Calcium Oxalate Crystal Yield in Various Gravity Environments

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ABSTRACT

An experimental chamber and hand-manipulated syringe apparatus were designed, tested, and utilized to assess calcium oxalate crystal yield in Terrestrial-g (1 g), micro-g (0.01 g), Lunar-g (0.16 g), and Martian-g (0.38 g). Aqueous solutions of calcium chloride (100 mM) and oxalic acid (200 mM) were mixed to precipitate calcium oxalate crystals. Gravitational differences were hypothesized to result in differences in the yield of crystal formation. These data are essential for efforts to better understand the correlation between calcium oxalate crystal formation and the production of kidney stones often associated with long-term space missions. The analyses of crystal formation produced in the micro-g ($\cong 0.01$ g) conditions of this study suggest that calcium oxalate monohydrate formation yield is slightly greater than those produced in Terrestrial-g conditions.

Key words: Calcium Oxalate; Microgravity; Kidney Stone; Secondary Plant Metabolite; Oxalic Acid; Calcium Chloride

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BACKGROUND

Calcium oxalate (CaC_2O_4 or CaOx) is an insoluble crystal, formed as a precipitate of calcium and oxalate ions, and is produced in both plants and animals. In plants, CaOx has multiple functions including: (1) defense from herbivores, causing irritation and burning sensations when consumed (Korth et al., 2006), (2) critical structural support in sclereids and cell membranes, (3) gravity perception in the form of statoliths (Audus, 1962), and (4) control of pH, ionic, and osmotic fluctuations (Raven et al., 2005). In animals, CaOx is generally considered pathological and often associated with renal edema and kidney stone formation (Dempsey et al., 1960). Most human diets are rich in oxalates (e.g., green leafy vegetables – spinach, chard, rhubarb, broccoli) and calcium (e.g., dairy products – milk, yogurt, cheese), and other metabolites (e.g., purine, uric acid) and toxins (e.g., ethylene glycol) that can contribute to renal pathologies (Franceschi and Horner, 1980; Hagler and Herman, 1973). Humans prevent CaOx accumulation by normal excretion (26-46 mg/day) of oxalate in urine. However, metabolic dyshomeostasis including increased urinary oxalate (hyperoxaluria), uric acid (hyperuricosuria), phosphorous (hyperphosphaturia), and decreased urinary excretion of citrate (hypocitraturia), can all lead to an increased risk of kidney stone formation in humans (Zerwekh, 2002).

Astronauts on long- and short-term spaceflights under microgravity often exhibit progressive and continuous negative calcium balance from bone loss (Miyamoto *et al.*, 1998; Whedon and Rambaut, 2006), increased urinary calcium and pH, and decreased urine volume (Whitson *et al.*, 1997; Whitson *et al.*, 1999; Whitson *et al.*, 2001). Decalcification of bones during extended-duration spaceflights has been demonstrated to occur at a rate of ~300 mg of calcium/day (Smith *et al.*, 2005). These biological factors can all contribute to the formation of harmful kidney stones in astronauts (Drinnan and Begoune de Juniac, 2013). Our hypothesis was that microgravity induces the formation of a preferred crystal structure (either mono or dihydrate calcium oxalate), and there could be a

difference in total crystal concentration when formed under various gravitational accelerations.

It has long been known that calcium oxalate can form either as a monohydrate (COM) or a dihydrate (COD) (Equations 1-2). The COM crystals, also known as whewellite, vary in size and morphology, and may have a spindle, oval and dumbbell, or elongated hexagonal structure (Monje and Baran, 2002). The COD crystals, or weddellite, are octahedral (square bipyramid) with eight faces and an envelope morphology. Most kidney stones contain about twice as much COM as COD (Petrova *et al.*, 2004; Wesson *et al.*, 1998). The COM crystal structures are found to have a high affinity for the surface of renal tubule cells (Peerapen and Thongboonkerd, 2011), suggesting that crystalline morphology and size may influence their deleterious effects.

Equation (1): Calcium oxalate monohydrate (COM)

Equation (2): Calcium oxalate dihydrate (COD)

MATERIALS AND METHODS

Initially, calcium chloride (CaCl_2) and sodium oxalate ($\text{Na}_2\text{C}_2\text{O}_4$) were used to form either COM or COD crystals; see Figure 1. This equimolar, equal-volume mixture yielded a precipitate that was too small to readily visualize, resulting in replacement of sodium oxalate with oxalic acid ($\text{H}_2\text{C}_2\text{O}_4$), and mixed at three concentrations (equimolar, 100, or 200 mM). Solutions were allowed to mix for several minutes, followed by vacuum filtering. Resulting crystals were oven-dried, mounted on slides, and visualized using light microscopy (Nomarski DIC 40x and 100x, Olympus BX45 with charge-coupled device (CCD) camera). Figure 2A shows the resulting mixture of COM (right-pointing arrow) and COD (left-pointing arrow) crystals from equimolar concentrations of calcium chloride and oxalic acid.

