Melamine Crystallization: Physicochemical Properties, Interactions With Other Lithogenic Salts and Response to Therapeutic Agents

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Purpose: There were reports of children in the People's Republic of China being hospitalized with renal stones and/or failure by September 2008, which were caused by melamine and its co-contaminant cyanurate. We investigated the physicochemical behavior of melamine, its interaction with other endogenous urine factors and the response to therapeutic agents in the renal environment in vitro.

Materials and Methods: A mixed suspension, mixed product removal system was set up for crystallization studies of melamine in urine. Crystallization kinetic parameters, including the nucleation and growth rates, and suspension density, were determined according to crystal number and size, as measured by a Coulter particle counter.

Results: Melamine crystallized out from urine under normal urinary conditions (pH 5.0 to 6.5) but crystallization was strongly inhibited at pH 4.5 or lower. Melamine significantly enhanced calcium oxalate precipitation while uric acid significantly decreased melamine crystallization. Bacteria mimicking urinary tract infection promoted melamine crystallization. Clinical relevant drugs, such as citrate and bicarbonate, significantly decreased melamine crystallization.

Conclusions: This implies that melamine crystallizes under normal urinary conditions and can interact with other lithogenic salts and pose a significant risk for other stones. Urinary tract infection promotes melamine crystallization. Citrate and bicarbonate therapy are effective prophylactic agents against melamine induced crystallization.

Key Words: kidney, calculi, melamine, crystallization, People's Republic of China

Abbreviations and Acronyms

B_o = nucleation rate
CaP = calcium phosphate
CaOx = calcium oxalate
M_τ = suspension density
MC = melamine cyanurate
MSMPR = mixed suspension mixed product removal
Ox = oxalate
UA = uric acid
UTI = urinary tract infection

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Since 2004, acute renal failure in animals has been known to be associated with adulterated pet food in Asia and the United States.[1] Melamine was found to be a contaminant in wheat gluten added to pet food. The co-contaminant cyanuric acid was also identified.[2]

In September 2008 there was an increased incidence of renal stones in infants and children admitted to hospitals in the People's Republic of China. Subsequently melamine was found to have been added to milk to increase the nitrogen content (66.6% by weight) during the classic crude protein test for dairy products using the Kjeldahl and Dumas method to increase the measured protein content. At that time 6 infants reportedly

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died and around 300,000 had urinary tract ailments, including kidney stones, of whom 850 are still being treated and 150 are seriously ill.[3]

In Hong Kong 15 children tested positive and more than 40,000 were screened and found to be healthy.[4,5] A total of 3,835 children attending Princess Margaret Hospital in Hong Kong were further investigated, of whom 22 (0.6%) showed a renal disorder that was not necessarily related to melamine.[6] Most children with melamine related stones were described as asymptomatic until renal abnormalities were severe enough to cause impaired renal function, by which time melamine and its crystalline calculi had caused damage.[7]

The physicochemical nature of melamine in body fluids (blood and urine) and its handling have been largely unknown until recently. Recent reports suggest that the melamine and cyanurate levels allowed by WHO should be lowered for infants (younger than 3 years) since their relative risk of renal stones is 1.7 compared to that of controls at the previously defined safe level of less than 0.2 mg/kg daily.[8]

Many in vitro methods are used to study urine crystallization. The MSMPR system that we previously established attains steady-state supersaturation and is a good model for in vivo renal crystallization studies.[9] This model is useful for investigating how melamine behaves by changing urinary factors such as supersaturation and pH, and the presence of other stone forming salts, including CaOx, CaP and UA, since they are important factors that may affect melamine crystallization.[10,11] Infection and commonly used treatments such as potassium citrate and sodium bicarbonate can also be tested by the system. This information would be vital since melamine is still allowed in food and meat for human consumption according to the United States Food and Drug Administration. Furthermore, wide use of melamine in fertilizers is a risk factor. We determined whether current allowable limits pose a risk.

MATERIALS AND METHODS

Urine and Reagent Preparation

The constituents of the urine used in this study, which was supersaturated and at normal urinary pH, were those described in the study by Kavanagh et al.[12] Artificial urine was freshly prepared daily. Analytical grade chemicals were dissolved in Milli-Q® water and pH was adjusted to 6.0 with hydrochloric acid. The final concentration of the major ions were Ca 6 mM, magnesium 3.0 mM, sodium 196 mM, potassium 82 mM, phosphate 23 mM, sulfate 20 mM, oxalate 1.2 mM and citrate 2.2 mM. Urine was buffered with disodium phosphate (22 mM).

Stock melamine solution (10 mM) was prepared by dissolving 1.26 gm melamine (Sigma-Aldrich®) in 1 l Milli-Q water. Stock cyanuric acid solution (10 mM) was prepared by dissolving 1.2908 gm cyanuric acid (Sigma-Aldrich) in 1 l Milli-Q water.

