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Ethylene glycol induces calcium oxalate crystal deposition in Malpighian tubules: a novel *Drosophila* model for nephrolithiasis/urolithiasis

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AUTHOR CONTRIBUTIONS

Y.H.C. took the primary role in writing the manuscript and provided statistical support. W.Y.L designed most of the experiments, with H.Y.C., F.J.T., H.P.L., C.H.C., and Y.J.L., and supervised the final experiments. W.C.C. obtained the funding and conceived, designed, and supervised the study. W.C.C. and W.Y.L. provided the original ideas for this study.

ABSTRACT

Urolithiasis, most commonly caused by excess calcium oxalate (CaOx), is a common disorder in humans. 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. Herein, we report a novel *Drosophila* model for the study of CaOx stone disease. Lithogenic agents, ethylene glycol (EG), hydroxyl-L-proline, and sodium oxalate, were fed to *D. melanogaster*. At different periods during the experiment, Malpighian tubules were dissected and a polarized light microscope was used to highlight the bi-refringent crystals of CaOx. Scanning electron microscopy and energy dispersive X-ray spectroscopy also confirmed that the crystal composition is predominately CaOx. Furthermore, administration of potassium citrate successfully reduced the quantity of EG-induced CaOx crystals and modulated the integrity of the crystals. An ideal animal model for nephrolithiasis/urolithiasis and bio-mineralization would effectively produce crystals in its urinary system and be adaptable to many lithogenic agents, efficient, and cost-effective while permitting convenient observation of crystal formation and genetic manipulation. The proposed novel *Drosophila* model might mimic the etiology and clinical manifestations of CaOx stone disease, and could be used for investigations of the pathophysiology of this disease as well as evaluation of therapeutic approaches.

Keywords: calcium oxalate; *Drosophila melanogaster*; ethylene glycol; nephrolithiasis/urolithiasis; potassium citrate

INTRODUCTION

Urolithiasis is a common human disorder affecting approximately 9.6% of the population in Taiwan and a range of 10-12% of the population in industrialized countries.[1,2] The progressive increase of the social cost for treatment of urolithiasis may be related to increased incidence of the disease and/or to an increase in costs for diagnosing and treating renal stones. Many factors can induce urolithiasis or cause a predisposition to its development. Such factors include dehydration, particular medications, alkaline urinary pH, hypercalciuria, hyperoxaluria, and a family history of the disorder.[3] In humans, calcium oxalate (CaOx) is the major component of uroliths, and CaOx stones constitute about 80% of all stones.[4] Therefore, most investigations of urolithiasis have focused on CaOx stones. While several species may be used as animal models for the study of human CaOx stone disease, an ideal animal model has yet to be identified.[5] The ideal animal model should mimic the true pathogenesis of the human disease and allow genetic manipulation with convenient observation of results as well as being cost-effective and easy to breed.[6]

Kidney stones in both humans and hyperoxaluric rats are located on renal papillary surfaces and consist of an organic matrix and crystals of CaOx and/or calcium phosphate (CaP). Hyperoxaluria can induce CaOx nephrolithiasis in both humans and rats. Dietary factors play an important role in kidney stone formation.[7,8] Oxalate metabolism is considered to be almost identical in rats and humans. Thus, there are many similarities between experimental nephrolithiasis induced in rats and human kidney-stone formation. A rat model of CaOx nephrolithiasis can be used to investigate the mechanisms involved in human kidney stone formation.[5,9] Thus the disease is experimentally induced and the rats are generally made hyperoxaluric either by administration of excess oxalate, exposure to the toxin ethylene glycol (EG),[10,11]

hydroxyl-L-proline (HLP),[12] sodium oxalate (NaOx),[13] or various nutritional manipulations. The advantages of the rat model include a high rate of stone formation, convenient animal breeding and a well-known anatomy. However, the costs of breeding and care of experimental rats have been increasing. In addition, the costs of performing gene knockout experiments in rats are also high and occasionally accompanied by technical difficulties. Other problems that may arise with the use of rats may include cases of normal animals having natural inhibitors of abnormal mineralization and cases of uncharacterized genetic promoters of metabolic pathways related to stone formation or destruction.

Dionne and Schneider have described two features of *Drosophila melanogaster* that make it particularly valuable as a model organism. First, the tools available for studying *Drosophila* are very accommodating for the study of basic biology. Second, *Drosophila* is a good model for researching human disease, such as cancer, neurological disorders, diabetes, and drug addiction, because any findings translate well into medicine.[14] All multi-cellular organisms have a specialized organ for concentrating and excreting wastes from the body. The kidneys in vertebrates and the Malpighian tubules in *Drosophila* accomplish these functions. Mammals and *Drosophila* have similar features during renal tubular development.[15,16] Matthew *et al.* have reported that Malpighian tubules act as a genetically tractable system in the regulation of ions and epithelial fluid transport.[17] Recently, investigations of the genomics of the Malpighian tubules have revealed far more extensive roles for these structures. The tubules have the capability to actively excrete a very broad range of organic solutes and xenobiotics such as insecticides.[18] The major excretory epithelia in insects are the Malpighian tubules and hindgut, which act in concert to form the functional kidney. Secretion of fluid and ions by Malpighian tubules may be followed

by re-absorption of water, ions, or useful metabolites.[19-21]

Recently described models of animal stone disease may help us better understand and ultimately treat nephrolithiasis in humans. Given the number of similarities between treatment patterns for humans and animals, many urologic human treatments are now being integrated into the treatment of domestic animals.[22] In our previous study, we used EG to induce kidney calculus formation in rats and identified examples of Chinese herbal formulas that can inhibit this effect.[10,11] Herein, we report a novel *Drosophila* animal model for the study of stone disease. Hyperoxaluria-causing agents in rat model, including EG, HLP, and NaOx, were employed as lithogenic agents. The present animal model effectively produces crystals in the *Drosophila* urinary system. In addition to being cost-effective, it is convenient to observe the crystals and perform genetic manipulations. We expect that this model is also adaptable to other lithogenic agents (such as melamine-tainted infant formula) and will be useful for investigations of the effects of new treatment agents for the prevention of CaOx crystal formation.

