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Melamine-Induced Urolithiasis in a *Drosophila* Model

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ABSTRACT: Melamine-tainted food can induce renal stones in both humans and animals. We have previously reported a novel *Drosophila* model for the study of renal stone disease. In addition to hyperoxaluria-causing agents, we also tested herein the effect of melamine on crystal formation in *Drosophila*. The results indicate that administration of melamine alone caused crystal formation in a dose-dependent manner. The crystals also appeared after ingestion of melamine for 3 weeks in the Malpighian tubules of *Drosophila* when viewed with polarized light. Administration of potassium citrate (K citrate) was found to significantly ameliorate the melamine-induced reduction of lifespan. However, administration of K citrate failed to reduce the quantity of crystals. Because calcium oxalate is not the major crystal induced by melamine, the predominant components of melamine-induced crystals and the potential crystal inhibitors warrant further investigation.

KEYWORDS: *Drosophila melanogaster, melamine, urolithiasis, potassium citrate*

INTRODUCTION

Melamine-contaminated milk powder causing infant fatal stone disease has evolved as a storm of stone disease in China since 2008.[1] Nearly 10 000 children suffering from urinary stone disease were reported to be related to the mis-ingestion of melamine-contaminated milk powder.[2,3] The addition of melamine/melamine analogues to food resulted in major outbreaks of poisoning associated with the melamine/melamine analogue in animals and humans. Although melamine/melamine analogues are unlikely to result in acute illness in animals, melamine alone can lead to stone formation with uric acid in children and animals.[4,5]

The stone composition of melamine induced in kidneys was studied in variable forms.[6] Melnick et al. reported that melamine causes urinary bladder stones in rodents and possibly transitional cell carcinoma.[7] Other types of urolithiasis may also relate to melamine from clinical studies. Wu et al. and Liu et al. studied the urinary concentration of melamine in uric acid and calcium stone patients and concluded that low-dose exposure (within the nanogram per milliliter level) of melamine may be responsible for both types of stone formation.[8,9] Melamine is excreted from kidneys as the original form.[10−12] Therefore, melamine may mix in the stone during its formation.

Recently, we have established a new physiological model with the fruit fly for urolithiasis.[13] The model has strengths, namely, the low cost of maintaining colonies and rapid deployment of new transgenic lines, but also weaknesses that may ultimately limit its usefulness, such as the mechanism of tubular fluid formation and difficulties in following plasma and urine biochemistries.[14] In the present study, we conducted a *Drosophila* study by applying our well-established novel model, fruit fly,[13] to investigate the possible mechanism of melamine-related urolithiasis.

MATERIALS AND METHODS

Fly-Rearing Conditions. Male wild-type flies, *Drosophila melanogaster* CS, were reared in plastic vials containing standard fly medium (yeast, corn syrup, and agar), at 25 °C and 60% humidity, with a 12 h light−dark cycle. The formula of the standard fly medium consisted of 6.7 g of agar, 21.7 g of yeast, 13.1 g of sugar, and 66.6 g of corn syrup with the addition of water to a final amount of 1 L. In the present study, the Taiwan-government-certified (without melamine and its analogue) materials were used. The solution was put in a microwave to heat, and 13.3 mL of 99% alcohol and 3.4 g of *β*-hydroxybenzoic acid methylester were added after cooling to 85 °C. Then, 10 mL of the medium was decanted into a 50 mL test tube and stored in a 4 °C freezer after the medium returned to room temperature (ready for use only within a 2 week interval).

