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A stone farm: development of a method for simultaneous production of multiple calcium oxalate stones in vitro

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Abstract We have previously shown how individual calcium oxalate stones of about 1 cm can be grown in vitro. While this proved a design concept, it was severely limited as an experimental tool because of the time required to undertake comparative studies. Here we describe a development of this system in which six parallel pairs of stone generators are supplied with feed solutions generating a medium that is supersaturated with calcium oxalate. Twelve stones were grown simultaneously in aseptically prepared artificial urine over a period of 32 days from 100 mg to about 250 mg. Flow rates, pH and $[Ca^{2+}]$ were stable and reproducible over the course of the experiment. Sodium azide (0.02%) was included in the growth medium of six stones and caused a modest decrease in growth rate from 5.5 to 3.4 mg/day. The experimental design is such that this was readily detectable both visually and statistically ($p < 0.001$). This multiple stone growing system (''a stone farm'') shows improved consistency and illustrates the statistical power of the technique. Azide has only a minor effect on the growth kinetics and can be used as an antibacterial agent in studies involving urinary macromolecules. The technique is suitable for practical and meaningful investigation of calcium oxalate stone formation in vitro.

Keywords Calcium · Calculi · Oxalate · Urolithiasis

Introduction

Most kidney stones have calcium oxalate (CaOx) as their primary component and many valuable urolithiasis studies have involved in vitro crystallization of CaOx. These experiments have been performed in inorganic solutions or urine and sometimes with cells in culture. The rationale for such studies is that they can be extrapolated to tell us something about stone formation in vivo. This is a questionable assumption, which has been described as a ''leap of faith'' [1]. Although stone formation may begin with crystallization, it is known that crystalluria occurs in healthy subjects with no evidence of stones [2]. To date, there has been no direct experimental evidence to link the processes behind crystallization and actual stone growth. Animal models have been helpful, as they appear closer to the natural process we are trying to understand [3], but these systems also suffer from a number of drawbacks. In particular, it is not possible to control the environment at the site of the developing calculus. There is therefore a need for an in vitro model system that will bridge the gap between microscopic crystallization and macroscopic stone formation.

We have previously described a method for in vitro CaOx stone generation which is based on the mixed-suspension, mixed-product removal (MSMPR) continuous crystalliser. This enabled us to grow CaOx stones in artificial urine (AU) to a clinically significant size (≈1 cm in diameter) and we showed that their growth rate was related to their surface area [4]. In this study [4], results on eight stones were presented, each one grown individually over periods of 4–16 days. These experiments proved the design concept for in vitro CaOx stone formation but were of limited value on a number of grounds. The main drawbacks were restriction of the aqueous medium to a defined inorganic solution, the unsuitability of the system for direct comparative studies and the time taken to produce enough stones to establish useful quantitative estimates of growth rates. Pilot studies [5] employing this method and including urinary macromolecules (UMM) gave further encouragement for this approach by showing that UMM caused a decrease in growth rate and promotion of a stone with greater structural integrity. However, this study did not

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take account of the possibility of infection. Here we address the deficiencies of the single stone generator and report on the refinement of this method and development for growth of multiple stones simultaneously (''a stone farm'').

Materials and methods

The MSMPR crystalliser for growth of individual stones [4] formed the basis of the system for simultaneous production of 12 stones (the stone farm). Six pairs of crystallization chambers were set up in parallel. Each pair consists of one of the previously described units, the outflow from which acts as the feed stock for a second unit. The details of the construction and the flow path are shown in Table 1 and shown diagrammaticaly in Fig. 1. The jacketed chambers [6] were made to special order (Radleys, UK). Temperature control of the chambers was by water circulation (Haake, DC10), taking care to keep the tubing length for each pair of chambers the same. At equilibrium, the chamber contents were at 37°C ($\pm$0.1°C). The chambers were closed to the atmosphere, but not hermetically sealed. Three 8-channel, peristaltic pumps (Watson Marlow, 205S) were used to control the flow through the system (each pump serves two pairs of chambers). The pumps were set to deliver the feed solutions at about 0.3 ml/min (8.5 rpm). Take-off tubes were positioned to control the chambers' volume at 20 ml. Their diameter was such that they could pump at a higher rate than the incoming feed. The chambers were located on individual magnetic stirrers (Electrothermal) with digital display of motor speed, set to 400 rpm. Each chamber contained a support for the growing stone, consisting of a concave PTFE disc (4×20 mm diameter, with a 2-mm diameter central hole) which was suspended from the chamber lid by three rods of PTFE sleeved stainless steel. The base of the support was 20 mm above the chamber bottom.

