (CaCO₃). All the cores were ground to about 100mg with abrasive paper. Their initial masses were obtained after soaking in AU for one hour before being dried as described above. Most stone growth experiments were conducted with the stones in 3 of the pairs of crystallizers receiving control feed solutions and the other 3 pairs receiving the same solutions supplemented with a test agent. In some cases the solutions feeding each set of 3 crystallizers was changed during the course of the experiment. In all cases the primary outcome was considered to be the comparison between the 6 control stones and the 6 supplemented stones while being grown simultaneously. As will be shown below, the growth followed in a surface-dependent manner and the results from the 6 stones growing under identical conditions were averaged and fitted to a growth equation, mass = k * time^{3/2} + c (where k is a growth rate constant and c is the initial mass). These are shown in the figures as ±95% confidence intervals (c.i.), derived from the curve fitting procedure (performed with FigSys, Biosoft, Cambridge, UK).

**Urinary Macromolecules And Nearly Whole Urine**

When urinary macromolecules (UMM) were used, these were obtained by tangential diafiltration across a filter with a nominal cut-off of 5kD. Urine was reduced in volume to 25% of its starting volume and then diafiltered with purified water until the conductivity was ≤1mS/cm [4]. This was stored frozen at -20°C until required. For use, sufficient UMM for one day was thawed and pooled and this was combined 1:1 with a feed solution (containing calcium) made up in half its usual volume. In this way, UMM are included at approximately their native concentration (without making allowance for any processing losses) and without the concentration of the major ions being altered.
When nearly whole urine was used it was stored at -20°C immediately after collection. For use, that sufficient for one day was thawed, centrifuged briefly (2000g for 5 minutes) and passed through a nylon sieve (20μm). Its pH was adjusted to 6.0 and its calcium adjusted to 6.67mM and 0.02% NaN₃ added. This urine was fed into the crystallizer chambers at 90% of the total flow, while the remainder of the flow was delivered by a 12mM oxalate solution in calcium deficient AU.

**Crystal Size Distribution**

The size distribution of crystals in suspension around the growing stones was examined just prior to changing feed solutions each day. A Coulter Multisizer was used with a 100μm orifice tube. The system was calibrated with 14μm diameter latex beads. Three consecutive readings of 1ml aspirations were averaged for each of the crystallization chambers. The particle number versus particle diameter data was transformed to ln(particle no.) versus particle size and the best fit straight line fitted to the data. The growth rate and nucleation rates were calculated from the slope and intercept of this line respectively [5].

**RESULTS**

**The Surface**

The raw data for the growth of twelve stones in one experiment are shown in Fig. 2A and the average for each arm of the same experiment in Fig. 2B. It is clear that the growth is not linear but gently accelerates. Analysis of the growth of 8 individual stones had previously led us to conclude that the growth is surface area dependent (i.e. mass = k * time$^{3/2}$ + c) [2]. This was based on comparing the goodness of fit of the data to a linear model (constant deposition, mass = k * time + c), a linear growth in diameter model (mass = k * time$^3$ +c) and the surface area growth model. We have repeated this analysis using the control data from 10 experiments (i.e. growth of 60 stones). The correlation coefficients for the surface area model were consistently higher than that of growth versus time or time$^3$ ($R^2 = 0.99$ vs 0.96 or 0.93 respectively). The best fits to the data from Fig. 2A are shown in Fig. 2C and the fits of the three different models to the control data are shown in Fig. 2D.

The growth of control stones in AU was relatively consistent between experiments when the core material was human stone fragments and even more so when marble chips were used as the core (Fig. 3). The slightly lower and more consistent growth with marble chips can probably be explained by their being slightly smaller and smoother than the human stone fragments.

While we had considered the possibility that the composition of the core material might influence the outcome of these experiments it has become clear that once an initial coating has been laid down, the central material no longer plays any part in the subsequent growth.
FIGURE 2. Open circles are control data (artificial urine); closed circles have 0.25μM phytate included. A, raw data, showing all 12 growth profiles; B, means ± s.d.; C, mean data with best fit to the surface area model; D, control data with best fits to surface area model (solid line) compared to linear growth and constant deposition models (dashed lines).

