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Review Article

Drosophila melanogaster as an Emerging Translational Model of Human Nephrolithiasis

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Abbreviations and Acronyms

ATPase = adenosine triphosphatase

CT = computerized tomography

UAS = upstream activation sequence

XDH = xanthine dehydrogenase

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Purpose: The limitations imposed by human clinical studies and mammalian models of nephrolithiasis have hampered the development of effective medical treatments and preventive measures for decades. The simple but elegant Drosophila melanogaster is emerging as a powerful translational model of human disease, including nephrolithiasis. It may provide important information essential to our understanding of stone formation. We present the current state of research using D. melanogaster as a model of human nephrolithiasis.

Materials and Methods: We comprehensively reviewed the English language literature using PubMed®. When necessary, authoritative texts on relevant subtopics were consulted.

Results: The genetic composition, anatomical structure and physiological function of Drosophila malpighian tubules are remarkably similar to those of the human nephron. The direct effects of dietary manipulation, environmental alteration and genetic variation on stone formation can be observed and quantified in a matter of days. Several Drosophila models of human nephrolithiasis have been developed, including genetically linked and environmentally induced stones. A model of calcium oxalate stone formation is among the most recent fly models of human nephrolithiasis.

Conclusions: The ability to readily manipulate and quantify stone formation in D. melanogaster models of human nephrolithiasis presents the urological community with a unique opportunity to increase our understanding of this enigmatic disease.

Key Words: kidney; nephrolithiasis; Drosophila melanogaster; malpighian tubules; disease models, animal

The development of effective medical therapies for the prevention and treatment of nephrolithiasis has been hindered by a lack of understanding of the fundamental mechanisms of the disease. Human clinical studies and mammalian models of nephrolithiasis are constrained by financial costs, ethical standards and protracted biological cycles. Furthermore, the genetic and physiological complexities of these models obscure the true impact of dietary manipulation, environmental alteration and genetic variation on the most basic processes underlying stone formation.

Drosophila melanogaster has been successfully used in the study of

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various human diseases spanning numerous organ systems. This versatile invertebrate is now emerging as a powerful translational model of human nephrolithiasis with an array of practical and functional advantages. Among these advantages are the low cost of acquisition and maintenance, and a brief life cycle that facilitates rapid, economical modeling. The D. melanogaster genome is highly conserved, fully characterized and easily exploited through a wide array of well established, sophisticated genomic tools. Mutant stock lines are readily available and affordable. Most importantly, Drosophila malpighian tubules are remarkably similar to human renal tubules in genetic activity, anatomical structure and physiological function, and yet their simplicity allows for direct observation and quantification of the effects of experimental conditions in ways that other model systems cannot.

We present a review of the current state of research using D. melanogaster as a model of human nephrolithiasis. A detailed description of the structure, function and genetics of the malpighian tubules is also included to highlight the current usefulness and prospective role of this novel invertebrate model.

MALPIGHIAN TUBULE STRUCTURE, FUNCTION AND GENETICS

The renal system of D. melanogaster is composed of 2 anatomically and functionally discrete organs, that is nephrocytes and malpighian tubules. Nephrocytes are specialized groups of cells clustered near the heart and esophagus that filter the hemolymph (circulatory fluid) of the fly and remove waste products in a manner analogous to the endocytic processes of podocytes in the human glomerulus.[1] The malpighian tubules are similar to the remainder of the human nephron and collecting duct. They generate urine via active transport of ions, water and organic solutes from the hemolymph into the malpighian tubule lumen.

D. melanogaster has 4 malpighian tubules, including 1 anterior and 1 posterior pair. Each pair of malpighian tubules coalesces into a common ureter at the junction of the midgut and hindgut (fig. 1). Like the human ureter, longitudinal and circular muscle layers surround the Drosophila ureter to facilitate urine peristalsis.[2] A single malpighian tubule is approximately 2 mm long with an inner luminal diameter of 17 μm (fig. 2).[3] The malpighian tubules can be divided into 3 genetically and physiologically distinct domains, including the initial, transitional and main segments. The main segment contains about 75 of the 100 to 150 cells that make up each tubule.[4] This segment, which is primarily responsible for Drosophila urine production, is