RESULTS

Crystal-inducing agents

A pilot study has been performed with many different lithogenic agents to establish our *Drosophila* model. In order to compare the effects of several experimental CaOx crystal formation models in *Drosophila* and to identify a simple and convenient model with significant CaOx crystal deposition in the Malpighian tubules, several lithogenic agents (including EG, HLP, and NaOx that were used in previous rat models) were fed to individual insects of the Canton-S (CS) strain of *Drosophila melanogaster*. At different periods during the experiment, Malpighian tubules were dissected and a polarized light microscope was used to highlight the

bi-refringent crystals of CaOx. **Fig. 1a** shows a view of the classical morphology of Malpighian tubules. The CaOx bi-refringent crystals appeared as early as 14 to 21 days after ingestion of EG, HLP, and NaOx in the Malpighian tubules of *Drosophila* when viewed with polarized light (**Fig. 1b**). Monohydrate CaOx crystals (clear or jewel-like gloss; 6-sided prisms or various forms) (**Fig. 1c**) are more common than the typical dihydrate CaOx crystals (Maltese cross or envelope-shaped crystals induced by feeding of a herbal formula) (**Fig. 1d**). Various forms of monohydrate CaOx crystals shapes were also identified. Free crystals were extensive, with many incorporated in casts. Their size is estimated to vary between about 5 to 20 μm. Most crystals were identified within the "enlarged initial (distal) segment" of the anterior Malpighian tubules. Crystal formation was evaluated using a polarized light microscope. **Fig. 1d** indicates the different degrees (-, +, ++, and +++) of CaOx crystal deposition in the Malpighian tubules. 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. To avoid inter-assay variability, all samples used for this scoring method were observed from samples under the same polarization conditions.

The optimized drug concentrations, crystal-inducing period, and incidence of crystal formation for different lithogenic agents are shown in **Table 1**. We successfully induced "moderate to strong" CaOx crystal formation in Malpighian tubules of *Drosophila* with the hyperoxaluria-causing agents, EG, HLP, and NaOx, in a dose-dependent manner. Since EG caused the most consistent results for the CaOx crystal deposition in the Malpighian tubules, we recommend using EG (0.5% in fly medium) as the major crystal-inducing agent. This dosage is similar to the inducing dose of 0.75% EG in drinking water used previously in the rat urolithiasis model.[10, 11]

Crystal identification

Qualitative analysis using energy dispersive X-ray spectroscopy (EDS or EDX) is a powerful tool in microanalysis. Elemental analysis in scanning electron microscopy (SEM) is performed by measuring the energy and intensity distribution of the X-ray signal generated by a focused electron beam.[23] 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. After removal of the Malpighian tubule tissue with lysis buffer containing 10% proteinase K (Invitrogen, USA), SEM reveals the crystal deposition inside the Malpighian tubules, and the EDS analysis successfully identifies the crystal composition. The predominant components are found to be carbon (C) (weight: 29.6 ± 8.3%; atom: 42.8 ± 8.7%), oxygen (O) (weight: 39.6 ± 5.7%; atom: 43.5 ± 6.7%), and Ca (weight: 30.8 ± 8.9%; atom: 13.7 ± 4.9%) (**Fig. 2**). The results of this microanalysis confirm that the crystal composition is predominately CaOx [chemical formula: CaC_2O_4 or $Ca(COO)_2$].

Drosophila lifespan

Renal stones lead to chronic kidney disease in humans and may be associated with an increased mortality rate. Since it is difficult to evaluate the levels of creatinine, and urea nitrogen as well as symptoms, behaviors,[24] and clinical characteristics in our *Drosophila* model, the relationship between different lithogenic agents-induced crystal formation and the lifespan of *Drosophila* were measured. Survival studies were performed to determine the impact of lithogenic agents on lifespan and mortality. The control insects had mean and maximum lifespan of 40.5 and 66 days, respectively. The mean lifespan was significantly reduced by administration of EG, HLP, and NaOx

(**Fig. 3a**). Furthermore, administration of EG, HLP, and NaOx had the effect of elevating the incidence of crystal formation in a dose-dependent manner (**Fig. 3b**). These data confirm that high-dose administration of EG, HLP, and NaOx causes significant reduction of the lifespan of *Drosophila*.

Inhibition of crystal formation and lifespan extension

Recent metabolic studies suggest that high doses of potassium citrate may be effective in reducing the risk of formation of stones risk due to alkali load and the citraturic response.[4] In a rat model of EG-induced CaOx nephrolithiasis, oral potassium citrate was found to be effective in preventing CaOx stone formation.[25] In the present study, we next investigated the effect of potassium citrate granules (Gentle Pharma, Yunlin, Taiwan) for the prevention of crystal formation in *Drosophila*. Potassium citrate granules are used as an effective treatment in humans. The results of this investigation indicate that administration of potassium citrate inhibits EG-induced CaOx crystal formation in a dose-dependent manner. The crystal deposit scores dropped significantly in study groups treated with potassium citrate (**Fig. 4a**). In parallel, administration of potassium citrate was found to significantly ameliorate the EG-induced reduction of lifespan of the treated insects (**Fig. 4b**). Administration of potassium citrate successfully reduces the quantity of CaOx crystals and modulates the integrity of the crystals. Smaller crystals (+ to ++; weak to moderate) of powdery shape were formed mainly in the vicinity of the "ureter" region of the Malpighian tubules (**Fig. 4c**). Additionally, administration of potassium citrate significantly inhibited both HLP and NaOx-induced crystal formation. However, administration of potassium citrate failed to ameliorate NaOx-induced reduction of lifespan (**Fig. 4d**).

Melamine-induced crystal formation

In addition to hyperoxaluria-causing agents, melamine-contaminated milk formula has been found to cause infant nephrolithiasis. We also tested the effect of melamine on crystal formation in *Drosophila*. The results indicate that administration of melamine caused crystal formation in a dose-dependent manner (**Supplementary Fig. 1b**). The crystals also appeared after ingestion of melamine in the Malpighian tubules of *Drosophila* when viewed with polarized light (**Supplementary Fig. 1a**). Administration of potassium citrate was found to significantly ameliorate the EG-induced reduction of lifespan. However, administration of potassium citrate failed to reduce the quantity of crystals (**Supplementary Fig. 1c**). Since CaOx is not the major crystals induced by melamine,[26] the predominant components of melamine-induced crystals and the potential crystal inhibitors warrant further investigation.

DISCUSSION

The data provide us with evidence that and *in vivo* CaOx stone disease can be efficiently studied using a *Drosophila* model. Like the previously employed rat model in combination with EG administration, our *Drosophila* model has proven effective for inducing the CaOx crystal formation process responsible for production of CaOx renal stones. Our novel *Drosophila* animal model satisfies the following criteria proposed by Wolkowski-Tyl *et al.*:[27] (i) the test animals should develop uroliths rapidly and reproducibly (ii) the symptoms should be ameliorated or prevented by drug treatments known to be effective in humans, and (iii) the drugs tested should be administered orally. In our investigation, the physical characteristics of calculi removed from human and *Drosophila* are found to be identical. Crystal formation in

Drosophila is found to be responsive to potassium citrate therapy, as it is in the case of human calcium-related urolithiasis.

