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# Melamine-Induced Urolithiasis in a *Drosophila* Model

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**Running Title:** Melamine-induced urolithiasis in *Drosophila*

# ABSTRACT

In 2008, a sharp increase of the number of children diagnosed with urinary calculi was observed in China, thousands of children were diagnosed as having melamine-contaminated food-induced urinary calculi. While several species are used for the study of urolithiasis, an ideal animal model has yet to be identified. The kidneys in vertebrates and the Malpighian tubules in *Drosophila* accomplish renal functions. We have previously reported a novel *Drosophila* model for the study of stone disease. In addition to hyperoxaluria-causing agents, we also herein tested 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 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. As calcium oxalate is not the major crystals 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 caused infant fatal stone disease has been evolved a storm of stone disease in Asia and United states since 2008 (*1-3*). Nearly ten thousand children suffered from urinary stone disease were reported to be related to the misingestion of melamine contaminated milk powder (*4, 5*). Studies of melamine associated with stone disease were reported worldwide (*6*). However, there were some arguments concerning stone or crystal formation related to ingest melamine alone or in combination with cyanuric acid (*7-9*). The recent outbreak in infants showed that melamine ingested in large doses may cause stones and illness without significant ingestion of other melamine-related chemicals. This may be due to increased uric acid excretion in infants and formation of melamine-uric acid stones. Melamine ingestion has been implicated in stone formation when co-ingested with cyanuric acid, but will cause urinary stones in infants when large amounts of melamine alone are ingested (*6*).

The stone composition of melamine induced in kidney were studied in a variable forms (*10*). Melnick *et al*. reported that melamine cause rodent’s urinary bladder stone and possibly transitional cell carcinoma (*11*). Other types of urolithiasis may also relate to melamine from clinical studies. Wu *et al*. and Liu *et al*. studied urinary concentration of melamine in uric acid and calcium stone patients and concluded that low-dose exposure (within ng/ml level) of melamine may responsible to both types stone formation (*12, 13*). Melamine is excreted from kidney as original form and may occupy half part of stone (*14-16*). Therefore, melamine may mix in the stone during its formation.

Recently, we have established a new physiological model with the fruit fly for urolithiasis (*17*). 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 (*18*). Since the stone composition contain melamine was not examined before and the mechanism is not studied yet, to address this question, we conducted an *Drosophila* study by applying our well established novel model – fruit fly (*17*) to investigate the possible mechanism.

## MATERIALS AND METHODS

**Fly Rearing Conditions.** Male wild type flies, *Drosophila melanogaster* CS, were were reared in plastic vials containing standard fly medium (yeast, corn syrup, and agar), at 25°C, 60% humidity with a 12-h light-dark cycle. The formula of standard fly medium consisted of agar 6.7 gram, yeast 21.7 gram, sugar 13.1 gram, and corn syrup 66.6 gram with the addition of water in a final amount of 1 liter. The solution was put in microwave to heat and add 13.3 ml 99% alcohol and 3.4 g β-hydroxybenzoic acid methylester after cooling to 85°C. Then, 10 ml of the medium was decanted into a 50-ml test tube and storage in 4°C freezer after the medium return to room temperature (ready for use only within a 2-week interval).

**Lithogenesis of Flies.** The details for the breeding lithogenic flies are according our previous study (*19*). In brief, 0.5% ethylene glycol (EG) (Sigma), different concentrations of melamine (Sigma), and nutritional manipulations 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 ≅ 100 for each group) were sacrificed under $CO_2$ narcotization, and the Malpighian tubules were dissected, removed, and processed for polarized light microscopy examination.

**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 with Kodak ProImage 100 film and the scales were obtained with the projection of a micrometric slide under the same conditions utilized in the illustrations.

**Electron Microscopy and EDS Microanalysis.** The crystals were also processed for further scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS or EDX) studies to analyze the compositions. Microanalyses were performed with a JEOL JSM-6360 SEM, with EDS, operated at an accelerated voltage of 20 kV. Pieces ($2 \times 2$ cm$^2$) of the slides containing the hyphae but lacking the culture medium were cut with a diamond cutter and fixed on a carbon support with carbon tapes. In order 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 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 *P* value in the log-rank test. All statistics were done by using the SigmaStat software (SPSS; Systat Software).

## RESULTS AND DISCUSSION

**Crystal-inducing Agents.** Lithogenic agent such as EG has been established in our *Drosophila* model (*17*) 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 melamine induced crystal in Malpighian tubules. 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. The melamine induced crystals were observed under polarized microscopy which was different to the 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 crystals under polarized microscopy seems more uniform and whitish. The distribution of melamine induced crystals appeared more concentrated and clusters than the CaOx. 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.

**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 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 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 1**). The results of this microanalysis suggest that the crystal compositions are mixture types and may include CaOx, calcium phosphate, uric acid, and melamine itself. CaOx is not the only type of crystals induced by melamine.