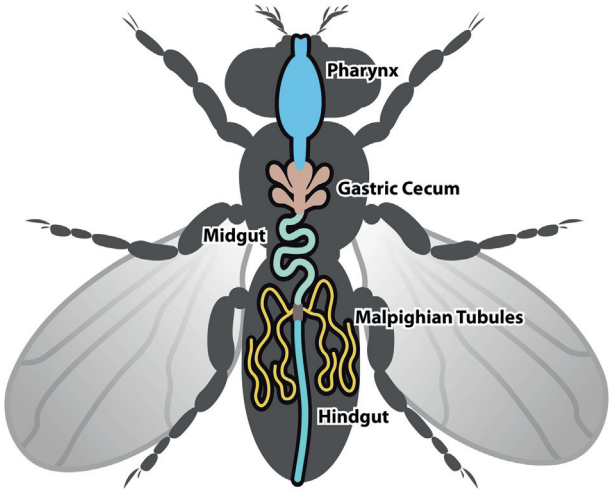

Figure 1. In D. melanogaster excretory tract 2 pairs of malpighian tubules, 1 anterior and 1 posterior, are each connected to gut by common ureter.

composed of 2 cell types, including principal cells and stellate cells. These cells are comparable in structure and function to the principal cells and intercalated cells of the human collecting duct tubules and they contain many homologous ion and organic solute transporters (fig. 3).

The larger principal cells, concentrated in the main segment of the tubule, contain basolateral ion cotransporters for Na^+, K^+ and Cl^- as well as a Na^+ dependent solute transporter and a Na^+ dependent Cl^-/HCO_3^- exchanger.[5] Ion transport at the apical cell membrane of the principal cell is accomplished via vacuolar-type H^+-ATPase, which pumps protons from the cell into the malpighian tubule lumen, providing the gradient necessary for secondary

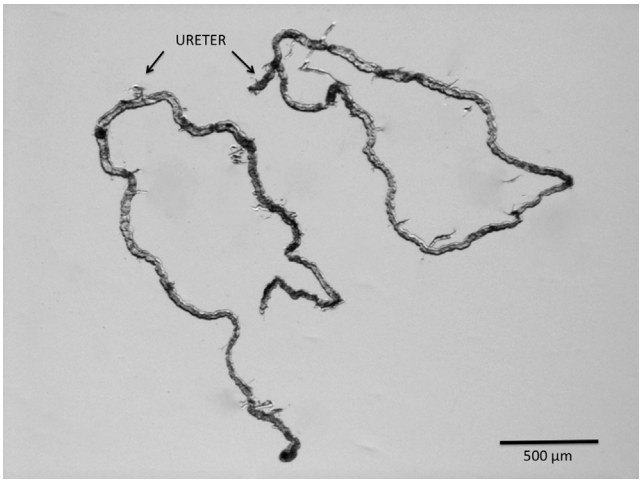

Figure 2. Photomicrograph shows 2 pairs of malpighian tubules dissected free from adult D. melanogaster. Each pair coalesces into common ureter.

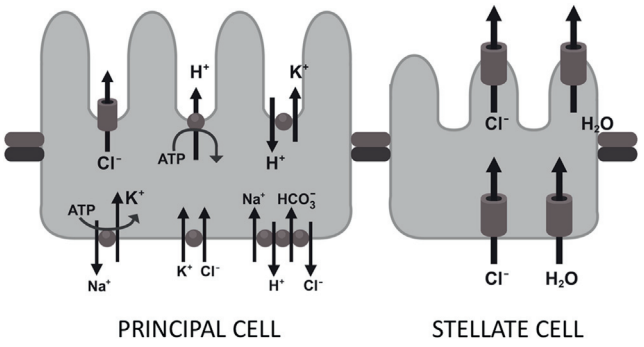

Figure 3. D. melanogaster malpighian tubule principal and stellate cells.

movement of Na^+ and K^+ into the lumen by the Na^+/H^+ and K^+/H^+ exchangers.[6] The stellate cells, which are more evenly distributed throughout the initial, transitional and main segments of the posterior malpighian tubules, provide mainly chloride transport and water conductance.[7]

The malpighian tubules regulate whole body calcium levels through the secretion and subsequent formation of intraluminal concretions that are variably composed of concentric rings of calcium, magnesium, potassium, carbonate, phosphate, chloride and an organic matrix of glycosaminoglycans or proteoglycans.[8] In flies fed a high calcium diet increased concretion formation causes malpighian tubule obstruction and distention.[9]