While yields were relatively high, the methodology was further refined to favor formation of predominantly COM crystals.

Figures 2B and 2C show the druse-like COM crystals formed from mixing equal volumes of 100 mM calcium chloride (pH 5.64) and 200 mM oxalic acid (pH 1.48) (reagent grade, Fisher Scientific). All solutions were prepared with double-distilled water using volumetric glassware. The vacuum filtration method was used to collect the crystals in Figures 2B and 2D; the in-flight apparatus was used for crystals in Figure 2C. Higher concentrations of calcium chloride (200 mM) and oxalic acid (100 mM) favored the formation of predominantly COD (Figure 2D). The 100 mM calcium chloride and 200 mM oxalic acid mixture was the standard solution used in this study to model COM crystal formation (that most associated with kidney stones) in the microgravity in-flight experiments.

The in-flight experiments were conducted at an altitude 26-36,000 feet above the Gulf of Mexico aboard NASA's Boeing-727 aircraft, officially nicknamed the "The Weightless Wonder" based at Ellington Field, Houston, Texas. Eight California State University, Fresno

Figure 1. Preliminary crystals formed by mixing sodium oxalate and calcium chloride. The scale bar represents 100 μm .

undergraduates participated in the design and data collection as a part of the NASA-Reduced Gravity Student Flight Opportunities Program. Performing up to 30 parabolic maneuvers per flight, this flight pattern produced 30 segments of microgravity (0.01 g) lasting ~25-30 seconds, and one to two segments of Lunar gravity (0.16 g), and Martian gravity (0.38 g), also lasting ~30 seconds (Figure 3). Two flights were conducted over two successive days; the flight number and the associated crystal yields are reported. Crystal precipitation reactions were conducted at each of these gravity environments using an apparatus that consisted of two 10 mL syringes, a three-way luer stopcock, and a 0.45 μm Swin-Lok filter holder (Fisher Scientific) (Figure 4). One syringe contained 2 mL of 200 mM oxalic acid and the other syringe contained 2 mL of 100 mM calcium chloride. During each of the 25-30 seconds of microgravity (0.01 g), Lunar gravity (0.16 g), or Martian gravity (0.38 g) flight segments, oxalic acid was injected into the adjoining syringe containing calcium chloride. The luer stopcock was turned to block solution transfer to one syringe after 5 seconds, to allow for precipitate formation, to facilitate the collection of the resulting crystals on the filter below the apparatus, and to subsequently void remaining solution volume into the attached waste bag. The chamber

allowed for three flight team members to simultaneously manipulate each of the syringe apparatus (Figure 4 and Figure 5, top panel). Flight safety conditions mandated that student-designed/built equipment required for each precipitation reaction be securely housed directly below the syringe apparatus (Figure 5). Filters were removed from the apparatus upon aircraft return to Ellington Field, dried, weighed, and were stored in glass vials for subsequent structure characterization via light microscopy upon return to California State University, Fresno.

Figure 2. Examples of crystals formed with: (a) Equimolar concentrations of oxalic acid and calcium chloride. The COM elongated, ‘orzo-shaped’ crystalline form is indicated by the right-pointing arrow and the COD octagonal, ‘envelope-shaped’ crystalline form is indicated by the left-pointing arrow. The COM form is more abundant in this equimolar mixture; (b) 200 mM oxalic acid and 100 mM calcium chloride collected using vacuum filtration; (c) Same reagent conditions as in Figure 2B, except synthesized in the luer lock 3-way syringe apparatus; (d) 100 mM oxalic acid and 200 mM calcium chloride. Each scale bar represents 10 μm .

Figure 3. The parabolic flight path of the NASA Zero-G (“Weightless Wonder”) aircraft.

The self-contained in-flight experimental chamber was designed using SolidWorks and constructed of an aluminum frame (1.27 m long $\times$ 0.508 m wide $\times$ 0.762 m tall), lined with silicon-sealed Lexan panels on all sides, and a top latched door (Figures 4 and 5). The chamber interior contained three separated foam blocks (green in Figure 5) with cut-outs to house individual syringe apparatus and covered with two sliding panels to contain the syringes after use throughout the flight. The reaction waste-collection bag was secured at the bottom of the chamber and connected to the syringes via polytubing. Six gloved-access holes were cut into the sides of the chamber; allowing for each of three flight team members to have simultaneous access to the chamber interior during the flight. Chamber and in-flight securing strap integrity were analyzed using the finite element method and were

submitted to and verified by NASA Johnson Space Center engineering staff.