Lithogenesis of Flies. The details for the breeding lithogenic flies are according to our previous study.[13] In brief, 0.5% ethylene glycol (EG) (Sigma), different concentrations (0.01, 0.05, 0.1, and 0.5%) of melamine (Sigma), and nutritional manipulations (6.7 g of agar, 21.7 g of yeast, 13.1 g of sugar, and 66.6 g of corn syrup with the addition of water to a final amount of 1 L) were added in the fly medium. The potassium citrate (K citrate) granules were kindly provided by Gentle Pharma (Yunlin, Taiwan). After 3 weeks, the flies ($n \cong 100$ for each group) were sacrificed under CO_2 narcotization, and the Malpighian tubules were dissected, removed, and processed for polarized light microscopy examination. The different degrees (−, 1+, 2+, and 3+) of crystal deposition in the Malpighian tubules were rated. Each blinded specimen was evaluated by three investigators who assessed crystal formation using a crystal score of 0 = none, 1 = weak, 2 = moderate, and 3 = strong.[13]

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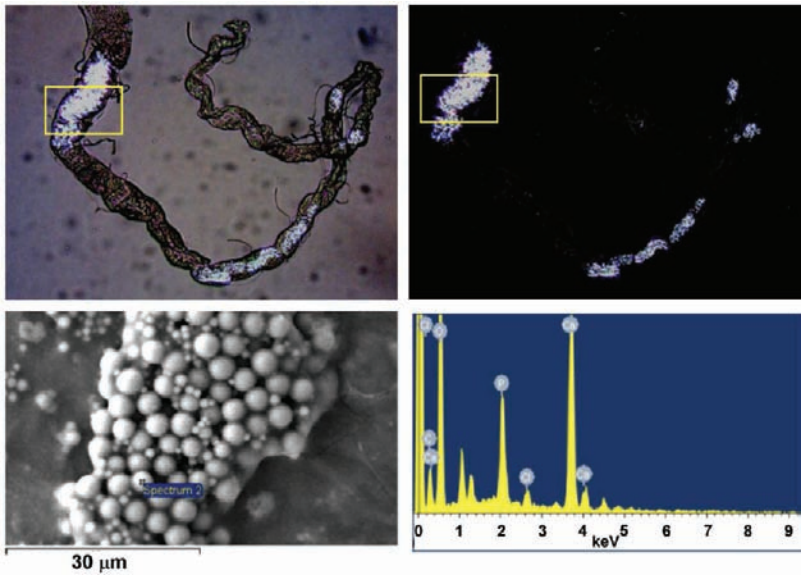

Figure 1. Melamine and EG induce crystal deposition in Malpighian tubules. A drawing of the Malpighian tubules shows that *Drosophila* has four tubules. Malpighian tubules remove extra salt, water, and nitrogenous wastes from hemolymph. Representative polarized microscopy photos for control and melamine (0.01, 0.05, and 0.1%)-/EG (0.5%)-induced crystal formation in Malpighian tubules.

Polarized Light Microscopy. The Malpighian tubules were dissected and immediately observed under normal and polarized white light with an Olympus BX51 optical microscope after the melamine crystal induction period. The relevant aspects were photographed, and the scales were obtained with the projection of a micrometric slide under the same conditions used in the illustrations.

Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS) Microanalysis. The crystals were also processed for further SEM and EDS studies to analyze the compositions. Microanalyses were performed with JEOL JSM-6700F SEM (Tokyo, Japan), with EDS, operating at an accelerated voltage of 20 kV. Pieces (12×12 mm^2) of the slides containing the samples were fixed on a carbon support with carbon tapes. To improve the image contrast, carbon was evaporated to form a thin (few nanometers) layer over the sample.

Fly Collection and Lifespan Assay. To set up lifespan assays, new emergents were collected under light CO_2 anesthesia. Foam plugs, instead of cotton plugs, were used, and the food vials were kept horizontally to avoid weaker flies being accidentally stuck to food or cotton plugs. Survivors in each vial were counted, and dead flies were removed daily. Survivorship was compared and tested for significance with log-rank tests. Lifespan curves were from pooled counts of a large number of vials ($n \cong 300$).

Statistical Analyses. One-way analysis of variation (ANOVA) was applied to detect overall differences among the groups. Bonferroni correction was applied for all multiple comparisons. Significantly different groups were compared pairwise by the Mann−Whitney *U* test for crystal scores. For comparison between two lifespan curves, we determined the *p* value in the log-rank test. All statistics were performed using the SigmaStat software (SPSS, Systat Software).