In addition to the shared structural and physiological properties, there is excellent conservation of the gene function profile of the malpighian tubules. The D. melanogaster genome comprises 3 pairs of autosomal chromosomes and 1 pair of sex chromosomes (fig. 4). While the entire Drosophila genome is approximately the size of a single human chromosome, there is remarkable conservation across species. On cross genomic analysis more than 70% of human disease loci were found to have a homologue in the D. melanogaster genome, including 68% of the genes linked to human cancer.[10,11]

The renal system is among the most highly conserved organ systems in the fly with dozens of Drosophila genes that correspond to genetic diseases of the human kidney (see table).[7,12,13] This conservation is dramatically shown by gene enrichment studies, in which expression levels of a gene in a specific tissue type are compared to gene expression levels in the entire organism. These data are used to infer tissue properties and physiological detail in experimental animals, and identify phenotypes suitable for studying human diseases. The D. melanogaster genome contains more than 200 genes with expression levels that are more than tenfold enriched in the malpighian tubules compared to the

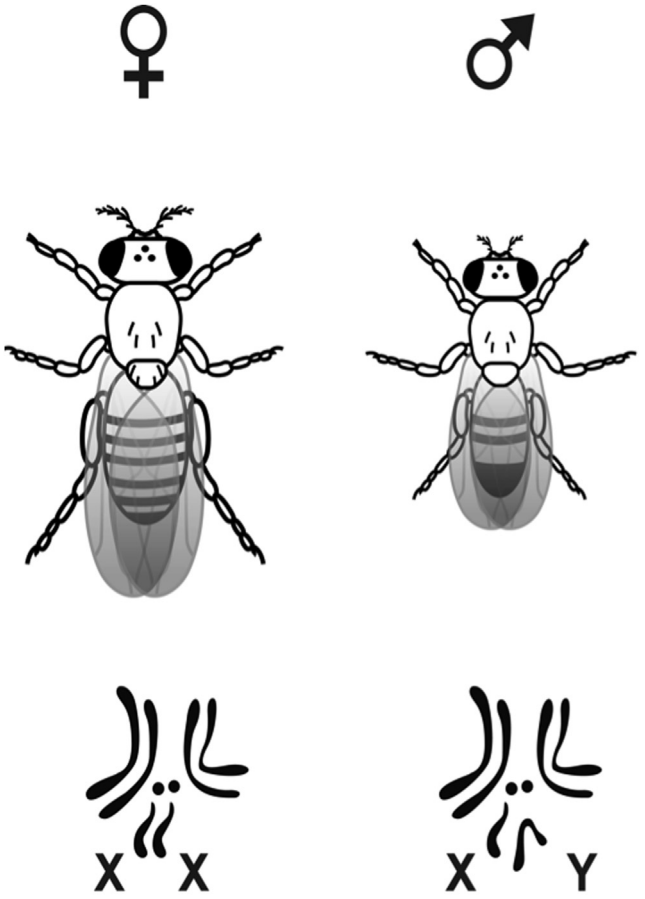

Figure 4. D. melanogaster is easily segregated visually by gender due to longer, pointed abdomen of female. Genome comprises 3 autosomal pairs and 1 sex pair.

entire fly. Of Drosophila genes with a human disease homologue 50 are enriched threefold or more in the malpighian tubules, implying conservation of function of the excretory systems of 2 species separated by millions of years of evolution (see table).[12,14]

DROSOPHILA MODELS OF HUMAN NEPHROLITHIASIS

The conservation of genetic composition and transporter protein structure as well as the similarities of physiological function of the malpighian tubules has facilitated the development of several Drosophila stone models, including those of genetically linked and environmentally induced nephrolithiasis. Most recently, a D. melanogaster model of calcium oxalate nephrolithiasis was described.