The first of each pair of chambers received two feed solutions at the same flow rate. Feed solution I was made up of 12 mM $CaCl_2$, 6 mM $MgCl_2$ and 4 mM K_3Citrate. Feed solution II contained 300 mM NaCl, 151 mM KCl, 40.4 mM $(NH_4)_2SO_4$, 10.2 mM Na_2HPO_4, 38.8 mM NaH_2PO_4 and 2.4 mM Na_2Oxalate (all chemicals of Analar grade, from VWR International; the water was distilled and further purified to a resistivity of 18 MΩ). Under aseptic conditions, feed solutions were filter sterilised (0.2 μm) into sterile glass bottles and stored at 4°C for up to 8 days. Air inlets for the feed solutions were protected by 0.2 μm hydrophobic filters. Mixing solutions I and II in equal proportions gives an artificial urine of pH 6.0, with essentially the same composition as used in earlier studies [6, 7, 8]. The salts were weighed out to within $\pm$0.1% and final volumes controlled gravimetrically to a tolerance of $<$0.05%, taking into account the densities of solution I and II (1.00 and 1.024 g/cc, respectively, determined empirically). Each batch of AU prepared was sufficient for 3 days. All the feed solutions in use at any one time were prepared from the same batch. NaN_3 was included (aseptically) in solution I (0.04% w/w) being supplied to three of the six pairs of chambers. Waste reservoirs were emptied daily and contained disinfectant (Virkon, VWR International) sufficient to make a 1% solution.

Twelve small fragments of renal stone from an individual patient (major constituent, calcium oxalate) were adjusted to 100 mg $\pm$2 mg, using abrasive paper. Their precise, wetted weight was obtained after soaking in artificial urine for 1 h and drying with absorbent paper. These fragments were used as the starting material in each crystallization chamber. They were positioned on their supports and the pumps were run for about 22–23 h, at which time the stones were removed and the system was flushed with 0.1 M HCl (to remove any encrustation), rinsed thoroughly with water before restarting with fresh feed solutions by priming each chamber with 10 ml of solution I and 10 ml of solution II and the stones replaced. Actual daily flow rates from each feed solution reservoir were calculated by weight change over the time the pumps were running. Approximately every other day, during the change of feed solutions, the stones were dried on absorbent paper and weighed. On most days, the pH and ionised calcium concentration ($[Ca^{2+}]$) of the contents of each chamber were measured (specific ion electrode) just before the change over period. $[Ca^{2+}]$ calibration standards were made up using the same stock of $CaCl_2$ used to make up feed solution I, dissolved in 0.15 M NaCl. Over 32 consecutive experimental days, the elapsed growing time was 716 h 20 min (29.85 days). Mean cross-sectional surface areas of the stones before and after the experiment were estimated from photographs using a graphics tablet and diameters were calculated as though this area was a circle.

The number of stones available does not permit us to assess objectively the data distributions. Statistical analyses over a number of time points have been taken to be normally distributed and, more conservatively, we have used non parametric tests to compare end points. Examination of pH, $[Ca^{2+}]$ and weights over time

Table 1 Details of flow path through one pair of crystallization chambers

Construction	Component	
0.2 μm, hydrophobic	Filter	Filter
PTFE 50×2.5*	Vent tube	Vent tube
500 ml glass	Reservoir, feed solution I	Reservoir, feed solution II
PTFE 200×2.5	Transfer tube	Transfer tube
Polycarbonate	3 way valve = 10 ml syringe	3 way valve =10 ml syringe
PTFE 300×2.5	Transfer tube	Transfer tube
PVC 430×1	Pump tube	Pump tube
PTFE 300×1	Transfer tube	Transfer tube
20 ml glass	Chamber A	
Water jacketed 37°C	Temperature controlled	
Magnetic stirrer 400 rpm	Stirred	
PTFE	Stone support	
PTFE 450×2.5	Take-off/transfer tube	
PVC 430×2.3	Pump tube	
PTFE 450×2.5	Transfer tube	
20 ml glass	Chamber B	
Water jacketed 37°C	Temperature controlled	
Magnetic stirrer 400 rpm	Stirred	
PTFE	Stone support	
PTFE/PVC 10×2.5/500×2.8	Take-off/transfer tube	
PVC 430×2.8	Pump tube	
PVC 1000×2.8	Transfer tube	
	Waste reservoir	