Administration of EG to rats is the most common method for inducing CaOx renal stones *in vivo*.[28] Other methods for experimentally inducing CaOx renal stones include adding HLP to drinking water or food, implantation of osmotic mini-pumps filled with oxalate, resection of ileum in combination with oxalate feeding, and intraperitoneal administration of oxalate or HLP.[29-32] Knight *et al.* have reported the data for HLP ingestion and urinary oxalate and glycolate excretion in human.[33] Their results revealed that the kidney absorbs significant quantities of HLP and glycolate. The Malpighian tubule has proved to be a good model for some human renal disease, and to act as an organotypic "testbed" for mammalian genes.[18] However, how similar is *Drosophila* metabolism to mammalian in the pathway where HLP is a precursor to glyoxylate, and the major site of metabolism of HLP in flies versus mammals are still unclear. Although the principal precursor of oxalate is believed to be glyoxylate, pathways in humans and flies resulting in glyoxylate synthesis are also not well defined; their metabolism to oxalate in this tissue warrants further consideration. By contrast, EG is broken down *in vivo* into four organic acids: glycoaldehyde, glycolic acid, glyoxylic acid, and oxalic acid. The oxalic acid subsequently precipitates as CaOx crystals in the kidneys.[34] The rat model has been criticized because of the side effect of metabolic acidosis induced by administration of EG. Nevertheless, Green *et al.* stated that metabolic acidosis does not always happen when the renal function is preserved.[35] Therefore, EG should be used when the rat's renal function is normal. Evaluation of the rat model may be complicated by impaired renal function due to drug or stone effects. As it is well established that EG leads to a high anion gap metabolic acidosis in patients, we should be cautious in dismissing the development

of a metabolic acidosis in our model. It is entirely possible that the negative correlation between EG dose and lifespan is due to either toxic effects of the EG in *Drosophila* and/or an EG dose-dependent induced metabolic acidosis. The positive correlation between EG dose and CaOx crystal formation could be driven by the decline in "renal" function as the severity of the acidosis or the degree of toxicity increases with an increase in EG dose. Given that potassium citrate is equivalent to a base load (from an acid-base point of view) and increases citrate excretion (from a risk of kidney stone formation point of view), it would be predicted that the metabolic acidosis would be less sever (and lifespan increased) and CaOx crystal formation decreased with potassium citrate administration.

The function of all animal excretory systems is to rid the body of toxins and to maintain homeostatic balance. Although excretory organs in diverse animal species appear superficially different, they are often built on the two common principles of filtration and tubular secretion/re-absorption. The *Drosophila* excretory system is composed of filtration Malpighian tubules.[36,37] Various insects contain crystalline structures such as CaOx or calcium urate that are predominantly localized in the Malpighian tubules.[38] In one notable example, the urine of plant-feeding caterpillars is loaded with granules and crystals of calcium carbonate and CaOx that are derived from the diet. These compounds are thought to either bind to or eliminate excess calcium or, in the case of CaOx, to neutralize and eliminate the oxalic acid.[38,39] Calcium salts have also been reported to be present in the shells of eggs of various insects, adding to their rigidity. Whereas other calcium salts such as calcium carbonate, calcium urate, or CaOx that have been observed in a variety of insects usually occur in the Malpighian tubules, calcium tartrate crystals have been identified in the mid-gut of the grape leafhopper. The accumulation of calcium salts in the

mid-gut of insects has been detected only in rare cases.[39,40] The primary excretory organs of insects are the Malpighian tubules and the rectum. The blind-ended Malpighian tubules empty into the intestinal tract at the mid-gut/hind-gut junction.[41] The site of the initial solid phase has long been the subject of debate. Our observations of the initial segment are in agreement with previous observations that interstitial crystals are located at, or adjacent to, the papillary tip. Randall's plaques, were found to be common in the tubules of insects with stone formations. On the other hand, Malpighian tubules secrete primary urine that is isosmotic to hemolymph and rich in KCl and/or NaCl. The processes of regulation of calcium and oxalate transport in Malpighian tubules remains unclear.[42] The molecular mechanisms underlying calcium and oxalate transport must be more clearly defined.

Because natural and experimental fly "urolithiasis" is similar to the human disease, the *Drosophila* model could be used to further define the pathogenesis of formation of CaOx calculi. Other lithogenic factors in addition to hyperoxaluria-causing agents need to be more clearly defined. For example, melamine-contaminated milk formula has been found to cause infant nephrolithiasis in some areas of China. Its combination with cyanuric acid causes crystallization in renal tubules. Recently, the assessment of melamine and cyanuric acid toxicity in cats and dogs has been reported.[43-45] Our *Drosophila* model can provide another complementary platform (as a substitute for "pets") to avoid experimentation on domestic animals for further examinations of the mechanisms of melamine-related urolithiasis. The model would also be useful in the evaluation of drugs, diets, or even herbal medicines[46] that might be used to prevent the formation or induce the dissolution of calculi. Potassium citrate is a well-known drug for the prevention of stone disease. However, it can only adjust the thermodynamic stability from one side

of solubility equilibrium. The solubility of a combination of potassium oxalate and calcium citrate is higher than the original ions from CaOx. Future trends in the treatment of CaOx stone disease should be focused on the other side of the solubility equilibrium i.e. the adjustment of oxalic acid levels. Selectivity of the response to different types of therapy implies that the *Drosophila* model may provide value in the development of new drugs. Thus, due to its simplicity and specificity, the model appears to offer advantages for studies of the mechanism, pathology, and treatment of calcium urolithiasis.

Urolithiasis is usually associated with metabolic abnormalities that may include hypercalciuria, hyperphosphaturia, hyperoxaluria, hypocitraturia, hyperuricosuria, cystinuria, a low urinary volume, and defects of urinary acidification.[47] The etiology of these metabolic abnormalities and of urolithiasis is multi-factorial and involves interactions between environmental, hormonal, and genetic determinants.[48] With the complete sequencing of the *Drosophila* genome, and the concurrent development of post-genomic technologies such as microarrays, proteomics, metabolomics, and systems biology, completely unexpected roles for the insect Malpighian tubule have emerged.[49] In addition to the classical role of osmoregulation, the Malpighian tubule is highly specialized for organic solute transport, as well as metabolism and detoxification. Our present *Drosophila* model may also be used as a platform for studies of lithogenic genetic factors[50] that play roles in idiopathic hypercalciuria or hyperoxaluria.[51]

A comparison of characteristics[52] of human and *Drosophila* urolithiasis/nephrolithiasis are provided in **Table 2**. Although *Drosophila* can be used as a model for urolithiasis, EG-induced crystal deposition in *Drosophila* and spontaneous urolithiasis in humans have some differences. The induced urolithiasis

was found to be more prevalent in human males and was accompanied by hypercalciuria and aciduria. Men are also more susceptible to stone formation than women, and hypercalciuria is perhaps the most common biochemical abnormality in patients with calcium urolithiasis. Calcium salts have also been reported in the eggs of various insects and add to the rigidity of the shell. Therefore, the male *Drosophila* rather than female may represent a more stable model for CaOx stone disease. On the other hand, pathological changes in the kidneys, which include renal injury and dysfunction, can lead to retention of crystals.[32] Since *Drosophila* is not appropriate for investigation of renal functions, appropriate evaluation methods must be further established. Additionally, whether the drug dosages scaled for administration to *Drosophila* can provide an approximation for the optimum therapeutic range for humans has yet to be clarified. Furthermore, because dietary factors play an important role in the crystal formation, the *Drosophila* medium should be conscientiously controlled to prevent contamination with potential lithogenic factors such as melamine-containing dairy products.