Genetically Linked Nephrolithiasis

Numerous inborn metabolic diseases, such as hyperoxaluria, cystinuria and hyperaminoaciduria (Fanconi syndrome), manifest as urinary stone disease in humans.[15] Although they are among the

Fly Gene	Malpighian Tubule Enrichment (fold)	Corresponding Human Disease	Human Gene Locus	Gene Function
CG7642	18	Xanthinuria type 1	2p23-p22	Xanthine oxidase
CG12602	2.3	Renal tubular acidosis, distal	7q33-q34	V-ATPase subunit
CG17369	3.7	Renal tubular acidosis	2cen-q13	V-ATPase subunit
CG1709	—	Renal tubular acidosis, distal	7q33-q34	V-ATPase subunit
CG4675	4.9	Renal tubular acidosis, proximal	4q21	Sodium borate co-transporter
CG4357	—	Gitelman syndrome	16q13	Sodium chloride co-transporter
CG5284	4.2	Bartter syndrome type 4, Dent disease	Xp11	Voltage sensitive chloride channel
CG31547	—	Bartter syndrome type 1	15q15-q21	Potassium chloride co-transporter
CG31116	11	Bartter syndrome types 2 and 4	3q27-q28	Voltage sensitive chloride channel
CG3926	9.7	Hyperoxaluria, primary type 1	27q36-37	Alanine-glyoxylate aminotransferase
CG6126	7.5	Hypouricemia	11q13	Urate transporter
CG9023	5.1	Diabetes insipidus, nephrogenic	9p13	Aquaporin water channel
CG17119	7	Cystinosis, nephropathic	17p13	Lysosomal cystine transporter

most rare causes of human nephrolithiasis, exploring the genetic basis of these diseases in Drosophila may reveal mechanisms underlying stone formation and its regulation that are common to other stone types.

Xanthinuria types I and II are human inherited autosomal recessive disorders caused by defective purine metabolism. Enzyme deficiencies result in total body accumulation and increased urinary excretion of xanthine. The disease commonly manifests as xanthine nephrolithiasis in children and adolescents.[16] Xanthinuria type I is caused by a deficiency in XDH, the enzyme responsible for converting hypoxanthine to xanthine and xanthine to uric acid. Cloning of the human genes for XDH (2p22/23) from 2 affected siblings led to identification of the mutations responsible for the classic form of human xanthinuria type I.[17]

Xanthinuria type II also results from XDH deficiency but involves deficiencies of additional enzymatic co-factors. The *rosy* and *maroon-like* mutant strains of D. melanogaster have genetic homologues of the altered human XDH genes and these flies similarly show whole body xanthine accumulation and the formation of xanthine concretions in the malpighian tubules.[18] The pathophysiology of xanthinuria type II was unknown until cloning of the *maroon-like* gene in Drosophila led to identification of the human homologue of this gene, the human molybdenum cofactor sulfurase gene.[19] In a manner similar to that in humans with xanthinuria, the accumulation of malpighian tubule concretions in the *rosy* and *maroon-like* models can be manipulated with dietary modification and alterations of transporter protein expression but, unlike in humans, the effects these changes in Drosophila can be readily observed and quantified in a matter of days (fig. 5).[20]

Environmentally Induced Nephrolithiasis

In 2007 a large-scale recall of pet food was initiated after several Chinese brands were found to be contaminated with the industrial chemical melamine. Outbreaks of renal failure and the subsequent death of thousands of dogs in Asia and North America led to the discovery of melamine induced nephrolithiasis and nephrotoxicosis as the cause.[21] Contamination of infant formula with melamine led to the death of 4 children and the hospitalization of more than 6,000 infants in China the following year.[22] As in the canine poisonings, most of the children presented with renal failure and melamine induced nephrolithiasis.

The human kidney excretes melamine intact and even low urinary concentrations of melamine can result in the formation of calcium oxalate, calcium phosphate or uric acid nephrolithiasis.[23] In the