RESULTS

The average mass of synthesized crystals from each of the gravity environments for each of the two successive flights are summarized in Table 1. Theoretical calculations based on the initial reactants/concentrations predicted a final mass of COM to be $\sim$ 30-60 mg. All of the in-flight crystals appeared to be greater in mass than those formed under Terrestrial gravity (1 g). The average mass of crystals precipitated in microgravity were the highest (108-220 mg), followed by that under Lunar gravity (82-216 mg), Martian gravity (69-194 mg), and lastly, Terrestrial gravity (70-77 mg). The relatively large variation in crystal mass obtained in each flight was likely due to the unavoidable manipulation of the syringe apparatus in the turbulent flight environment.

Figure 4. Schematic diagram of experimental rig. Top: Schematic drawing of the syringe apparatus used in the experiment. Bottom: CAD drawing of the experimental glove box for the formation and capture of crystals formed during the two flights. Glove box for experimental procedures and manipulations.

Figure 5. Photograph of the experimental rig. The image on the bottom shows the top view looking into the chamber. The layout of the syringes is shown for each of the three users.

Figure 6. Microscope light images of calcium oxalate crystals formed under varying gravity conditions: 6A and 6B (Terrestrial gravity (1 g)); 6C and 6D (microgravity (0.01 g)); 6E and 6F (Lunar gravity (0.16 g)); and, 6G and 6H (Martian gravity (0.38 g)). Scale bar is 100 μm for 6A, 6C, 6E, and 6G. Total magnification of 400x. Scale bar = 25 μm for 6B, 6D, 6F, and 6H.

Table 1. The average mass of synthesized crystals from each of the gravity environments (control, micro, lunar, and Martian), for each of the two successive flights. The number of trials (n) is given for each flight and gravity environment condition. The average mass (in gram units) and standard deviation (S.D.) are listed.

Condition	Control (1 g)		Micro (~0.01 g)		Lunar (.16 g)		Martian (0.38 g)	
Flight #	1	2	1	2	1	2	1	2
# of trials (n) for each flight	2	1	12	14	4	6	3	6
Average mass (g) ±S.D.	0.077 ±0.03	0.070 n/a	0.108 ±0.04	0.220 ±0.10	0.082 ±0.04	0.216 ±0.07	0.069 ±0.03	0.194 ±0.06

The images of the crystals formed in-flight and Terrestrial gravity (1 g) are shown in Figure 6 (A-H). The Terrestrial gravity (1 g) (control) crystals in Figures 6A and 6B are characteristic COM druse whewellites, resembling COMs naturally found in cacti (Monje and Baran, 2002), each ~10-15 μm in diameter. The crystals produced at microgravity (0.01 g) are shown in Figures 6C and 6D. The crystals formed under Lunar gravity (0.16 g) (Figures 6E and 6F) and Martian gravity (0.38 g) (Figures 6G and 6H) also present the characteristic COM druse morphology.

DISCUSSION

The calcium oxalate crystals formed under microgravity showed only slight differences in crystal size, but not in morphology, when compared to crystals formed under Terrestrial gravity (1 g). Our hypothesis that the CaOx crystal yield should be greater in microgravity was confirmed in this study, as the yield of COM crystals in microgravity was slightly greater than that under Terrestrial gravity (1 g).

The thermodynamically preferred state of CaOx is the COM structure, though the microgravity environment was hypothesized to influence the yield. The results of this study showed a slight increase in yield of COMs formed under microgravity and these data may provide for a better understanding of kidney stone formation under such conditions. Studies using multiple concentrations of calcium chloride and oxalic acid are necessary to further characterize formation of the preferred structures. The kinetic parameters of each reaction performed in this study may also have had an effect, as the time

period (~10 seconds) of each gravity environment, particularly at microgravity, may not have been sufficient to fully reveal actual gravity effects on CaOx structure formation (Jung et al., 2011). A longer reaction time may be necessary to fully model crystal formation in the microgravity environment found in long-term space missions, and should be incorporated in subsequent in-flight studies. Conducting these precipitation reactions over a span of hours, or even days in a simulated or actual microgravity environment (with multiple reaction concentrations and an automated mixing system), is the next logical step for our undergraduate student team investigation.

It should also be noted there may be major differences between synthetic *in vitro* and *in vivo* formed biogenic crystals (da Costa et al., 2009). We cannot rule out other biological factors (e.g., nanobacteria in the human body or other urinary molecules) (Çiftçioglu et al., 2005) that likely influence the formation and subsequent damage caused by CaOx crystals. Our follow-up studies will include infrared spectroscopy (IR), scanning electron microscopy (SEM), and x-ray diffraction (XRD) to confirm the morphology of the precipitated crystals formed in this study and will likely also include use of fluorescence techniques (Hernandez-Santana et al., 2011) to identify the presence/absence of calcium containing crystals. These data are essential for the success of future long-term manned space exploration missions.

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