RESULTS AND DISCUSSION

Crystal-Inducing Agents. Lithogenic agents, such as EG, have been established in our *Drosophila* model[13] and as a contrast study with melamine for the present study. At different periods during the experiment, Malpighian tubules were dissected and a polarized light microscope was used to observe the crystals. Figure 1 shows a view of the morphology pattern of the melamine-induced crystal in Malpighian tubules. After 3 weeks, there were multiple different diameter spherical crystals in Malpighian tubules. Different concentrations of melamine (0.01, 0.05, and 0.1%) induced crystals in Malphigian tubules of *Drosophila*, which reveals a dose-dependent manner (Table 1). The melamine-induced crystals were observed under polarized microscopy, which was different from the control or EG-induced birefringent crystals of monohydrate calcium oxalate (in a clear or jewel-like gloss; six-sided prisms or various forms). The color of melamine-induced globular crystals under polarized microscopy seems more uniform and whitish. The distribution of melamine-induced crystals appeared more concentrated and clustered than CaO_x. Their size is estimated to vary approximately between 5 and 20 μm. Most crystals were identified within the distal segment of the anterior Malpighian tubules. In the *Drosophila* model, following melamine and cyanuric acid co-ingestion, the flies die before crystal formation. Thus, the melamine and cyanuric acid co-ingestion-induced urolithiasis could not be completed in the present study.

Table 1. Analysis of Crystal Deposit Scores in Experimental *Drosophila*[a]

group	mean of scores
control	0.12 ± 0.04
melamine	
0.01%	0.31 ± 0.08[b]
0.05%	0.48 ± 0.10[b]
0.1%	0.85 ± 0.17[b]
0.1% melamine + 2% K citrate	0.76 ± 0.23[b]

[a]Values are expressed as the mean ± standard deviation (SD). [b]$p < 0.05$ in comparison to the control group (Mann−Whitney *U* test).

Crystal Identification. Qualitative analysis using EDS is a powerful tool in microanalysis. Elemental analysis in SEM is performed by measuring the energy and intensity distribution of the X-ray signal generated by a focused electron beam. In addition to the use of the polarized light microscope for assessing crystal refraction, SEM and EDS were also used to identify the relative elemental composition of the crystals (Figure 2). After removal of the Malpighian tubule tissue with lysis buffer containing 10% proteinase K (Invitrogen, Carlsbad, CA), SEM reveals the crystal deposition inside the Malpighian

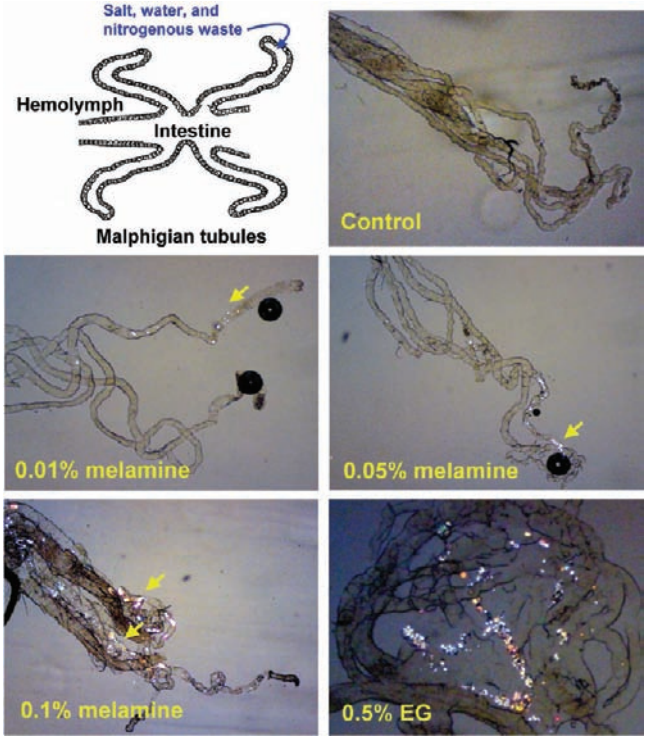

Figure 2. Analyses of melamine-induced crystals. Representative polarized microscopy photos, SEM images, and EDS spectra of a grain present at the top of Malpighian tubules under melamine treatment are shown. After Malpighian tubule tissue was removed with lysis buffer, SEM shows an internalization view. The surface shows adherence with protruding crystals. EDS spectra were recorded at 20 kV. The asterisk shows the location where the beam was focused. Scale bar = 30 μm.