MSMPR System for Melamine Crystallization

Two decreased size (20 ml) crystallizers[9] in parallel (test and control chambers, respectively) were developed for crystallization studies. The crystallizer is a 2-layer glass beaker with 37C water passing through the in-between layer to perform experiments throughout at 37C. A closely fitting lid covered the opening of the crystallizer to minimize evaporation. Four openings were made for the inlet of urine, melamine solution, cyanuric acid solution and

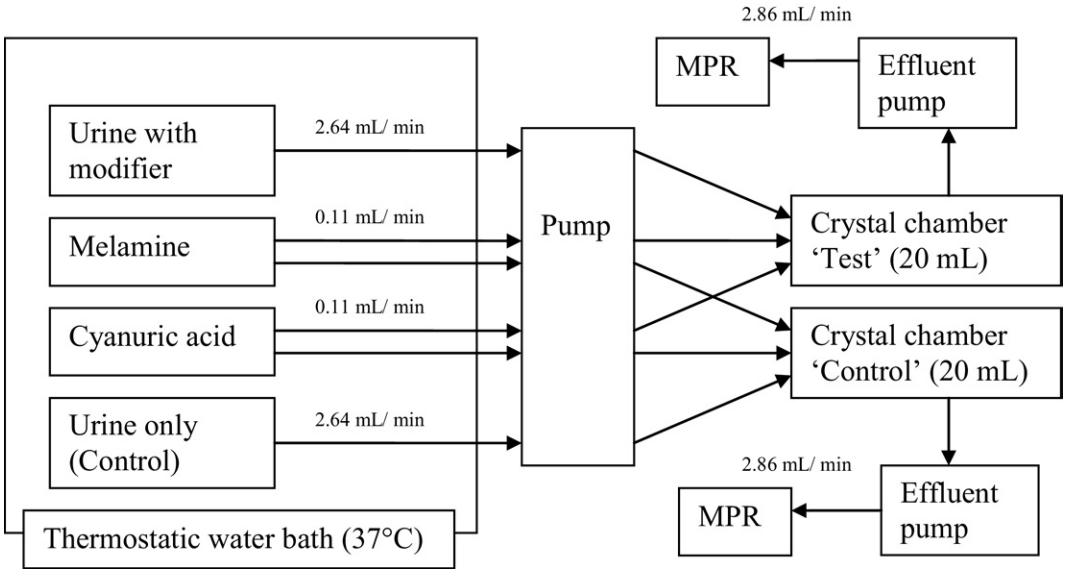

Figure 1. MSMPR crystallizer. Test and control thermostatically controlled chambers were run simultaneously by peristaltic pump. *MPR*, MSMPR.

outlet tubes for mixed product removal. The suspension inside the crystallizer was mixed by a magnetic stirrer with the magnetic plate under the crystallizer. A flow rate of 2.64 ml per minute for urine and 0.11 ml per minute for melamine and cyanuric acid solutions was applied so that the flow rate at the crystallizer outlets was the sum of the 3 feed solutions, that is 2.86 ml per minute. Thus, a proportion was achieved of 92% urine and 2, 4% feed solutions of melamine and cyanuric acid, respectively (fig. 1). We used the equations and derivation of Söhnel and Garside for the parameters of growth rate in μm per minute, B_o in number per minute per ml and M_τ in mmol/l or in mM based on specific salt density.[13]

The 2 chambers were run simultaneously for 7 to 8 residence times[12] to equilibrate the MSMPR system. This is the average time that crystals remained in each chamber. Ten consecutive measurements of crystal number and size were made per chamber at 7-minute intervals using the Multisizer™ 3 Coulter Counter particle size analyzer. Using the Multisizer, particle number data were collected from 300 size channels distributed over 12 log scales. When plotted as ln N, where N represents the number per ml, and asking whether the number of crystals per unit volume is greater than size L, MSMPR data should show a straight line, and the growth rate and B_o can be calculated from the slope and intercept, respectively. $M\tau$ was estimated using the equation, $M\tau = \pi\rho B_o G^3\tau^4/D \times 10^{-9}$, where ρ represents crystal density in gm/cm^3, G represents the growth rate, D represents the molecular weight of the crystalline species measured and τ represents residence time, as shown by V/Q, where V represents chamber volume (20 ml) and Q represents the total flow rate (2.86 ml per minute).