*Tubing dimensions are length × internal diameter (millimetres)

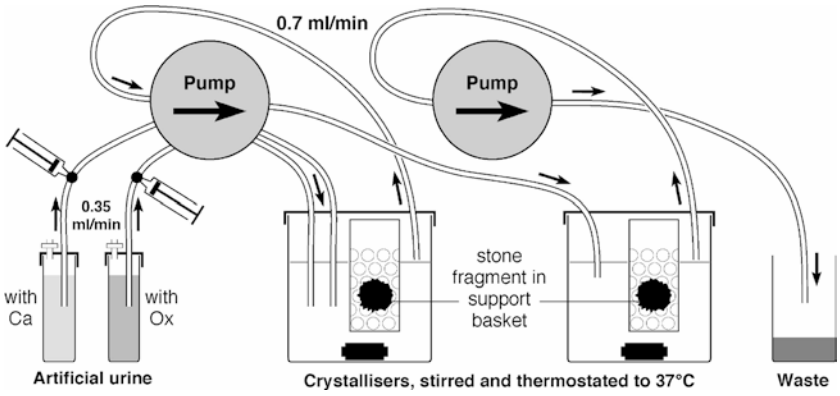

Fig. 1 Diagrammatic representation of one of the pairs of stone generators

(within-subjects variables), distinguishing between different chambers and presence or absence of azide (between-subjects factors) were performed using a general linear model with repeated measures (SPSS version 10.1). Individual stone growth rates were estimated by linear regression. Cross-sectional areas and diameters of the stones and their weight at the end of the experiment were compared by Man-Whitney U tests.

Results

Over 32 continuous days, the mean flow rate for the 12 channels was 0.307 ml/min with an intra feed-reservoir c.v. of $\leq 2.9\%$ and a daily inter feed-reservoir c.v. of $\leq 3.3\%$. There was no difference in flow rate between feed-reservoirs I and II ($p = 0.178$), between reservoirs supplying azide treated stones and controls ($p = 0.831$) or any significant change over the course of the experiment ($p = 0.050$) (Fig. 2).

The pH values in the 12 crystallization chambers, measured on 23 of the 32 days, ranged from 5.93 to 6.20. There was a statistically significant ($p < 0.001$) rise in pH during the course of the experiment but this amounted to an average of only 0.0008 pH units/day and 0.026 pH units overall. The pH of the azide-containing chambers was slightly lower than those without azide (means ± s.d. of 6.00 ± 0.04 and 6.07 ± 0.06, respectively, $p = 0.006$) (Fig. 3) and the pH of the second chamber of each pair was marginally higher than the first (means ± s.d. of 6.06 ± 0.07 and 6.01 ± 0.04, respectively, $p = 0.032$).

The ionised calcium in the chambers did not differ between azide treated or untreated ($p = 0.343$) or between the first and second chambers of each pair ($p = 0.696$). There was, however, a very small but significant ($p = 0.032$) change over time, amounting to an average decline of 0.01 mmol/day (< 1 part in 200) (Fig. 4).

The initial weights of the 12 stone fragments ranged from 98.5 to 102.6 mg with a mean of 101.4 mg (± 1.2, s.d.). The mean weight of the six azide-treated stones after 32 days of growth (elapsed time = 29.85 days) was 199.9 mg compared with 268.1 mg without azide ($p < 0.001$) (Fig. 5). Growth over the 32 days was significantly different between the first and second chambers of each pair ($p < 0.001$) (Table 2). The 12 stones all appeared to gain weight linearly with time (individual correlation coefficients ranged from 0.89 to 0.99 and were not improved by other reasonable models [4] such as weight vs. time^3 or weight vs. time$^{3/2}$).

The mean cross-sectional area of the initial stone fragments was 28.8 mm^2 (corresponds to a circle of diameter 5.6 mm). At the end of the experiments the area of the stones without azide increased by 131% to

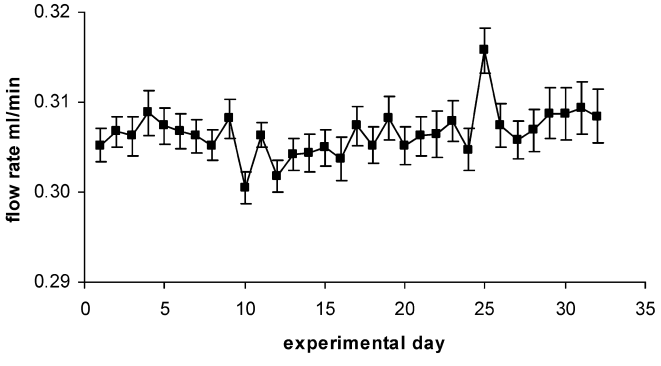

Fig. 2 Mean flow rates (± sem) of the 12 feed solutions through the stone farm over the course of the experiment