Some limitations should be considered in this study. The translation of our obtained results using the proposed model to the humans is rather difficult. There are two main concerns. One is that the absorption, metabolism and excretion of a given substance using an insect model can be totally different to those of mammals and consequently the results may be not comparable. The second aspect is related to the composition of fluids in Malpighian tubules of insects and the urine composition of mammals. Obviously the crystallization of CaOx in a fluid strongly depends on its supersaturation but also depends on the other components of such fluids and on their ionic strength. Since *Drosophila* is not appropriate for investigation of renal functions, appropriate evaluation methods must be further established.

In summary, from the study of fruit fly, we have made well progress toward validation of an original research method for studying *in vivo* CaOx stone disease. The use of hyperoxaluria-causing for inducing CaOx crystal deposition in the Malpighian tubules of *Drosophila* has the potential to allow *Drosophila* to be used as novel cost-effective urolithiasis model.

MATERIALS AND METHODS

Fly stocks and rearing conditions

Wild type flies, *Drosophila melanogaster* CS, were used in these experiments. Flies were reared in plastic vials containing standard fly medium (yeast, corn syrup, sugar, and agar), at 25°C, 50-60% humidity with a 12 hours light-dark cycle.

Lithogenesis of flies

The experimental model of CaOx crystal formation was produced in both male and female CS *Drosophila* as described below. Different concentrations of EG, HLP, NaOx, or various nutritional manipulations were added in the fly medium (wt/vol). 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 crystals were also processed for further SEM and EDS studies. The potassium citrate (K-Citrate) granules were kindly provided by Gentle Pharma (Yunlin, Taiwan).

Polarized light microscopy

After the CaOx crystal induction period, the Malpighian tubules were dissected and immediately observed under normal and polarized white light with an Olympus BX51

optical microscope. The relevant aspects were photographed 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

Microanalyses were performed with a JEOL JSM-6700F SEM, with EDS, operated 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. 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 ($n \cong 150$).

Statistical Analyses

One-way ANOVA was applied to detect overall differences among the groups; for all multiple comparisons, Bonferroni correction was applied. 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).

DISCLOSURE

All the authors declared no competing interests.

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FIGURE LEGENDS

Figure 1. Hyperoxaluria-causing agents-induced CaOx crystal deposition in Malpighian tubules. (a) A drawing and of the Malpighian tubules. *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. (b) Representative polarized microscopy photos for EG-, HLP, and NaOx-induced CaOx crystal formation in Malpighian tubules. (c) Monohydrate CaOx crystals and dihydrate CaOx crystals. (d) The different degrees (-, +, ++, and +++) of CaOX crystal deposition in the Malpighian tubules for semi-quantification of crystal formation (arbitrarily crystal score: 0 = no, 1 = weak, 2 = moderate, and 3 = strong crystal formation).

Figure 2. SEM and EDS microanalysis for CaOx crystals. Representative SEM images and EDX spectrums of a grain present at the top of Malpighian tubules under EG 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 inset photo shows the polarized microscopy image of the crystal sample, the arrow shows the location where the beam was focused; EDS spectra obtained with the beam focused at points in the crystal sample. The predominant components were found to be C, O, and Ca. Scale bar = 60 μm.

Figure 3. High-dose hyperoxaluria-causing agents induced of *Drosophila* lifespan reduction and increased incidence of crystal formation. (a) Cumulative survival distributions by hyperoxaluria-causing agents EG, HLP, and NaOx administration.

Hyperoxaluria-causing agents-treated flies showed significant lifespan reduction in a dose-dependent manner compared with control (n ≅ 150 for each group, $P < 0.0001$). (b) The relation of mean lifespan and incidence of CaOx crystal formation in hyperoxaluria-causing agents-treated male *Drosophila* (n ≅ 100 for each group, $^{*}P < 0.01$ compared to control). In this and the following figures, only male flies were used, with their numbers indicated in parentheses for each experiment.

Figure 4. Inhibition of crystal formation and lifespan extension by potassium citrate treatment. (a) Dose-dependent effects of potassium citrate on 0.5% EG-induced CaOx crystal formation (n ≅ 100 for each group, the results for least 8 separate experiments are expressed as mean ± SD. $^{*}P < 0.001$ compared to control; $^{\#}P < 0.001$ compared to EG-treated group). (b) Lifespan extension of 0.5% EG-treated flies by potassium citrate treatment (n ≅ 150 for each group, $P < 0.0001$). (c) Representative polarized microscopy photos for potassium citrate-remedied CaOx crystal formation in Malpighian tubules. Enlarged photo shows the powdered small crystals in the "ureter" site. (d) Effects of potassium citrate on HLP and NaOx-induced crystal formation (n ≅ 100 for each group, the results for least 8 separate experiments are expressed as mean ± SD. $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to HLP or NaOx-treated group); lifespan extension of HLP-treated flies by potassium citrate treatment (n ≅ 150 for each group, $P < 0.05$).