MSMPR System Optimization

Cyanuric acid is a 1% to 5% contaminant during melamine production and it may contribute to urinary crystallization. Thus, the system was optimized for melamine crystallization by testing for different percents of melamine to cyanuric acid (0% to 100%). The optimum ratio of melamine and cyanuric acid was 1:1 (fig. 2). The minimum concentration (0 to 10 mM) of

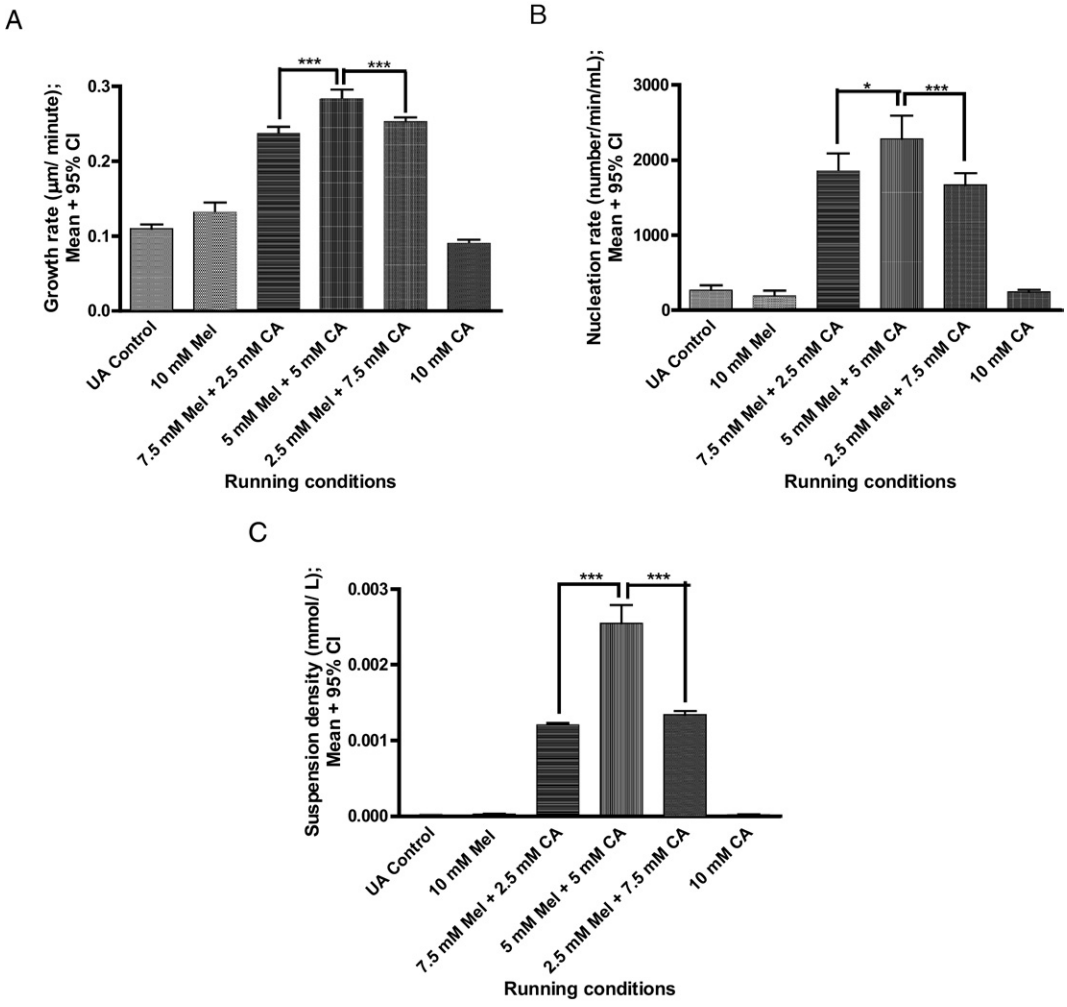

Figure 2. Mean + 95% CI melamine (*Mel*) and cyanuric acid (*CA*) growth rate (*A*), B_o (*B*) and M_τ (*C*). Different melamine and cyanuric acid ratios dissolved in UA were added to test chamber. Artificial urine was used for control. Single asterisk indicates $p < 0.05$. Triple asterisks indicate $p < 0.001$.

melamine and cyanuric acid in a 1:1 ratio for melamine crystallization was 5 mM (fig. 3).

Melamine

Physicochemical behavior. Urinary pH was adjusted from 3.5 to 6.7. Urinary ionic strength, which affects urine supersaturation, was adjusted with respect to sodium by adding 0.5× to 10× sodium chloride, where 1× equals 160 mM. Urinary sodium can be between 15 and 250 mol daily depending on salt and hydration levels.

Interaction with other lithogenic salts. To study the effects of other lithogenic ions, including CaOx, CaP and UA, on melamine crystallization we set up the MSMPR system. 1) For CaOx 5.77 mM Ca chloride and 1.15 mM sodium Ox were added separately to the chambers by 2 inlet tubes simultaneously. 2) To test urine 0.2× and 1× UA were added, where 1× equals 2.925 mM UA. 3) To test urine 0.2× to 1× CaP were added, where 1× equals 4 mM calcium chloride and 25 mM disodium hydrogen phosphate.