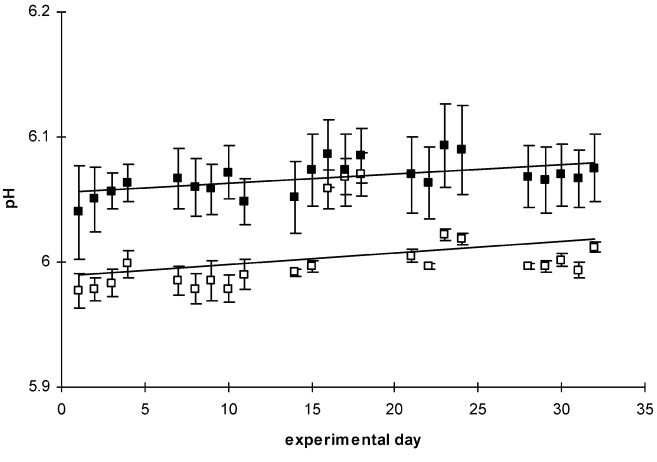

Fig. 3 Mean pH (± sem) in crystallization chambers, with (□) or without (■) added azide

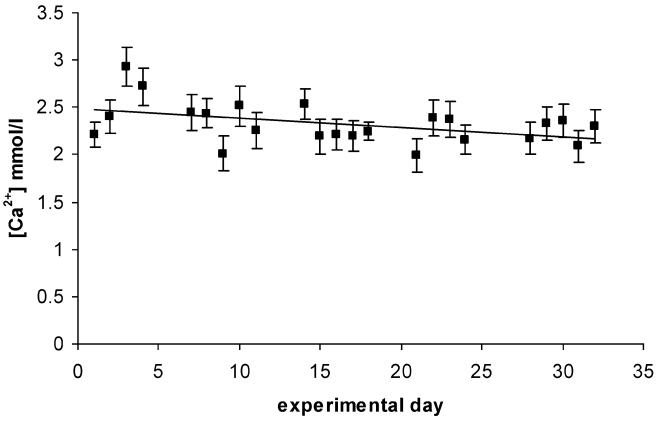

Fig. 4 Mean ionised calcium concentration (±sem) in the crystallization chambers over the course of the experiment

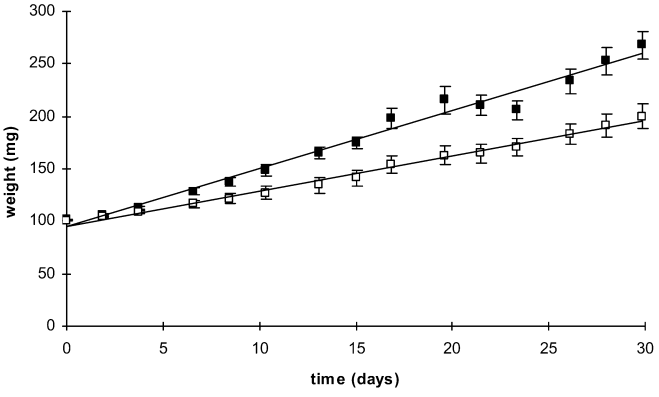

Fig. 5 Mean weight of stones (±sem) over almost 30 days growth in artificial urine with (□) or without added azide (■)

Table 2 Final weights and growth rates of stones after 32 days growth in artificial urine with or without added NaN_3

Control				Azide			
Chamber A		Chamber B		Chamber A		Chamber B	
mg	mg/day	mg	mg/day	mg	mg/day	mg	mg/day
264	5.05	305	6.76	188	3.04	218	3.90
246	5.09	308	7.00	164	2.16	216	4.04
226	4.02	259	5.22	175	2.46	239	4.52

57.4 mm^2 (d = 8.6 mm) while those with azide increased in area by 77% to 43.9 mm^2 (d = 7.5 mm). These differences are visibly clear, albeit small (Fig. 6), and statistically significant ($p = 0.004$, Mann-Whitney U test).