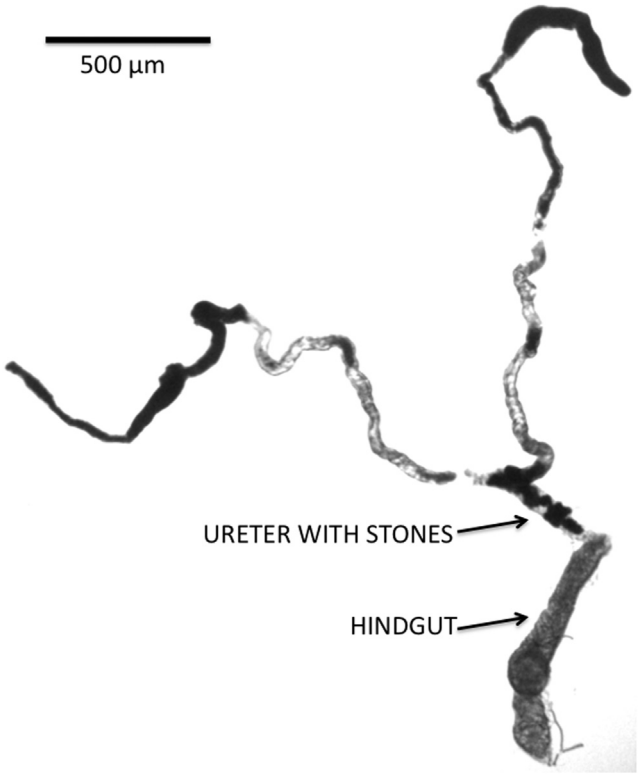

Figure 5. Xanthine stones in malpighian tubule lumina of *rosy* mutants deficient in XDH.

Drosophila model of melamine induced nephrolithiasis flies fed food stock containing increasing levels of melamine formed crystals in the malpighian tubules in a dose dependent manner in only 21 days.[24] Simple light microscopy was used to qualify the degree of crystal formation in the translucent malpighian tubules and assess the effects of dose variation and the administration of stone inhibiting substances. Scanning electron microscopy revealed that the crystals were variably composed of calcium, oxygen, phosphate and carbon. A dose dependent decrease in the life span from a mean of approximately 40 days to less than 10 days was also noted in flies fed increasing concentrations of melamine. Attempted mitigation of the effects of melamine with potassium citrate resulted in a reversal of the effects on the D. melanogaster life span but no significant effect on malpighian tubule crystal formation. As with other Drosophila models of environmentally induced nephrolithiasis, the melamine induced nephrolithiasis Drosophila model has limited applicability to the formation of idiopathic calcium oxalate nephrolithiasis in humans since the crystals are neither calcium oxalate nor uric acid.

Several other lithogenic substances have been used to develop environmentally induced models of human nephrolithiasis in rats, including ethylene glycol, hydroxyl-L-proline, vitamin D and various oxalate containing compounds.[25,26] In rat models of environmentally induced nephrolithiasis lithogenic substances must be administered daily via gastric tube after dissolution in salad oil, via intraperitoneal injection and/or via subcutaneous injection for up to 28 days. Direct observation of the effects of the various experimental conditions on crystal formation in the murine renal tubules requires polarized light microscopy after rat kidney excision, sectioning and complex staining.[25,26]

Similar models of environmentally induced nephrolithiasis in D. melanogaster are considerably less cumbersome. Dose dependent calcium oxalate crystal formation is readily visible as early as 14 days in the malpighian tubules of flies fed a diet containing increasing concentrations of ethylene glycol, hydroxyl-L-proline or sodium oxalate. A dose dependent decrease in life span from a mean of 40 days in controls to less than 10 days in flies fed 1% ethylene glycol can also be easily demonstrated in Drosophila. Adding potassium citrate to the lithogenic diet of these experimental flies resulted in an obvious decrease in the formation of calcium oxalate crystal in the malpighian tubules in all experimental models and resulted in an increased life span in the ethylene glycol model.[27]

Several of these Drosophila models of environmentally induced nephrolithiasis demonstrate calcium oxalate crystal formation in the malpighian tubules. As with genetically linked nephrolithiasis, these Drosophila models are not entirely representative of idiopathic calcium stone formation in humans but common mechanisms of crystal formation and inhibition may be defined. Moreover, the practical advantages of D. melanogaster allow rapid evaluation of scores of target genes and stone related metabolic pathways, and facilitate efficient large-scale screening of contributory or preventive compounds.

Calcium Oxalate Nephrolithiasis

The most common type of human kidney stones is largely composed of calcium oxalate and likely results from a combination of genetic and environmental factors, including diet. Calcium oxalate stones also form in humans due to well-defined monogenic mutations involving cell membrane transporters, ion receptors and transmembrane ion channels. These diseases include hypercalciuric metabolic conditions such as Dent disease and Bartter syndrome, and hyperoxaluric metabolic disease, including the various forms of primary hyperoxaluria.[28,29] Lastly, calcium oxalate stones can form secondarily in patients with other metabolic diseases, such as those linked to hyperuricosuria.[30] The recently developed fruit fly model of calcium oxalate nephrolithiasis, Drosophila Prestin, represents a major step toward describing the basic mechanisms underlying the formation of the most common type of human nephrolithiasis.