Response to UTI. The MSMPR system was set up to mimic UTI by adding Escherichia coli (ATCC™ 25922) to test urine. A 1.5×10^8 cfu/ml stock E. coli suspension was freshly prepared by suspending E. coli in normal saline to the 0.5 McFarland turbidity standard (Key Scientific, Stamford, Texas) with 0.132 absorbance at 600 nm, as measured by a spectrophotometer. A clinically associated infective dose of E. coli (1×10^5 cfu/ml urine), and 10 times lower and higher doses were used by diluting stock E. coli in test urine.

Response to therapeutic agents. Known therapies used for stone disease, including potassium citrate and sodium bicarbonate, were tested to observe effects on melamine crystallization. The MSMPR system was set up appropriately. 1) Potassium citrate (1× to 10×) was added to test urine, where 1× equals 2.17 mmol/l. 2) Sodium bicarbonate (12.5 to 50 mmol/l) was prepared by diluting 1,000 mmol/l sodium bicarbonate stock solution, that is 8.4% weight per volume sodium bicarbonate intravenous infusion (Braun, Melsungen, Germany), in test urine.

Data and Statistical Analysis

Prism®, version 4.03 for Windows® was used for all statistical analyses. The slope and y intercept of the plot of ln (N) against crystal size was tested by linear regression for each measurement. Linear plots with an r^2 correlation coefficient of greater than 0.95 represented a realistic growth rate and B_o (absent aggregation). One-way ANOVA was used to compare mean differences with $p < 0.05$ considered statistically significant. All significant ANOVA test results were analyzed by the Dunnett multiple comparisons post test.

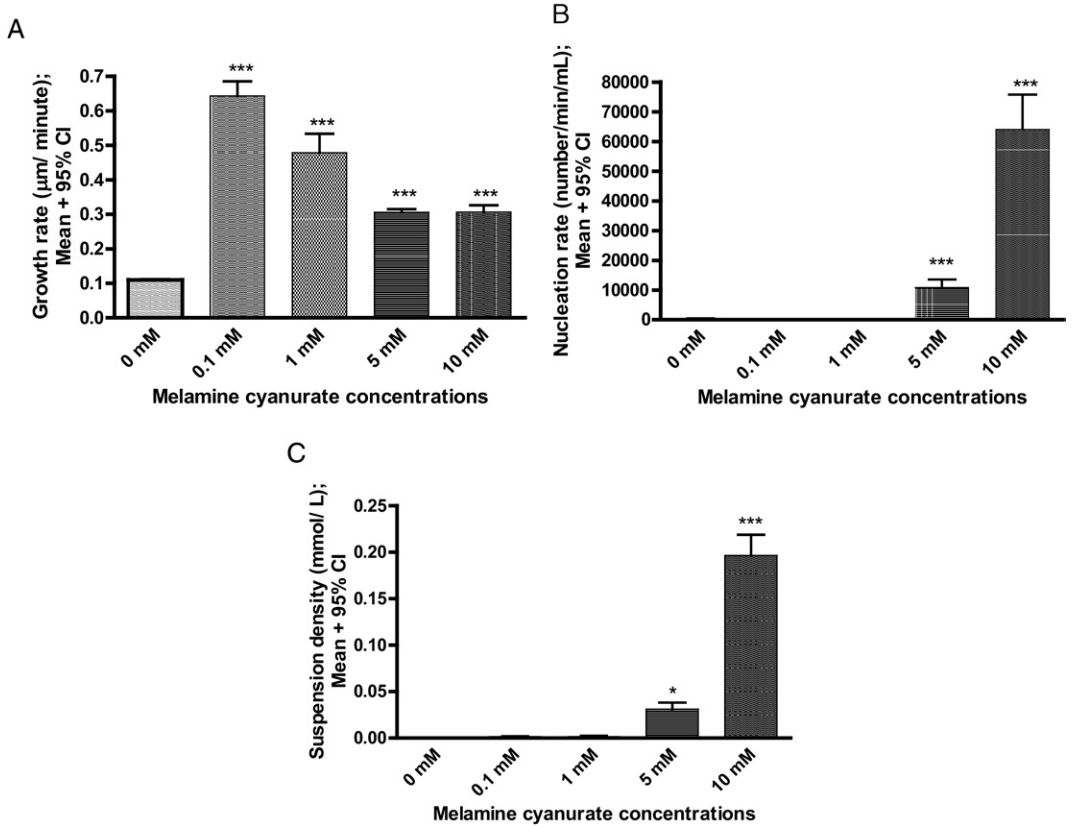

Figure 3. Mean + 95% CI growth rate (*A*), B_o (*B*) and M_τ (*C*) at 0 to 10 mM MC dissolved 1:1 in UA for test. Artificial urine was used for control. Single asterisk indicates $p < 0.05$ vs 0 mM control chamber. Triple asterisks indicate $p < 0.001$ vs 0 mM control chamber.