Discussion

We have previously shown that CaOx stones grown individually in artificial urine in an MSMPR system followed surface area dependent growth kinetics [4]. In the same system, inclusion of UMM from male stone formers inhibited the growth rate and appeared to change the kinetics to a linear pattern of weight deposition. The appearance of these stones was very similar to naturally occurring renal calculi and offered further justification for this approach to in vitro stone generation [5]. The MSMPR system was chosen for these studies because of its widely accepted value in crystallization studies and urolithiasis [1, 7, 8]. This in part stems from the continuous flow through the crystallization chamber, allowing an equilibrium supersaturation to become established which is dynamically similar to the renal system. Furthermore, it has been suggested that the particle size distribution of calcium oxalate crystalluric urine could result from two MSMPR systems operating in series, perhaps analogous to the ducts of Bellini and the renal pelvis [9]. This concept inspired our use of two consecutive stone growth chambers, which might be thought of as crude analogues of calculi growing in two separate regions of the urinary system, one distal to the other, e.g. calyceal and ureteric stones. More prosaically, the introduction of the second chamber allows a second stone to be grown without increasing the requirements for feed solutions or otherwise greatly increasing the practical difficulties. By multiplying this system up to six pairs of stone generators, we anticipated that this would enable us to improve the reproducibility of growth rates and that the numbers would be sufficient for contemporaneous test of potential modifiers of macroscopic stone growth against a suitable control.

When grown individually [4, 5], practical constraints meant that experiments were sometimes interrupted for 1 or 2 days. By contrast here, the stone farm was operated continuously, except for the 1–2 h per day required for cleaning, etc. The potential for infection, especially when UMM were added, was not addressed in the individual experiments. In the stone farm, the feed AU was sterilised and a number of other precautionary measures were introduced. The consistency of the pH within the crystallisation chambers, after the feed solutions had been circulated for 20–23 h, suggests that even in the absence of azide, infection is not likely to be a problem. Furthermore, we have shown here that inclusion of sodium azide has only a modest effect on stone growth and is therefore suitable for inclusion as an antimicrobial. This will be particularly important in future experiments in which urinary macromolecules will be included.

The inter-experiment reproducibility of stones when grown individually in AU was poor (cv = 40%), which was put down, in a large part, to variations in flow rate of feed solutions through the system [4]. When operating the stone farm, we monitored flow rates daily, for all 24 feed solutions, allowing early detection of wear and tear of pump tubes; the resulting control was much improved. Similarly, regular monitoring of the pH and $[Ca^{2+}]$ in the chambers suggests the inter-batch variation in making up the feed solutions will not be a significant source of experimental variation. The statistically significant but quantitatively trivial changes in pH