REFERENCES

1. Lee YH, Huang WC, Tsai JY, *et al.* Epidemiological studies on the prevalence of upper urinary calculi in Taiwan. *Urol Int* 2002; **68:** 172-177.
2. Moe OW. Kidney stones: pathophysiology and medical management. *Lancet* 2006; **367:** 333-344.
3. Broadus AE, Thier SO. Metabolic basis of renal-stone disease. *N Engl J Med* 1979; **300:** 839-845.
4. Tracy CR, Pearle MS. Update on the medical management of stone disease. *Curr Opin Urol* 2009; **19:** 200-204.
5. Khan SR. Animal models of kidney stone formation: an analysis. *World J Urol* 1997; **15:** 236-243.
6. Dow JA. Model organisms and molecular genetics for endocrinology. *Gen Comp Endocrinol* 2007; **153:** 3-12.
7. Borghi L, Schianchi T, Meschi T, *et al.* Comparison of two diets for the prevention of recurrent stones in idiopathic hypercalciuria. *N Engl J Med* 2002; **346:** 77-84.
8. Curhan GC, Willett WC, Speizer FE, *et al.* Comparison of dietary calcium with supplemental calcium and other nutrients as factors affecting the risk for kidney stones in women. *Ann Intern Med* 1997; **126:** 497-504.
9. Evan AP, Bledsoe SB, Smith SB, *et al.* Calcium oxalate crystal localization and osteopontin immunostaining in genetic hypercalciuric stone-forming rats. *Kidney Int* 2004; **65:** 154-161.
10. Tsai CH, Chen YC, Chen LD, *et al.* A traditional Chinese herbal antilithic formula, Wulingsan, effectively prevents the renal deposition of calcium oxalate crystal in ethylene glycol-fed rats. *Urol Res* 2008; **36:** 17-24.
11. Tsai CH, Pan TC, Lai MT, *et al.* Prophylaxis of experimentally induced calcium oxalate nephrolithiasis in rats by Zhulingtang, a traditional Chinese herbal formula. *Urol Int* 2009; **82:** 464-471.
12. Khan SR, Glenton PA, Byer KJ. Modeling of hyperoxaluric calcium oxalate nephrolithiasis: experimental induction of hyperoxaluria by hydroxy-L-proline. *Kidney Int* 2006; **70:** 914-923.
13. Poonkuzhali B, Saraswathi CP, Rajalakshmi K. Effect of uric acid on sodium oxalate-induced urolithiasis in rats--biochemical and histological evidences. *Indian J Exp Biol* 1994; **32:** 20-24.
14. Dionne MS, Schneider DS. Models of infectious diseases in the fruit fly Drosophila melanogaster. *Dis Model Mech* 2008; **1:** 43-49.
15. Singh SR, Hou SX. Lessons learned about adult kidney stem cells from the malpighian tubules of Drosophila. *J Am Soc Nephrol* 2008; **19:** 660-666.

16. Jung AC, Denholm B, Skaer H, *et al.* Renal tubule development in Drosophila: a closer look at the cellular level. *J Am Soc Nephrol* 2005; **16:** 322-328.
17. MacPherson MR, Pollock VP, Broderick KE, *et al.* Model organisms: new insights into ion channel and transporter function. L-type calcium channels regulate epithelial fluid transport in Drosophila melanogaster. *Am J Physiol Cell Physiol* 2001; **280:** C394-407.
18. Dow JA, Davies SA. The Malpighian tubule: rapid insights from post-genomic biology. *J Insect Physiol* 2006; **52:** 365-378.
19. O'Donnell MJ, Maddrell SH. Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female Drosophila. *J Exp Biol* 1995; **198:** 1647-1653.
20. O'Donnell MJ, Ianowski JP, Linton SM, *et al.* Inorganic and organic anion transport by insect renal epithelia. *Biochim Biophys Acta* 2003; **1618:** 194-206.
21. Beyenbach KW. Transport mechanisms of diuresis in Malpighian tubules of insects. *J Exp Biol* 2003; **206:** 3845-3856.
22. Robinson MR, Norris RD, Sur RL, *et al.* Urolithiasis: not just a 2-legged animal disease. *J Urol* 2008; **179:** 46-52.
23. Rio MC, de Oliveira BV, de Tomazella DP, *et al.* Production of calcium oxalate crystals by the basidiomycete Moniliophthora perniciosa, the causal agent of witches' broom disease of Cacao. *Curr Microbiol* 2008; **56:** 363-370.
24. Dankert H, Wang L, Hoopfer ED, *et al.* Automated monitoring and analysis of social behavior in Drosophila. *Nat Methods* 2009; **6:** 297-303.
25. Yasui T, Sato M, Fujita K, *et al.* Effects of citrate on renal stone formation and osteopontin expression in a rat urolithiasis model. *Urol Res* 2001; **29:** 50-56.
26. Chen WC, Wu SY, Liu HP, *et al.* Identification of melamine/cyanuric acid-containing nephrolithiasis by infrared spectroscopy. *J Clin Lab Anal* 2010; **24:** 92-99.
27. Wolkowski-Tyl R, Chin TY, Popp JA, *et al.* Chemically induced urolithiasis in weanling rats. *Am J Pathol* 1982; **107:** 419-421.
28. Hennequin C, Tardivel S, Medetognon J, *et al.* A stable animal model of diet-induced calcium oxalate crystalluria. *Urol Res* 1998; **26:** 57-63.
29. Tawashi R, Cousineau M, Sharkawi M. Calcium oxalate crystal formation in the kidneys of rats injected with 4-hydroxy-L-proline. *Urol Res* 1980; **8:** 121-127.
30. O'Connor RC, Worcester EM, Evan AP, *et al.* Nephrolithiasis and nephrocalcinosis in rats with small bowel resection. *Urol Res* 2005; **33:** 105-115.

31. Marengo SR, Chen DH, MacLennan GT, *et al.* Minipump induced hyperoxaluria and crystal deposition in rats: a model for calcium oxalate urolithiasis. *J Urol* 2004; **171:** 1304-1308.
32. Khan SR. Renal tubular damage/dysfunction: key to the formation of kidney stones. *Urol Res* 2006; **34:** 86-91.
33. Knight J, Jiang J, Assimos DG, *et al.* Hydroxyproline ingestion and urinary oxalate and glycolate excretion. *Kidney Int* 2006; **70:** 1929-1934.
34. Leth PM, Gregersen M. Ethylene glycol poisoning. *Forensic Sci Int* 2005; **155:** 179-184.
35. Green ML, Hatch M, Freel RW. Ethylene glycol induces hyperoxaluria without metabolic acidosis in rats. *Am J Physiol Renal Physiol* 2005; **289:** F536-543.
36. O'Donnell MJ. Too much of a good thing: how insects cope with excess ions or toxins in the diet. *J Exp Biol* 2009; **212:** 363-372.
37. Denholm B, Skaer H. Bringing together components of the fly renal system. *Curr Opin Genet Dev* 2009; **19:** 526-532.
38. Teigler DJ, Arnott HJ. Crystal development in the malpighian tubules of Bombyx mori (L.). *Tissue Cell* 1972; **4:** 173-185.
39. Boll S, Schmitt T, Burschka C, *et al.* Calcium tartrate crystals in the midgut of the grape leafhopper. *J Chem Ecol* 2005; **31:** 2847-2856.
40. Waku Y, Sumimoto K. Metamorphosis of midgut epithelial cells in the silkworm (Bombyx Mori L.) with special regard to the calcium salt deposits in the cytoplasm. I. light microscopy. *Tissue Cell* 1971; **3:** 127-136.
41. Singer MA. Dietary protein-induced changes in excretory function: a general animal design feature. *Comp Biochem Physiol B Biochem Mol Biol* 2003; **136:** 785-801.
42. Dube K, McDonald DG, O'Donnell MJ. Calcium transport by isolated anterior and posterior Malpighian tubules of Drosophila melanogaster: roles of sequestration and secretion. *J Insect Physiol* 2000; **46:** 1449-1460.
43. Puschner B, Poppenga RH, Lowenstine LJ, *et al.* Assessment of melamine and cyanuric acid toxicity in cats. *J Vet Diagn Invest* 2007; **19:** 616-624.
44. Thompson ME, Lewin-Smith MR, Kalasinsky VF, *et al.* Characterization of melamine-containing and calcium oxalate crystals in three dogs with suspected pet food-induced nephrotoxicosis. *Vet Pathol* 2008; **45:** 417-426.
45. Dobson RL, Motlagh S, Quijano M, *et al.* Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. *Toxicol Sci* 2008; **106:** 251-262.
46. Butterweck V, Khan SR. Herbal medicines in the management of urolithiasis:

alternative or complementary? *Planta Med* 2009; **75:** 1095-1103.

47. Stechman MJ, Loh NY, Thakker RV. Genetics of hypercalciuric nephrolithiasis: renal stone disease. *Ann N Y Acad Sci* 2007; **1116:** 461-484.
48. Khan SR, Canales BK. Genetic basis of renal cellular dysfunction and the formation of kidney stones. *Urol Res* 2009; **37:** 169-180.
49. Dow JA. Insights into the Malpighian tubule from functional genomics. *J Exp Biol* 2009; **212:** 435-445.
50. Sayer JA. The genetics of nephrolithiasis. *Nephron Exp Nephrol* 2008; **110:** e37-43.
51. Devuyst O, Pirson Y. Genetics of hypercalciuric stone forming diseases. *Kidney Int* 2007; **72:** 1065-1072.
52. Klausner JS, Osborne CA, Griffith DP. Canine struvite urolithiasis. *Am J Pathol* 1981; **102:** 457-458.

Table 1. Incidence of crystal formation for different lithogenic agents in *Drosophila*

	Crystal Formation (%)		
	Male	**Female**	**Total**
Control	6.6 ± 3.6	46.8 ± 3.4	24.3 ± 3.0
EG			
0.1%	54.8 ± 9.4*	67.0 ± 4.9*	61.3 ± 2.8*
0.5%	94.2 ± 1.5*	98.4 ± 0.1*	97.7 ± 1.4*
0.75%	100.0 ± 0.0*	100.0 ± 0.0*	100.0 ± 0.0*
1%	100.0 ± 0.0*	100.0 ± 0.0*	100.0 ± 0.0*
HLP			
0.01%	4.6 ± 7.5	90.3 ± 8.3*	38.5 ± 3.1*
0.1%	9.0 ± 5.7	91.2 ± 8.2*	42.4 ± 9.9*
1%	15.3 ± 2.8*	91.6 ± 11.7*	48.7 ± 7.5*
NaOx			
0.01%	49.6 ± 3.5*	98.0 ± 3.4*	73.8 ± 4.3*
0.05%	91.0 ± 5.3*	96.0 ± 3.5*	93.7 ± 4.5*

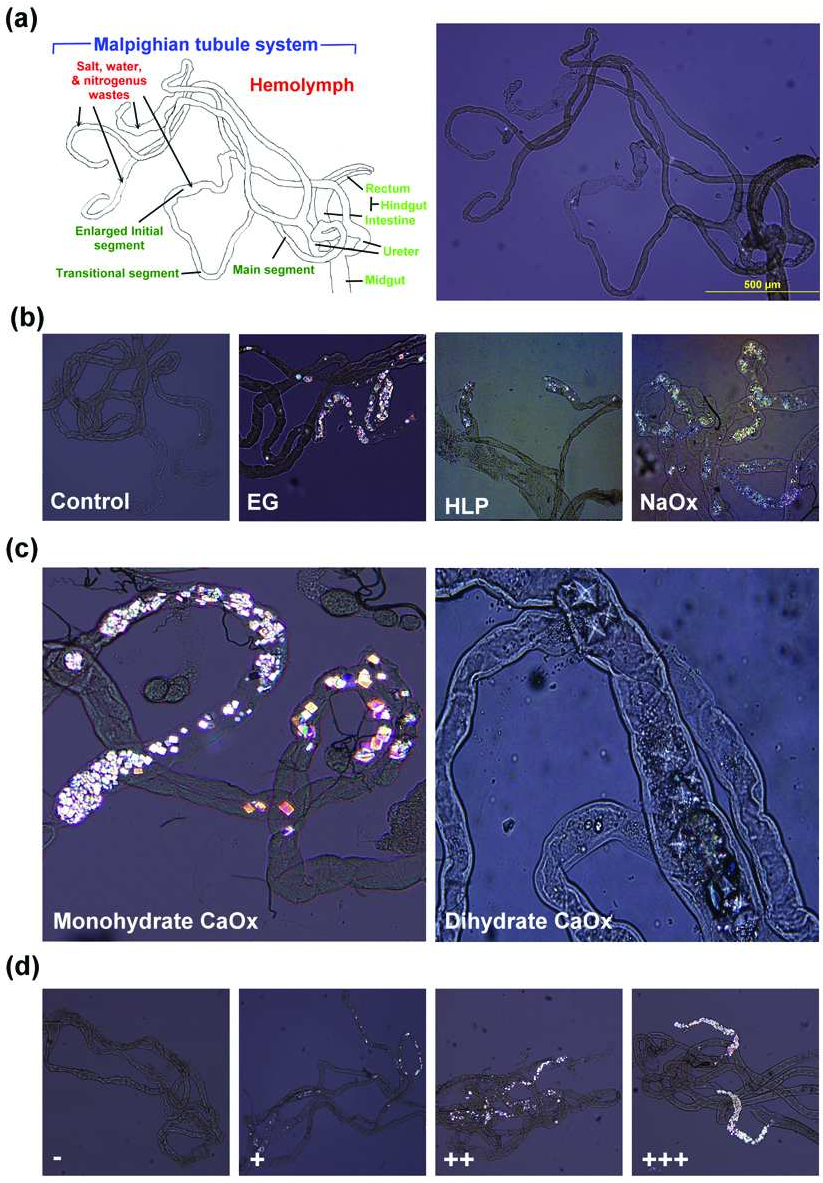
EG: ethylene glycol; HLP: hydroxyl-L-proline; NaOx: sodium oxalate.