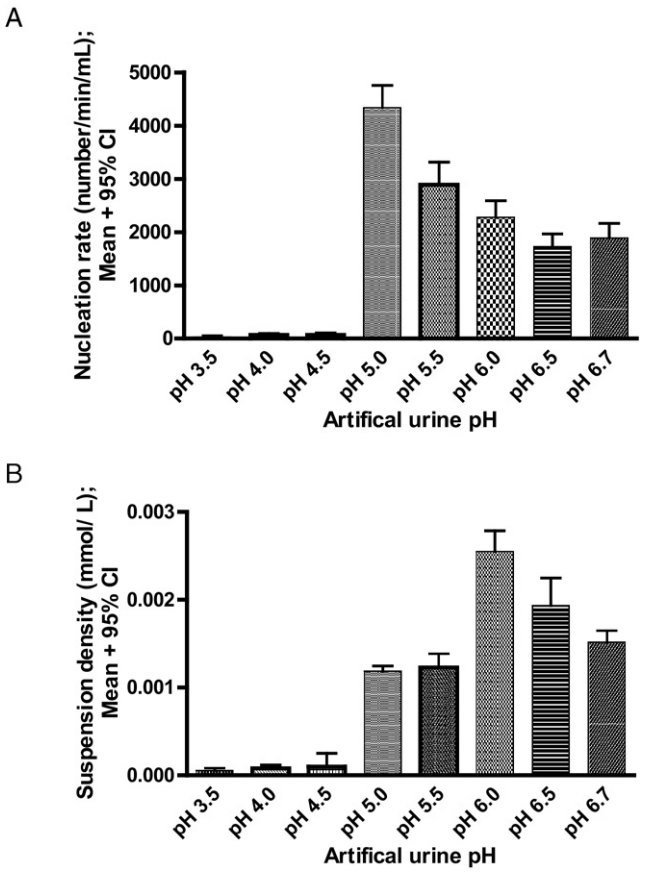

Figure 4. Mean + 95% CI urinary pH effect on B_o (*A*) and M_τ (*B*) of MC crystals. MC (5 mM) was dissolved in UA for test. Artificial urine was used for control. Artificial urine pH was prepared from phosphate buffer and adjusted with HCl to desired pH.

RESULTS

MC Crystallization Physicochemical Aspects

MC crystallization is concentration dependent. The minimum concentration that triggered crystallization was 5 mM (1:1 ratio) and at 10 mM it was 6 times more (fig. 3). At different pH values melamine crystallized out from urine under normal urinary conditions of pH 5.0 to 6.5 but crystallization was strongly inhibited at pH 4.5 or lower (fig. 4). At pH higher than 6.7 CaP crystals started to precipitate and pH data beyond pH 6.7 included each crystal type. When varying ionic strength based on the sodium concentration, we observed no difference or trend in melamine crystallization (data not shown).

Melamine Crystallization and Other Endogenous Urine Factors

The effects of melamine, cyanurate and MC on CaOx crystallization were profound (fig. 5). Melamine, cyanurate and MC enhanced B_o and decreased the growth rate, implying that they lead to the formation of smaller CaOx crystals, as observed microscopically. Each promoted CaOx crystallization. The effect of MC (1:1) on promoting CaOx crystallization was much higher than that of melamine and cyanurate alone at 5 and 10 mM.

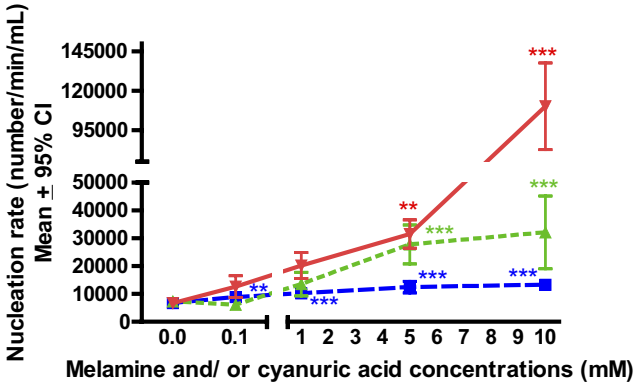

Figure 5. Effect of melamine (blue curve), cyanuric acid (green curve) and MC (red curve) on CaOx crystal B_o. Each constituent (5 mM) was dissolved in UA. Artificial urine was used for control. $CaCl_2$ (5.77 mM) and Na_2Ox (1.15 mM) were applied separately to test and control crystallizers to induce CaOx crystallization. Double asterisks indicate $p < 0.01$ vs 0 mM control. Triple asterisks indicate $p < 0.001$ vs 0 mM control.