The Drosophila Prestin gene model of human calcium oxalate nephrolithiasis was developed by selective knockdown of Slc26a6, a luminal oxalate transporter in the malpighian tubules.[31] In humans Slc26 anion transporters are distributed in tissues throughout the body and perform various physiological functions.[32] The D. melanogaster genome contains 9 homologues of the 11 human Slc26 transporters. The Drosophila Prestin gene (CG5485) codes for proteins homologous to the human Slc26a5 and Slc26a6 transporters.[31] The functions of Slc26a5/a6 in humans include Cl^-/HCO_3^- exchange and oxalate secretion in the kidney as well as the regulation of oxalate secretion in the small intestine (Cl^-/oxalate^{2-} exchange).[32,33] As in humans, the Slc26a5/a6 transporter homologues in Drosophila are found in various tissues, including the malpighian tubules, and they also mediate Cl^-/oxalate^{2-} exchange.[31]

To accurately model oxalate excretion in the human renal tubules selective knockdown of Slc26a5/a6 in the D. melanogaster malpighian tubules was performed using the GAL4/UAS system.[34] The GAL4/UAS system accomplishes tissue specific gene knockdown by inserting the transcription

activating driver (GAL4 driver) into one line of flies and then crossing these flies with another line that contains the corresponding promoter region (the UAS responder) and the downstream desired gene sequence. This selective knockdown results in loss of function in the desired tissue (the malpighian tubule), while maintaining normal gene function throughout the rest of the organism.

The fidelity of the Drosophila Prestin model of human calcium oxalate nephrolithiasis was demonstrated through the directly observable effects of dietary manipulation on fly stone formation and the resultant mitigation of these effects brought about by the selective knockdown of Slc26a5/a6. For example, calcium oxalate crystals are not normally visible in the malpighian tubules of wild-type D. melanogaster fed a standard diet but adding sodium oxalate to the diet of wild-type Drosophila larvae results in the formation of calcium oxalate crystals in the malpighian tubules within 2 days. Adult flies fed an oxalate rich diet produce calcium oxalate crystals in 6 to 12 hours (fig. 6).[35] This calcium oxalate crystal formation can be quantified by birefringence microscopy of the dissected tubules or by micro-CT of whole flies (fig. 7). Dynamic in vitro formation of calcium oxalate crystals was also observed in real time in dissected wild-type adult malpighian tubules submerged in a sodium oxalate bath.[33] On the other hand, selective Slc26a5/a6 knockdown in the malpighian tubules of the Drosophila Prestin model resulted in a statistically significant decrease in the intraluminal formation of calcium oxalate crystals.[35]