Citrate

The control AU contains 2mM citrate. When this was increased to 6mM there was significant inhibition and this was also the case when UMM from stone formers or healthy controls were also included (Table 1) and see [4] for more details.

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<th>2mM citrate</th>
<th>6mM citrate</th>
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<tr>
<td>AU</td>
<td>0.01078 ± 0.00058</td>
<td>0.00230 ± 0.00024</td>
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<tr>
<td>AU + UMM (MC)</td>
<td>0.00405 ± 0.00025</td>
<td>0.00143 ± 0.00013</td>
</tr>
<tr>
<td>AU + UMM (MSF)</td>
<td>0.00271 ± 0.00011</td>
<td>0.00107 ± 0.00008</td>
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Phytate

Inositol hexaphosphate (phytate) is reported to be an effective calcium oxalate and calcium phosphate crystallization inhibitor, *in vitro* and in simple salt solutions [6-9]. Based on these reports, we chose 5μM as our starting concentration to test phytate in the stone farm. This gave almost complete inhibition in AU. Reducing the phytate to 2.5μM also gave nearly 100% inhibition and only by decreasing the concentration to 0.25μM did we begin to see a dose dependent effect with the inhibition being 51% and 55% in two separate experiments (Fig. 4). When we performed an experiment with 0.25μM phytate in AU in which UMM from healthy male controls was included, we found no evidence of any inhibition of stone growth and it was only by increasing the phytate to 2.5μM that we saw evidence of growth inhibition. This amelioration of the inhibition of phytate by UMM was not simply a non specific protein effect as 0.25μM phytate was almost equally effective in the presence of albumin at 100μg/ml (40% inhibition) as with artificial urine alone (Fig. 4). Similarly, we also found that, in an *in vitro* crystallization assay [10], the effective concentration of phytate in artificial urine was about one order of magnitude lower than in human urine [11]. Interestingly, the concentration required for equivalent effects on stone growth and crystallization activity was about 2 orders of magnitude lower for both stone growth in artificial urine and stone growth in artificial urine plus UMM (compared to human urine) [11].

Macromolecules

Human serum albumin and mucin at 100μg/ml and UMM at approximately native concentrations, all in AU and 90% human urine were tested in a series of eight stone
farm experiments; in each case the contemporaneous control was artificial urine. This showed that neither albumin nor mucin had any significant effect, while UMM and nearly whole urine almost completely abolished stone growth (Fig. 5). In six other experiments, macromolecules in artificial urine were used as control arms of experiments testing, for example, the effects of citrate or phytate. The results of these reinforce the above conclusion, especially when taking into account another six AU control experiments (Fig. 5).

**FIGURE 4.** Growth rate constants (± 95% c.i.) for control stones grown in AU (open circles) and with added phytate (closed circles). UMM are urinary macromolecules.

**Enlargement Mechanism**

There are essentially two mechanisms by which stones could enlarge (or by a combination of both). These are through direct crystal growth at the stone surface or by aggregation and incorporation of crystals which developed in suspension [12]. If the first of these were the principal mechanism then it might be expected that, when conditions favor crystal growth, then there will be a lower nucleation rate [5, 13] and crystal growth at the surface would be high and stone enlargement maximised. Conversely, if conditions favor low growth rate (and a high nucleation rate) of suspended crystals, then the crystal growth at the surface would also be slow. Under either of these circumstances we would expect to observe a positive correlation between stone growth rate and crystal growth rate and a negative correlation between stone growth rate and nucleation rate. If the second of these mechanisms (i.e. aggregation) predominates, then the opposite would be expected, namely that the stone
growth rate would be positively correlated to the nucleation rate and inversely correlated to the crystal growth rate.

FIGURE 5. Growth rate constants (± 95% c.i.) for control stones grown in artificial urine (AU) (open circles) and with added macromolecules (solid symbols). HSA is human serum albumin, HU is human urine, MC are male controls, MSF are male stone formers, CF are cystic fibrosis patients and UMM are urinary macromolecules.