However, as little as 0.1 mM melamine enhanced CaOx precipitation and resulted in significant B_o

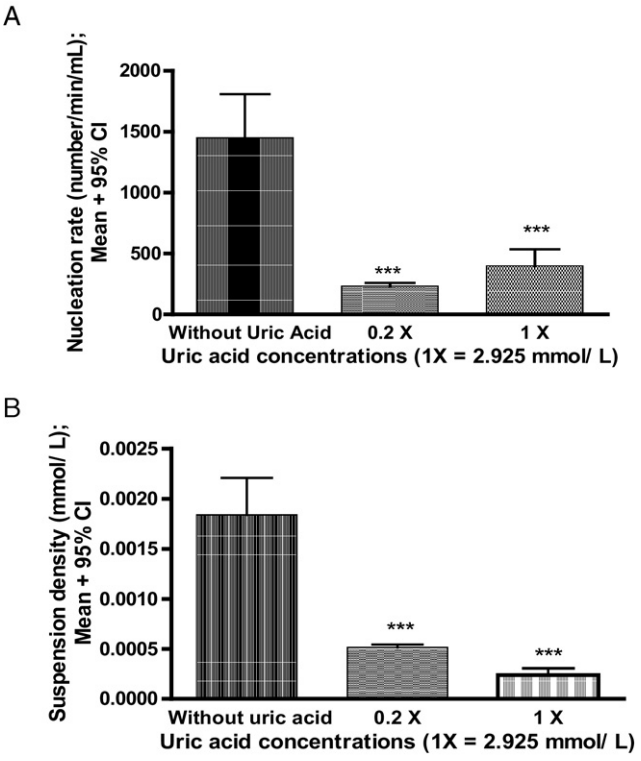

Figure 6. Mean + 95% CI UA inhibitory effect on MC crystal B_o (*A*) and M_τ (*B*). UA or artificial urine as control was added to UA. MC (5 mM) was applied to test and control chambers. Asterisks indicate $p < 0.001$ vs no UA.

changes compared to that of CaOx crystallization alone (fig. 5). This is an important finding. While it required a higher concentration of cyanurate (1 mM) and MC (5 mM) to promote CaOx precipitation, only 0.1 mM melamine in urine increased CaOx precipitation.

MC crystallization, B_o and M_τ were significantly decreased by adding UA (fig. 6), suggesting that MC crystallization was inhibited by UA. However, adding CaP to urine had no profound result on melamine crystallization. No trends were observed in the crystallization growth rate or M_τ (data not shown).

The presence of E. coli mimicked UTI and decreased B_o but enhanced the growth rate and M_τ (fig. 7), indicating MC promotion. Microscopic examination revealed larger melamine crystals.

Melamine Response to Therapeutic Agents

Citrate and bicarbonate were associated with decreased MC crystallization through different mechanisms. Increasing citrate resulted in a significant decrease in B_o and M_τ but had no effect on the growth rate (fig. 8, *A*). This effect was thought to be caused by the chelation of other ions (Ca), which decreased overall urine supersaturation to inhibit crystallization. Increasing bicarbonate concentration caused a significant increase in B_o with a decreased growth rate and M_τ (fig. 8, *B*) suggesting that bicarbonate inhibited MC crystallization due to the formation of smaller melamine crystals.

DISCUSSION

We began with some fundamental questions on the optimal proportion of melamine and cyanurate, and the pH and ionic strength at which crystallization would occur. That is, does urine provide an optimal condition for melamine and/or MC crystallization?

By studying various proportions of melamine to cyanurate we found that melamine crystallization was maximal for B_o, the growth rate and M_τ at a 1:1 ratio (fig. 2). Previous studies showed that the cyanurate-melamine complex was formed directly by simultaneous deposition of cyanuric acid and melamine.[14] A heat stable, solid 1:1 melamine-cyanurate complex was formed by hydrogen bonding after mixing melamine and cyanurate in aqueous solution.[15] This led to the formation of large, stable, structurally defined aggregates at equilibrium. Melamine and cyanurate alone showed little crystallization, as indicated by the growth rate and B_o. This suggests that melamine crystallization requires cyanurate, as supported by other findings.[16–18]

Since cyanurate is usually present as a contaminant in melamine only,[19] the cyanurate concentration is not high. This may explain why the overall stone incidence detected in the affected children is still not high. However, melamine crystallization is concentration dependent and so younger children are more vulnerable to melamine stone formation due to the relative higher intake of the melamine tainted milk product.[20]