tubules and the EDS analysis identified the crystal composition. The predominant components are found to be carbon (C, ~10.25%), oxygen (O, ~75.07%), phosphate (P, ~4.03%), chloride (Cl, ~0.71%), and calcium (Ca, ~9.94%) (Table 2).

Table 2. SEM Reveals the Crystal Deposited Inside the Malpighian Tubules, and EDS Analysis Identified the Crystal Composition[a]

element	atom (%)
C	10.25
O	75.07
P	4.03
Cl	0.71
Ca	9.94
total	100.00

[a]The predominant components were found to be carbon (C), oxygen (O), phosphate (P), chloride (Cl), and calcium (Ca).

The results of this microanalysis suggest that the crystal compositions may be mixture types. CaO_x is not the only type of crystals induced by melamine. The analysis of light elements [including nitrogen (N)] presents a special challenge for EDS. Some of the problems are due to inherent physical effects, while others are technical in nature, relating to the design of the instrument used for analysis and the measurement procedure. The EDS is comprised of a liquid nitrogen dewar, Si crystal, and pre-amplifier. These signals are amplified in the detector and sent to the analysis electronics in the computer for further processing. Liquid nitrogen cools and, therefore, stabilizes the electronic properties of the crystal, improving the measurements of minute X-ray signals. The light element N might be sheltered from liquid nitrogen or atmospheric N_2 in our EDS system. This might be the possible reason that N was not found in the EDS analysis.

***Drosophila* Lifespan.** Renal stones lead to chronic kidney disease in humans and may be associated with an increased mortality rate. Because it is difficult to evaluate the levels of creatinine and urea nitrogen as well as symptoms, behaviors, and clinical characteristics in the *Drosophila* model, the relationship between melamine-induced crystal formations and the lifespan of *Drosophila* was measured. Survival studies were performed to determine the impact of melamine on lifespan and mortality. The mean lifespan was significantly reduced by administration of melamine (0.01, 0.05, 0.1, and 0.5%) (Figure 3). These data confirm that administration of melamine may cause significant reduction of the lifespan of *Drosophila*.

Effects of K Citrate on Lifespan and Crystal Formation. Recent metabolic studies suggest that K citrate may be effective in reducing the risk of formation of stones because of alkali load and the citraturic response.[15] In a rat model of EG-induced CaO_x nephrolithiasis, oral administration of K citrate was found to be effective in preventing CaO_x stone formation.[16] Additionally, our previous study shows that K citrate was found to significantly ameliorate the EG-induced reduction of lifespan and inhibit EG-induced CaO_x crystal formation of the flies.[13] In the present study, we next investigated the effect of K citrate granules for the lifespan and crystal formation in *Drosophila*. The results of this investigation indicate that administration of 2% K citrate significantly ameliorates high-dose melamine-induced reduction of lifespan. However, it failed to inhibit melamine-induced crystal formation (Figure 3 and Table 1). Because most of the flies fed with 0.5% melamine die before crystal formation (3 weeks), the crystal formation experiment for 0.5% melamine cannot be completed. Deficiency of the K base in the diet increases the net systemic acid load imposed by the diet. Because K citrate is an alkalizer, the alkalinity and aging mechanisms might be involved in K-citrate-related longevity.[17] However, the exact mechanism needs to be further investigated.

Previous studies show that the composition of melamine-induced crystals was variable to include uric acid, calcium phosphate, and CaO_x.[18−20] Li et al. studied refractory melamine-related renal calculi by computed tomography, and blood biochemical parameters found that it contained a >10.88% calcium level.[21] They also used Fourier transform infrared spectroscopy to analyzed stone composition and found the stones contained both uric acid and calcium compounds.[22] Our results indicate that administration of melamine caused crystal formation in a dose-dependent manner (0.01, 0.05, and 0.1%) and mixed stone is the most possible type in melamine-induced stones.