***Drosophila* Lifespan.** Renal stones lead to chronic kidney disease in humans and may be associated with an increased mortality rate. As 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* were 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 (**Figure 3**). These data confirm that high-dose administration of EG and 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 risk because of alkali load and the citraturic response (*20*). In a rat model of EG-induced CaOx nephrolithiasis, oral administration of K-Citrate was found to be effective in preventing CaOx stone formation (*21*). Additionally, our previous study shows that K-Citrate was found to significantly ameliorate the EG-induced reduction of lifespan and inhibit EG-induced CaOx crystal formation of the flies (*17*). 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**).

The results indicate that administration of melamine caused crystal formation in a dose-dependent manner. The compositions of crystal in this model show a mixed stone which might include uric acid, CaOx, and not surprising, melamine. This study used only melamine without adding cyanuric acid and can also show crystals in Malphigian tubules. However, K-Citrate failed to exert an inhibitory effect on melamine-induced crystal formation in this study..

It has been reported that the major composition of melamine-induced stone in infant urinary calculi was uric acid (*22, 23*). Large and long term ingestion melamine alone may cause crystal and stone formation in infant. They proposed the mechanism possibly due to increase uric acid excretion (*3*). Kobayashi *et al*. studied melamine combined with cyanuric acid induced rat's crystal formation and found the major element composition was nitrogen without calcium (*11*). The cause of renal failure was tubule occlusion. However, melamine alone can induce crystal formation if long-term ingestion. Therefore, timing may play a role in the formation of crystal type (*24*). There was small size of crystal in Malphigian tubules of our study. The composition of melamine-induced crystals was variable to include uric acid, calcium phosphate, and CaOx (*25-27*). Li *et al*. studied refractory melamine-related renal calculi by computed tomography and blood biochemical parameters found it contained >10.88% calcium level (*28*). They also used Fourier transform infrared to analyzed stone composition and found the stones contained both uric acid and calcium compounds (*29*). Nevertheless, mixed stone is the most possible type in melamine-induced stones. Clinical therapeutic effect of K-Citrate was studied by Gao *et al*. (*30*). They concluded that K-Citrate can significantly increase the successful expulsion rate and time of melamine-induced urinary calculi.

Our study has some advantages and limitations. We have applied a new animal model which easily provided a large amount of animal number more than rats. The experimental period time was short and the crystals were easily to be observed and calculated. The crystal components in Malphigian tubules were easily to be detected through SEM with EDS. Besides, the results of our study were consistent with studies from rats and human. However, flies are invertebrate animal which may not fully responsible 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 to those of mammals. Furthermore, the mechanisms for the crystal formation (such as different pH for CaOx or uric acid crystal formation) are still unclear. As *Drosophila* is not appropriate for investigation of renal functions, appropriate evaluation methods must be further established. Further studies are warrant to confirm the insect experiment.

In conclusion, melamine alone can induce crystal formation in this animal model. The composition of crystal in Malphigian tubules of *Drosophila* was mixed types which may contain CaOx, calcium phosphate, uric acid, and melamine itself. Our results indicate that long term and large amount melamine ingestion alone may induce crystal in animal which may provide further evidence of melamine caused variable type of stones in human.

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# FIGURE LEGENDS

**Figure 1. Melamine and EG induced crystal deposition in Malpighian tubules.** (a) A drawing of the Malpighian tubules shows that *Drosophila* has four tubules; the anterior pair and the posterior pair. Each tubule has distinct morphologic regions: initial, transitional, main segments, and lower tubule. The two tubules in each pair merge together at ureters and connect to the gut at the midgut-hindgut boundary. Representative polarized microscopy photos for melamine- and EG-induced crystal formation in Malpighian tubules.

**Figure 2. SEM and EDS microanalysis for melamine-induced crystals.** Representative polarized microscopy photos, SEM images, and EDX spectrums of a grain present at the top of Malpighian tubules under melamine treatment. After removing Malpighian tubule tissue with lysis buffer, SEM shows internalization view. Surface shows adherence with protruding crystals. EDS spectra were recorded at 20 kV. The asterisk shows the location where the beam was focused; EDS spectra obtained with the beam focused at points in the crystal sample. Scale bar = 30 μm.

**Figure 3. Effects of K-Citrate on Lifespan and Crystal Formation**. (A) Effect melamine and K-citrate on lifespan of *Drosophila* (n ≅ 150 for each group, $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to 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 least 8 separate experiments are expressed as mean ± SD. *$P < 0.05$ compared to control).

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**Table 1.** SEM reveals the crystal deposited inside the Malpighian tubules, and the EDS analysis identified the crystal composition. The predominant components were found to be carbon (C), oxygen (O), phosphate (P), chloride (Cl), and calcium (Ca).

| Element | Atom (%) |
|---|---|
| **C** | 10.25 |
| **O** | 75.07 |
| **P** | 4.03 |
| **Cl** | 0.71 |
| **Ca** | 9.94 |
| **Total** | 100.00 |