Values were expressed as means ± SEM. Statistical evaluation was performed using one-way ANOVA followed by Bonferroni test.

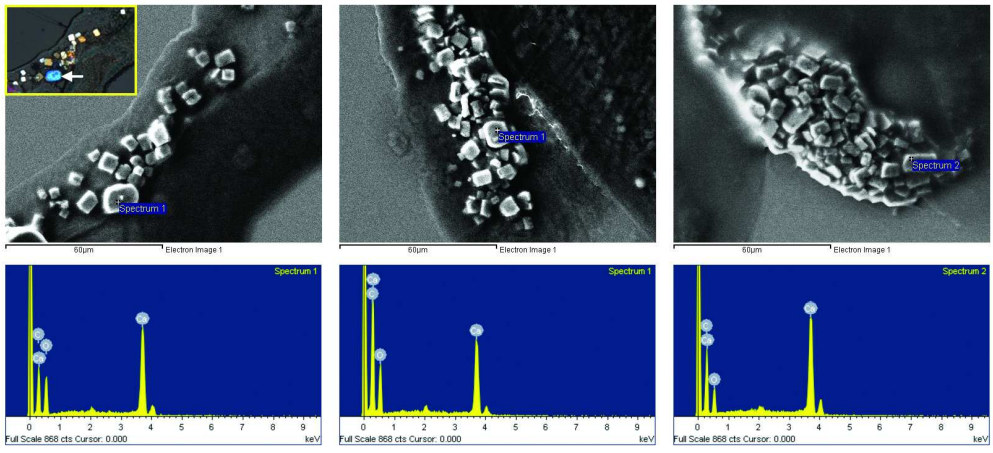
*A probability, *P*, value of < 0.05 was considered significant compared to control.

Table 2. Characteristics of human and *Drosophila* urolithiasis/nephrolithiasis

I. Similarities between human and *Drosophila* urolithiasis/nephrolithiasis
A. Overall prevalence and incidence (for male) of urolithiasis rate (~10%)
B. Dietary factors play an important role in stone/crystal formation
C. The stone/crystal can be ameliorated or prevented by oral drug treatment
D. Familiar physiology function of kidneys and Malpighian tubules
II. Differences between human and *Drosophila* urolithiasis/nephrolithiasis
A. Increased prevalence in male of human but in female of *Drosophila*
B. The symptom, complication, and mortality are inconsistent
C. Distinct drug pharmacokinetic and pharmacodynamic
D. Discrepant anatomy conformation between kidneys and Malpighian tubules



Hyperoxaluria-causing agents-induced CaOx crystal deposition in Malpighian tubules
70x101mm (300 x 300 DPI)

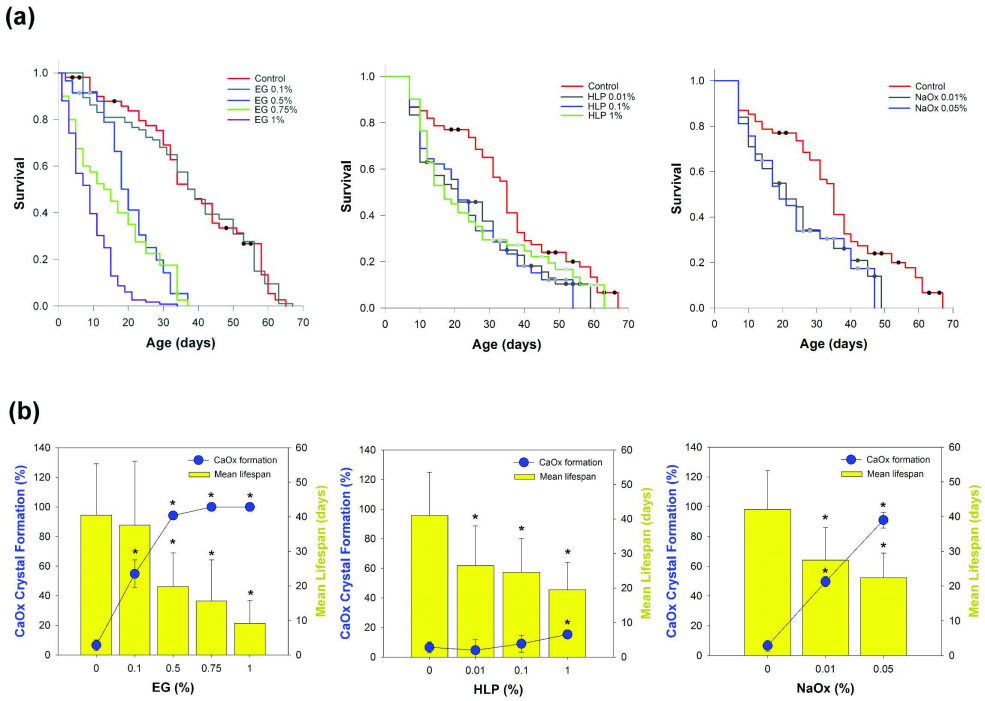


Element	Weight (%)	Atom (%)
C	22.33	33.91
O	44.86	51.15
Ca	32.81	14.93
Totals	100.00	100.00

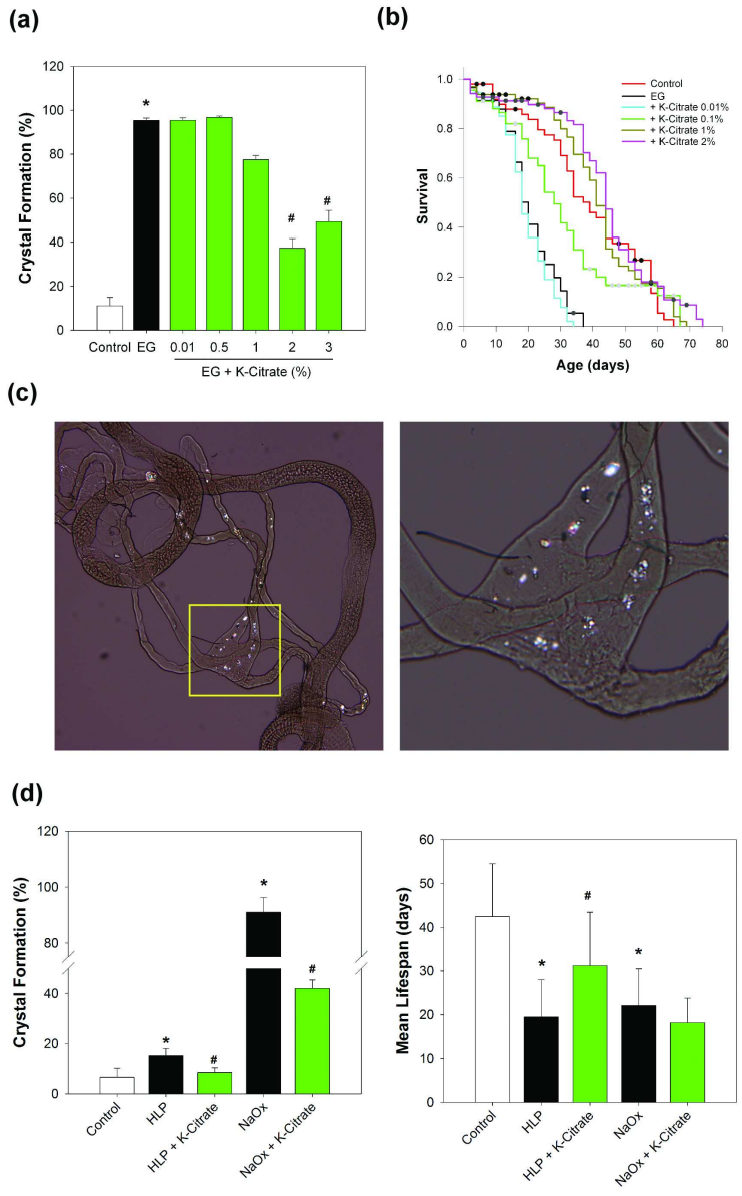
Element	Weight (%)	Atom (%)
C	38.62	51.35
O	40.33	40.26
Ca	21.06	8.39
Totals	100.00	100.00