In order to try to discriminate between these two mechanisms we have examined the relationships between stone growth rate, crystal growth rates and crystal nucleation rates over a period of 26 different growth periods. These involved a series of AU experiments in which control stones were grown with 6mM calcium and 1.2mM oxalate as the input concentrations and test stones were grown with various lower calcium and oxalate concentrations. Although not all of these experiments were contemporaneous, they were all carried out with marble chips as the core material, with which the inter-experimental variability is relatively small. We knew from previous work [5] that changing the input concentrations of calcium and oxalate would bring about a variation in the crystal size distribution of the suspended crystals which, in turn, would correspond to different crystal growth and nucleation rates. We observed significant negative correlation between nucleation rate and crystal growth rate ($p<0.001$) (Fig. 6A), consistent with our previous findings [5, 13] and a significant positive correlation between stone growth rate and crystal nucleation rate.
(p<0.001) and a significant negative correlation between stone growth rate and crystal growth rate (p=0.02) (Fig. 6B and C).

This suggests that the primary enlargement mechanism for stones grown in the stone farm is through aggregation rather than direct surface growth.

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\text{CONCLUSIONS AND IMPLICATIONS}
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For the first time, we are in a position to grow calcium stones reproducibly, \textit{in vitro}, under controlled conditions. The stone farm is suitable for making quantitative measurements and comparisons and we can use the system with nearly whole urine or incorporate urinary macromolecules. In the sense that it operates as a continuous flow system, with a realistic flow rate and solution supersaturation, it is a representative model of the renal system. On the other hand, the residence times are longer than would be the case in a kidney (because the volume of the crystallization chamber is larger than the renal spaces) and the input amount of oxalate is above what would normally be found in urine. Whatever its strengths and weaknesses, it is only an \textit{in vitro} model and any conclusions must be tempered by this recognition. Nevertheless it does act as bridge between purely \textit{in vitro} crystallization systems and the \textit{in vivo} pathological process we are trying to probe.

While the stone farm findings with citrate confirmed the inhibitory activity of citrate they did not reveal any particularly new insights. The results with UMM, nearly whole urine, albumin and mucin are also broadly in line with expectations derived from crystallization experiments, but it is worth noting just how difficult it is to get significant stone enlargement with urinary constituents present. Taken together with other findings from the stone farm and MSMPR crystallization studies we can conclude that this inhibition is effective at the stone surface in addition to any solution/suspension effects.

Our results on the dependence of the enlargement rate on the surface area and the primary importance of aggregation over growth as the accretion mechanism complement each other and are consistent with a two stage aggregation process of
collision and consolidation. The likelihood of collisions will be governed by the suspension crystal density and the exposed surface area of the stone, while the efficiency of converting collisions into consolidated material will reflect the ability to form a mechanical bridge [14, 15]. In previous MSMPR crystallization studies under very similar conditions to the stone farm with nearly whole urine, there was a significant crystal density despite the strong inhibition compared to AU [5, 13]. The almost complete elimination of stone enlargement by nearly whole urine therefore suggests that macromolecules from urine prevent suspended crystals that come into contact with the stone from permanently fusing to the surface.

We can similarly argue that the pronounced stone growth inhibition by phytate is also achieved by reducing the efficiency of consolidating suspended crystals into the stone, rather than being simply a consequence of crystallization inhibition. This conclusion follows from our observation that crystallization inhibition requires a much higher concentration of phytate than is effective in inhibiting stone enlargement [11]. A simple explanation for the amelioration of the response to phytate by UMM also follows from the concept that both phytate and UMM are effective because they are active at the interface between colliding crystals from the suspension and the stone surface. If the UMM are sufficient to partially or nearly saturate all available sites, then a much higher phytate concentration will be required for further effective inhibition than if the UMM were absent.

These in vitro stone growth experiments demonstrate that stone enlargement, while necessarily involving crystallization, is not simply a direct reflection of that crystallization activity. Stone enlargement is also critically dependent on the interaction at the interface between suspended crystals and the stone surface.

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**REFERENCES**