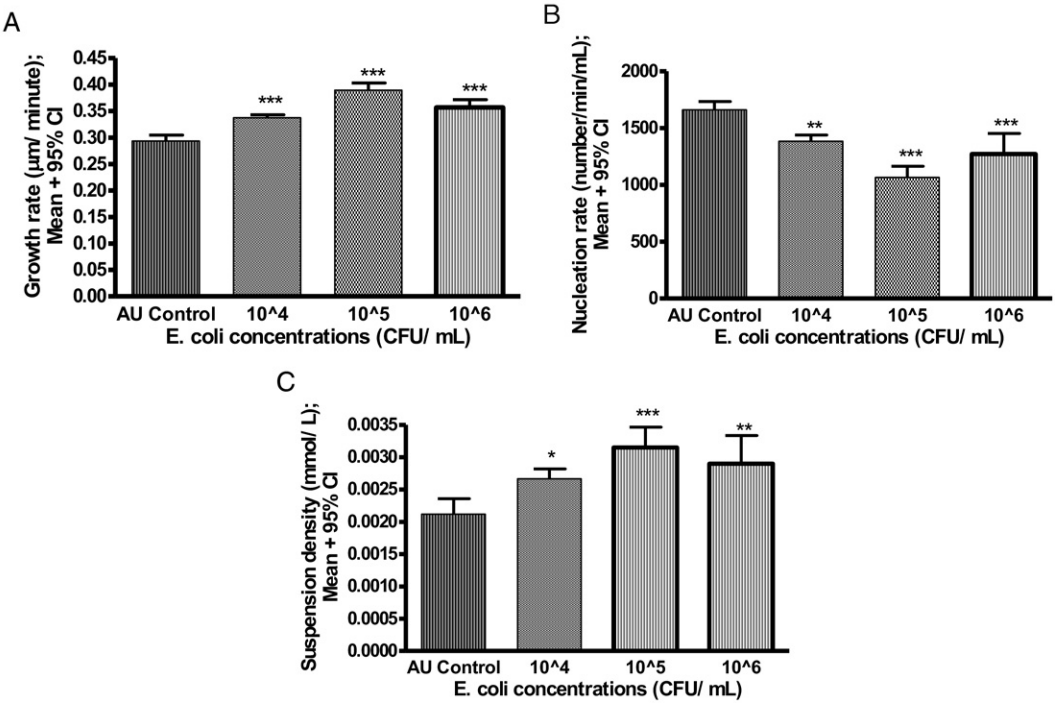

Figure 7. Mean + 95% CI urinary E. coli effect on MC crystal growth rate (*A*), B_o (*B*) and M_τ (*C*). Clinically relevant E. coli dose (1×10^5 cfu/ml urine) or artificial urine as control was added to UA. MC (5 mM) was applied to test and control chambers. Single asterisk indicates $p < 0.05$ vs control. Double asterisks indicate $p < 0.01$ vs control. Triple asterisks indicate $p < 0.001$ vs control.

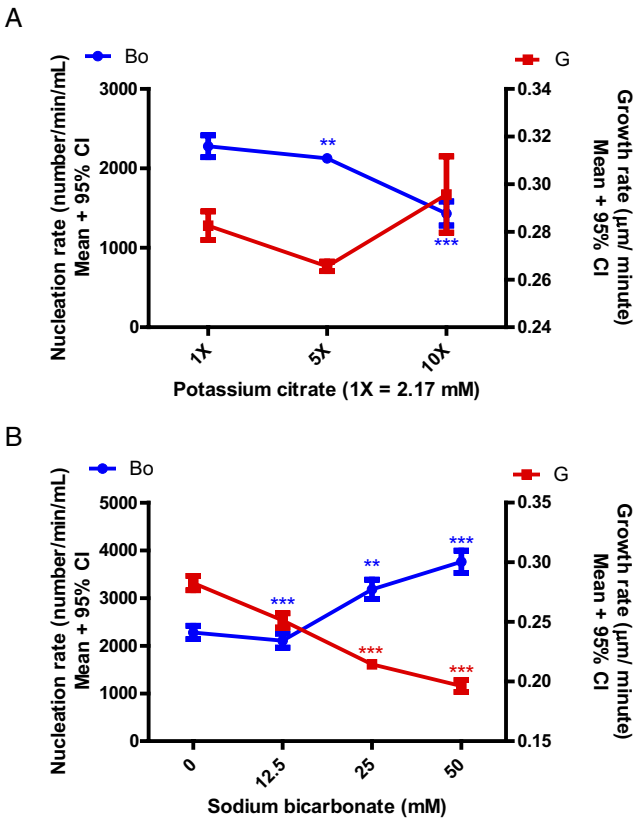

Figure 8. Mean + 95% CI effect of therapeutic potassium citrate (*A*) and sodium bicarbonate (*B*) on MC crystal B_o (*Bo*) and growth rate (*G*). Relevant therapeutic concentration was added to UA or artificial urine as control. MC (5 mM) was applied to test and control chambers. Double asterisks indicate $p < 0.01$ vs 1× potassium citrate or 0 mM control for sodium bicarbonate. Triple asterisks indicate $p < 0.001$ vs 1× potassium citrate or 0 mM control for sodium bicarbonate.