Element	Weight (%)	Atom (%)
C	27.86	43.11
O	33.58	39.01
Ca	38.56	17.88
Totals	100.00	100.00

SEM and EDS microanalysis for CaOx crystals
136x85mm (300 x 300 DPI)



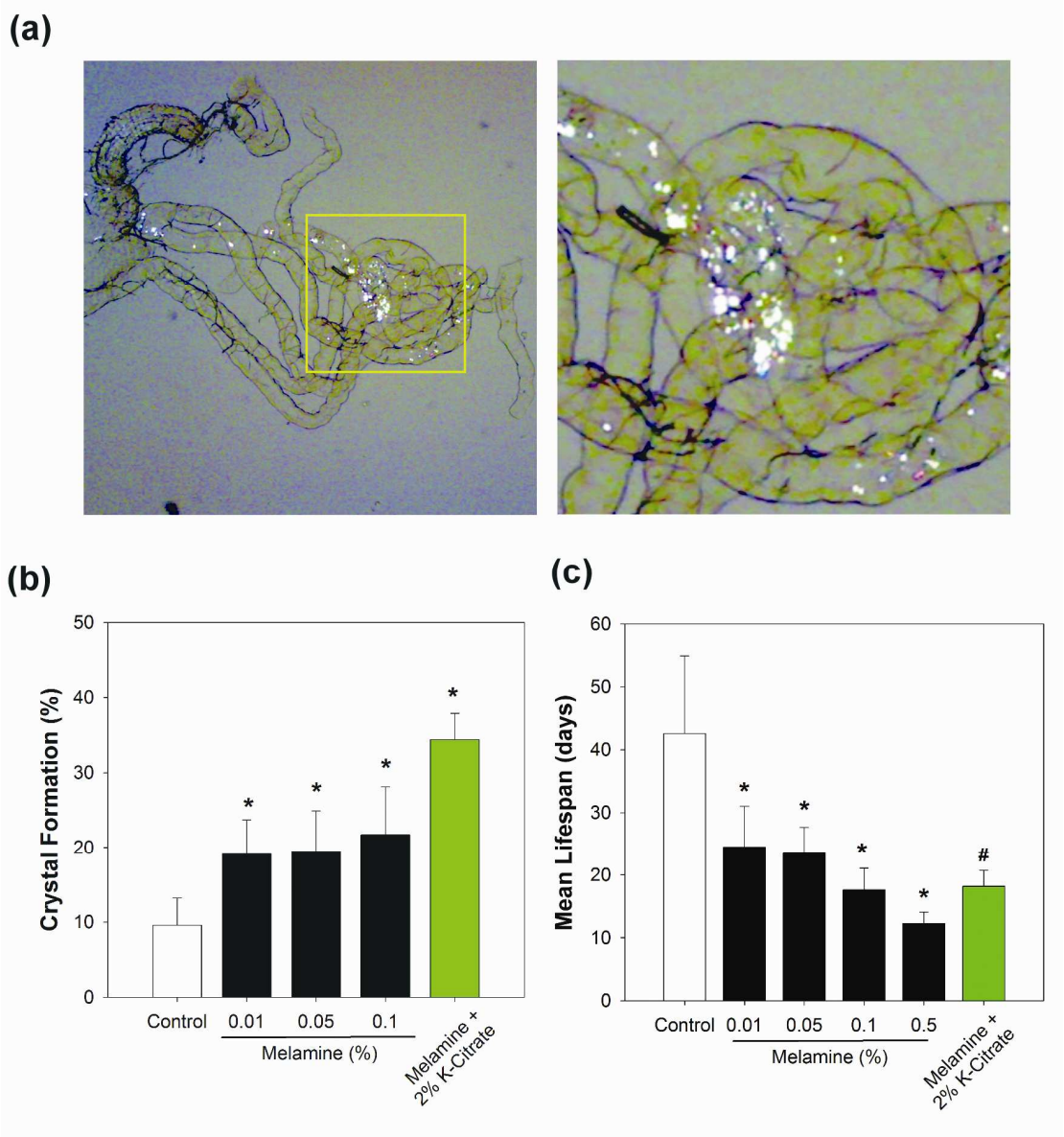
High-dose hyperoxaluria-causing agents induced of Drosophila lifespan reduction and increased incidence of crystal formation
197x141mm (300 x 300 DPI)



Inhibition of crystal formation and lifespan extension by potassium citrate treatment
146x236mm (300 x 300 DPI)

FIGURE LEGEND (Supplementary Data)

Figure 1. Melamine-induced crystal deposition in Malpighian tubules. (a) Representative polarized microscopy photos for melamine-induced crystal formation in Malpighian tubules. (b) Dose-dependent effect of melamine-induced crystal formation and effect of potassium citrate (n ≅ 100 for each group, the results for least 8 separate experiments are expressed as mean ± SD. *$P < 0.05$ compared to control). (c) Effect melamine and potassium 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).


(a)
(b)
Crystal Formation (%)
0
10
20
30
40
50
Control
0.01
0.05
0.1
Melamine (%)
Melamine + 2% K-Citrate
(c)
Mean Lifespan (days)
0
10
20
30
40
50
60
Control
0.01
0.05
0.1
0.5
Melamine (%)
Melamine + 2% K-Citrate