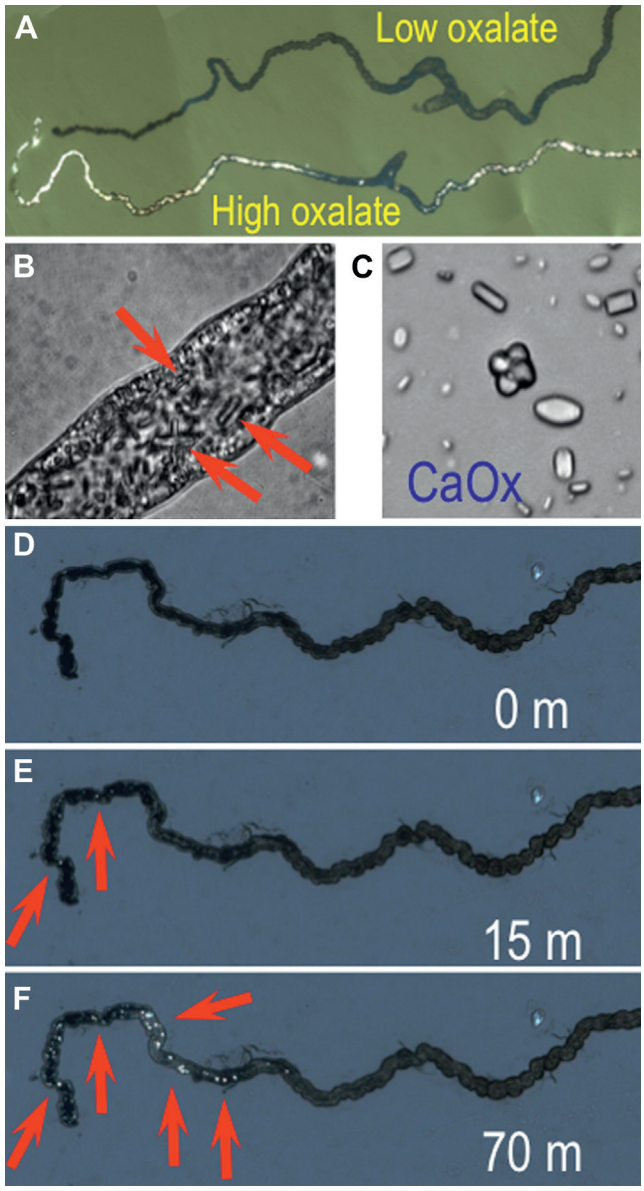

Figure 6. *A,* calcium oxalate crystal accumulation in D. melanogaster fed diet supplemented with high sodium oxalate vs control fed low sodium oxalate. Reduced from ×10. *B,* intraluminal calcium oxalate crystals. Reduced from ×40. *C,* crystals at higher magnification. Reduced from ×40. *D* to *F,* crystal formation (arrows) in dissected malpighian tubules in sodium oxalate bath. Reduced from ×10. Reprinted with permission.[35]

LIMITATIONS OF DROSOPHILA MODELING OF HUMAN NEPHROLITHIASIS

Foremost among the perceived limitations of Drosophila as a model for the study of human nephrolithiasis is the difference in the anatomical arrangements of the renal and excretory systems. The renal system of D. melanogaster is aglomerular and filtration products are not directly delivered to the tubules from an intimately associated glomerulus. In Drosophila 2 anatomically separate, functionally distinct cell populations are responsible for hemolymph filtration and ion transport. As described, ion transport is accomplished in Drosophila by the principal and stellate cells of the malpighian tubules. The nephrocytes perform many functions of the vertebrate glomerulus, including hemolymph filtration and detoxification. Nephrocytes have a complex, folded plasma membrane that forms multiple channels bordered by narrow slits, which serve as filtration junctions. In a manner similar to the podocytes of the glomerulus these small slits allow the nephrocytes to filter materials from the hemolymph based on particle size and charge, and then sequester the filtered materials via endocytosis. These mechanisms can be readily visualized in a nephrocyte cell culture using variable sizes of fluorescently labeled dextrans and by feeding Drosophila larvae with toxins such as silver nitrate.[1]

As with the malpighian tubules, numerous genetic homologues of the human glomerulus have been identified in the Drosophila nephrocyte and