Kobayashi et al. studied that melamine combined with cyanuric acid induced crystal formation in rats and found that the major element composition was nitrogen without calcium.[7] However, melamine alone can induce crystal formation during long-term ingestion. Therefore, timing may play a role in the formation of the crystal type.[23] A clinical therapeutic effect of K citrate was studied by Gao et al.[24] They concluded that K citrate can significantly increase the successful expulsion rate and time of melamine-induced urinary calculi. The present

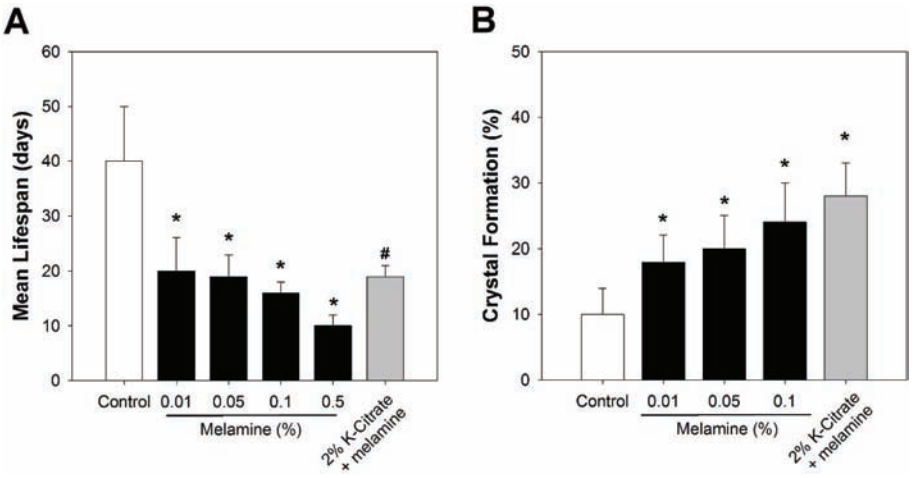

Figure 3. Effects of K citrate on lifespan and crystal formation. (A) Effect of melamine and K citrate on the lifespan of *Drosophila* ($n \cong 150$ for each group; ∗, $p < 0.05$ compared to the control; #, $p < 0.05$ compared to the 0.5% melamine-treated group). (B) Dose-dependent effect of melamine-induced crystal formation and effect of K citrate ($n \cong 100$ for each group; the results for at least eight separate experiments are expressed as the mean ± SD; ∗, $p < 0.05$ compared to the control).

study used only melamine without adding cyanuric acid and can also show crystals in Malphigian tubules of *Drosophila*. However, K citrate failed to exert an inhibitory effect on melamine-induced crystal formation in this study.

Our study has some advantages and limitations. We have applied a new animal model that easily provided a large amount of animal numbers more than rats. The experimental period time was short, and the crystals were easy to observe and calculate. The crystal components in Malphigian tubules were easily to detect through SEM with EDS. Besides, the results of our study were consistent with studies from rats and humans. However, flies are invertebrate animals that may not be fully comparable to mammals. The translation of our obtained results using the proposed model to the humans is rather difficult. For example, the absorption, metabolism, and excretion of a given substance using an insect model can be totally different from those of mammals. Furthermore, the mechanisms for the crystal formation (such as different pH values for CaO_x or uric acid crystal formation) are still unclear. Because *Drosophila* is not appropriate for investigation of renal functions, appropriate evaluation methods must be further established.

In conclusion, melamine alone can induce crystal formation in this animal model. The composition of crystal in Malphigian tubules of *Drosophila* was mixed type. Our results indicate that a long-term and large amount of melamine ingestion alone may induce crystals in animals, which may provide further evidence of melamine causing variable types of stones in humans.

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Notes

The authors declare no competing financial interest.

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