Therapeutic agents that aid in kidney stone prevention by urine alkalization or acidification may also be used for melamine stones. Melamine crystallization is inhibited under acidic conditions (pH less than 4.5), which is not clinically practical. However, such crystallization is also decreased at pH 6 to 6.5. Thus, alkalization to hold more melamine and other salts such as UA would become clinically relevant. Beyond pH 6.7 CaP would begin to precipitate. Therefore, optimal conditions to decrease supersaturation and crystalline components must be considered.

Since urine is a complex liquid containing various ions, and its composition and ionic strength may vary, we also investigated the effects of ionic strength while keeping the ratio at 1:1 and pH at 6.0. The nonsignificant results of changing urinary ionic strength on melamine crystallization can be attributed to the fact that urine is supersaturated with other lithogenic ions and any change in ionic strength is subtle.

Consuming a subtoxic load of melamine may carry an increased risk of CaOx stone formation since 0.1 mM melamine in urine can enhance CaOx precipitation, as evidenced by our results. This correlates with another study showing that exposure to even a low melamine dose results in a higher risk of UA and Ca urolithiasis in adults.[21] As a result, it may be worthwhile to screen older children and even adults for renal stones after melamine exposure.

Structurally melamine and cyanurate share a good epitaxial relationship with endogenous UA in human urine. This suggests that given the correct pH and supersaturation, the precipitation of either ion would cause precipitation of the other ion. Since optimal melamine precipitation was in the same pH region as that of UA, it is not surprising that UA also precipitates out by epitaxy. Thus, UA can interact with melamine crystallization by co-precipitating with melamine so that MC crystallization is inhibited.

Cyanuric acid is an inhibitor of hepatic UA oxidase that leads to enhanced serum UA.[22] Excessive UA excreted in renal tubules can compete with cyanuric acid for melamine binding. Clinicians noted that UA co-precipitated with melamine in infants and young children in a molar ratio of 1.2 to 2.1 UA to 1 melamine.[23] Adding CaP to urine did not have a profound effect on melamine crystallization. Thus, it is not suggested that CaP interacts with melamine crystallization.

UTI is one of the most common bacterial infections in children.[24] At least 8% of girls and 2% of boys have UTI in childhood.[25] Since melamine stones developed in infants and children, UTI may contribute to melamine crystallization. E. coli is the main bacterium isolated from urine in about 75% of UTI cases.[26] The presence of E. coli mimicked UTI and promoted melamine crystallization. Larger crystals were observed under microscopic examination, which may encourage blockage of the narrow urinary tract. We recommend that active UTI be treated to decrease the risk of crystal formation.

Current therapy for urolithiasis includes drugs that chelate lithogenic ions and alkalize or acidify urine pH to prevent precipitation. We studied the 2 clinically relevant therapeutics potassium citrate and sodium bicarbonate, which were also administered to children with melamine stones. Urinary citrate forms a soluble complex with Ca by chelation, which inhibits the formation and propagation of Ca containing crystals.[27] Citrate and bicarbonate enhance urinary pH[28] to treat UA stones (alkaline treatment). Each was associated with decreased melamine crystallization through different mechanisms. Increasing citrate decreased overall urine supersaturation to inhibit crystallization through the chelation of other ions (Ca). Bicarbonate inhibited melamine crystallization by the formation of smaller melamine crystals. In biological crystallization this is also a strategy to reduce overall supersaturation and nucleate numerous

small crystals while at the same time inhibiting growth. This allows crystals to be small enough to pass through the urinary tract without causing obstruction.

CONCLUSIONS

A treatment plan for melamine exposure is proposed for patients with acute melamine exposure. Besides the cessation of melamine intake, potassium citrate and sodium bicarbonate can be applied clinically with good efficacy in children[29,30] with melamine stones. To avoid CaOx and CaP formation in neutral and alkaline urine after treatment adequate hydration can help decrease the urinary concentration of melamine and, thus, melamine, CaOx and CaP crystal formation. Treating active UTIs is advisable. Therapy should also include diuretics to flush out crystals and dilute urine as well as lithogenic ions. Exposure to relatively small doses of MC poses a risk of other urolithic stones.

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