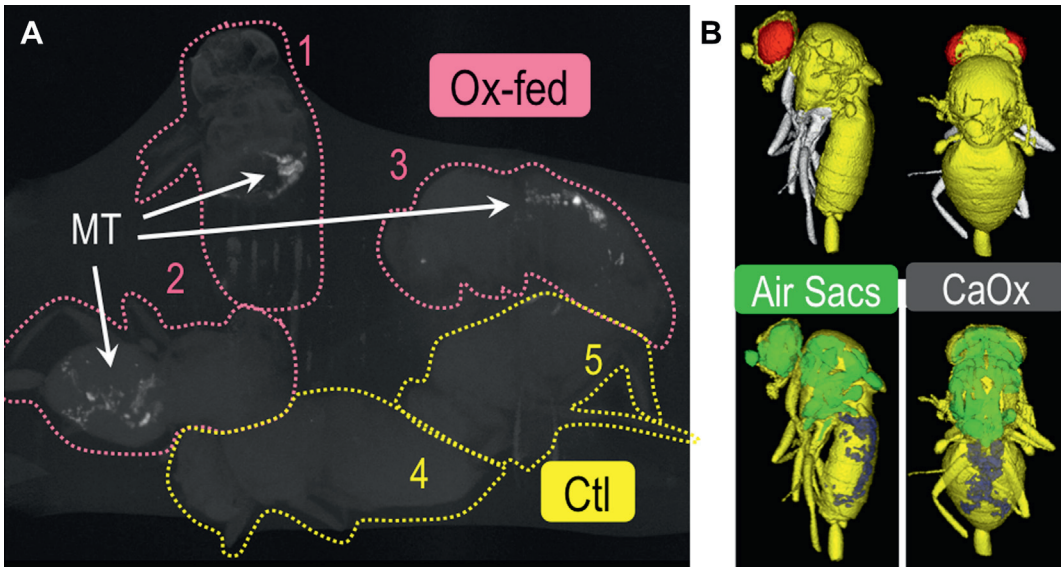

Figure 7. *A,* micro-CT reveals calcium oxalate crystal accumulation in 3 D. melanogaster adults fed diet supplemented with sodium oxalate vs 2 controls. *B,* surface renderings constructed from micro-CT. *Ox-Fed,* oxalate fed flies. *Ctl,* control flies. *MT,* malpighian tubules.

diseases such as human congenital nephrotic syndrome can be recapitulated in fly models.[1] Thus, rather than imposing limitations, the anatomical separation of filtration and ion transport functions in D. melanogaster presents opportunities for detailed, single tissue modeling of diseases affecting analogous vertebrate renal structures. This single tissue modeling of stone formation in Drosophila may provide the basis for subsequent experiments in more complex, expensive mammalian models, including humans.

The commingling of waste products from the malpighian tubules and the gut in the common cloaca of D. melanogaster was cited as a drawback of using invertebrates for modeling human nephrolithiasis.[36] This perceived limitation is readily overcome by combining the simplicity of D. melanogaster morphology with several well developed experimental techniques. The 2 pairs of malpighian tubules are easily dissected free from their attachments to the gut by severing the common ureter. These isolated tubules can then be anchored with a thin steel pin and submerged in medium containing experimental compounds or hemolymph harvested from relevant model adult flies or larvae. The dissected tubules continue to secrete fluid for more than 5 hours and the resultant secretory products can be collected for subsequent analysis in a manner analogous to human 24-hour urine collection.[3]

Among other challenges inherent to the D. melanogaster models of human renal function and nephrolithiasis formation are the differences in calcium homeostasis mechanisms. For example, as an invertebrate, D. melanogaster lacks the skeletal calcium stores that are an integral part of maintaining calcium balance in humans. Although calcium metabolism in D. melanogaster is well characterized and some mechanisms have been studied, the full potential of the model has not been realized. For example, 25% to 30% of the calcium content of the whole adult Drosophila is stored as calcium containing concretions in the distal segment of the anterior pair of malpighian tubules. Variations in dietary calcium are mitigated in D. melanogaster by combined tubular secretion of free calcium in urine and the sequestration of insoluble calcium in luminal concretions.[9] As in human nephrolithiasis, the pH dependent formation of these luminal concretions can be inhibited by common medications such as acetazolamide and hydrochlorothiazide.[37]

Current Drosophila models of human nephrolithiasis are limited to genetically linked, environmentally induced stone formation and a new model of calcium oxalate stone formation. To our knowledge the areas of uric acid and magnesium ammonium phosphate stone formation have not been studied. This may be because the focus has been on idiopathic calcium stones, particularly calcium oxalate stones, or the etiology of these 2 stone types (uric acid and infection stones) has been better studied in humans. Bacterial infection models in Drosophila may not replicate changes in the urinary milieu caused by the urea-splitting organisms that culminate in human infection stones. Intestinal bacterial infections in Drosophila models do not

cause immediate death but rather an indolent process similar to that in humans. Thus, this may be another area of stone research that could be exploited using the fly model in the future. Uric acid stones would ostensibly be simpler to model than infection stones and they may represent an avenue of additional research along with infection stones.

CONCLUSIONS

The technologies and techniques of the surgical treatment of nephrolithiasis have evolved greatly in the last 20 years but little progress has been made in developing effective medication for prevention or treatment. Conventional medical therapy for nephrolithiasis only alters the gross urinary constituents to decrease the risk of stone formation but stone formation is actually the end point of a complex, still poorly understood pathophysiological process. Since contemporary medications do not directly target these fundamental mechanisms, many drugs have undesirable side effects or limited short-term and long-term efficacy. New cost-effective, efficacious, readily deployable therapeutic strategies are needed. The disease models and genomic tools of D. melanogaster present the urological community with a unique opportunity to develop novel therapies for nephrolithiasis through increased understanding of the pathophysiology of this disease in its various manifestations.

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