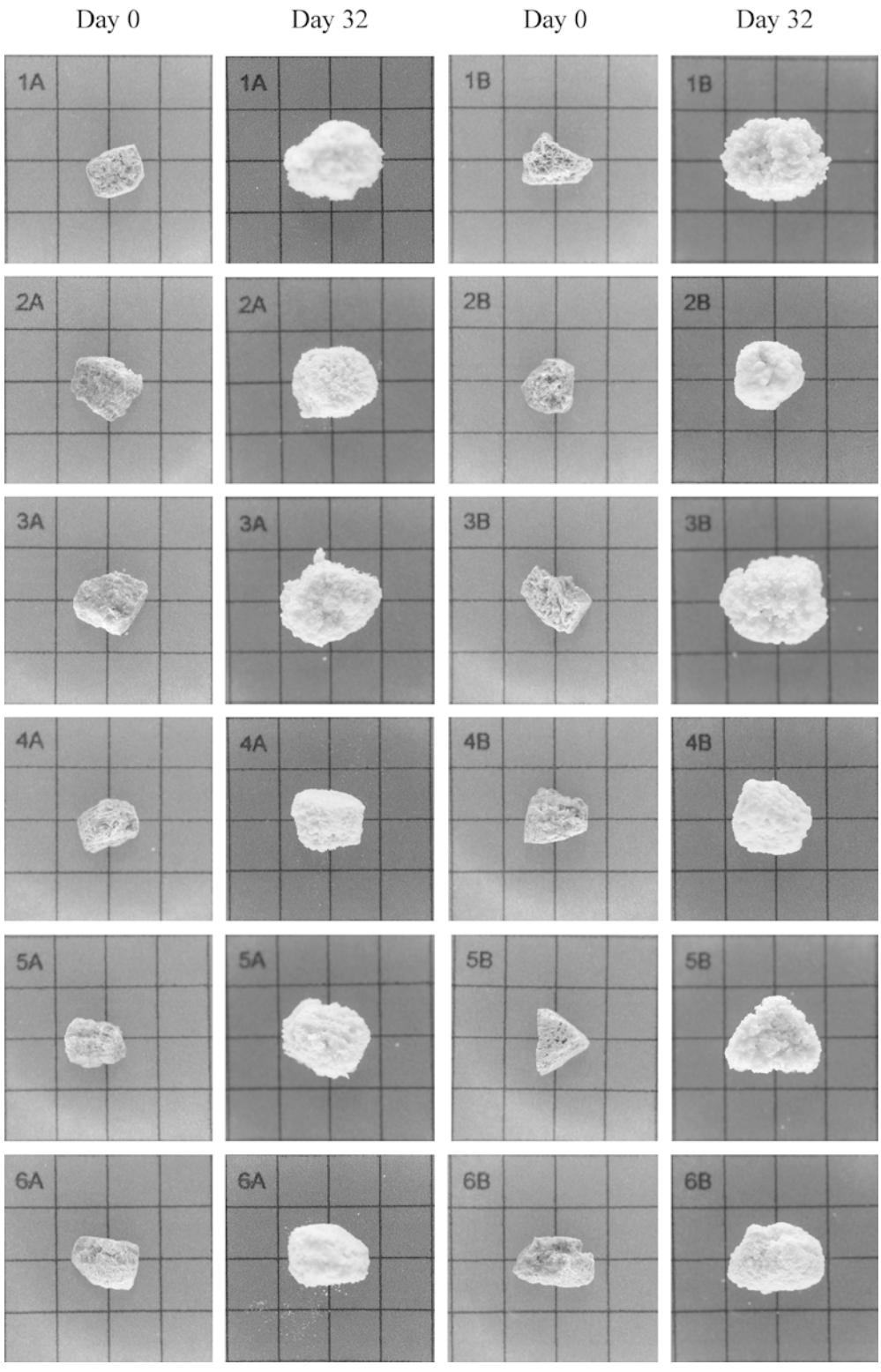

Fig. 6 Stone fragments at the start and end of the experiment. *A* and *B* refer to the chamber occupied (first or second in series). Stones 1, 3 and 5 were grown without added azide; stones 2, 4 and 6 were grown with added azide. Grid squares are 5 mm

and $[Ca^{2+}]$ during the course of the experiment might be due to systematic changes in the feed solutions or the standards used to calibrate the electrodes. The small rise in pH between the first and second chambers, which was more pronounced when azide was absent, might be a reflection of the slightly increased growth rate in the second chamber (which was also more obvious in the azide-free chambers). The experimental variation in weight between stones in comparable chambers and with the same feed solutions, increased as the stones enlarged. Over the four recordings in the last 7 days, for the four groups of three stones, the % c.v. was between 4 and 10% (average 7%). This is a considerable improvement on our previous experience and it is clear that this sys-

tem is sensitive to small changes in growth rate that might be brought about by experimental intervention.

Apart from the improved consistency, another difference between the stone farm and our earlier results is the growth rate. In our pilot study, the eight individually grown stones averaged 38 mg/day, accelerating through the course of each experiment [4]. Here, the 12 stones averaged 4.4 mg/day, at a constant rate. A probable cause of this difference is the change of support for the stone in the crystallization chambers. Previously, we used a perforated basket, which we have changed to a simple suspended support. This variation was introduced in order to make construction of comparable supports possible and to simplify the process of removal for weighing. The more open position of the stones means that they are more exposed to the turbulence generated by the stirrers. In preliminary experiments with the stone farm (data not shown) we found the distance between the stone support and the stirrers had a significant impact on growth rates. The lower growth rate in the current system is actually more representative of in vivo stones. The diameter changes (Fig. 6) correspond to volume changes of approximately 4–8 mm^3/day and 1–10 mm^3/day is considered realistic [10].

The stone farm incorporates many improvements compared to our earlier design and fulfils our objectives of providing a suitable experimental system to test the effect of potential modifiers of stone growth. It is not, however, a trivial undertaking; in the course of the 32-day experiment described here, 158.4 L of artificial urine passed through the system exposing the stone to 38.0 g of Ca and 17.1 g of oxalate. The total stone weight gain was 1.59 g, which corresponds to about 5% of the available oxalate.

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