Methods for Screening Antiurolithiatic Potential

Gifty Ann Alex\textsuperscript{1*}, Sumi James\textsuperscript{2}, Sherly Eapen\textsuperscript{3} and Dr. Santhosh M. Mathews\textsuperscript{4}

\textsuperscript{1} M.Pharm Student, Department of Pharmacology, Pushpagiri College of Pharmacy, Thiruvalla, Kerala, India.
\textsuperscript{2} Associate Professor, Department of Pharmacology, Pushpagiri College of Pharmacy, Thiruvalla, Kerala, India.
\textsuperscript{3} Assistant Professor, Department of Pharmacology, Pushpagiri College of Pharmacy, Thiruvalla, Kerala, India.
\textsuperscript{4} Principal, Pushpagiri College of Pharmacy, Thiruvalla, Kerala, India.

ABSTRACT

Urolithiasis is a widespread health issue with a high recurrence rate. The complex process of kidney stone formation, also known as urolithiasis, is brought on by a series of physicochemical processes, including supersaturation, nucleation, growth, aggregation, and retention of crystals inside the kidney. Numerous \textit{in vitro} and \textit{in vivo} models of urolithiasis have been created by scientists in an effort to mimic human urolithiasis. \textit{In vivo} models depict the pathological implications of urolithiasis, while \textit{in vitro} models allow the study of renal stone production. The current review article gives a general overview of the various \textit{in vitro} and \textit{in vivo} models for the assessment of urolithiasis.

KEYWORDS: Urolithiasis, Calcium oxalate, Ethylene glycol.

INTRODUCTION

No regional, cultural, or racial group is immune to the development of urinary tract stones, with urolithiasis accounting for more than 80\% of all stones. The prevalence of urinary stone disease is thought to be around 12\% of the population, with a recurrence rate of 70–80\% in men and 47–60\% in women. It is generally accepted that the events leading to crystal nucleation, aggregation, and development of insoluble particles play a role in the mechanism of calcific stone formation. Although the majority of people cannot develop urolithiasis due
to the crystallisation inhibitory power of urine, which is on the low side for stone formation, urine is oversaturated with the common minerals that cause stones to form.\[1\]

Although the renal pelvis is where most kidney stones are detected, the stone-forming process actually begins in the nephrons. The renal biopsies of a patient with type I primary hyperoxaluria revealed intracellular calcium oxalate (CaOx) intranephronic crystals. These crystals frequently precipitate in the tubular fluid of both stone-forming and non-stone-forming individuals, and they are safe as long as they are eliminated through the urine. Due to its slow growth rate, a single microcrystal is typically too tiny to occlude the tubular lumen. However, its excessive urine levels can cause stones to develop. The etiopathogenesis is complicated and includes genetic, dietary, socioeconomic, environmental, metabolic, and anatomical and infectious variables.\[2\]

For evaluating distinct mechanism profiles of testing extracts or compounds against various pathophysiological conditions as well as for urolithiasis, numerous in vitro and in vivo (animal models) studies are used. These models most accurately depict the test sample's preventative and curative effects. Before doing in vivo experiments, in vitro studies offer a preliminary analysis. Additionally, these investigations offer simplified setups with a tiny amount of the test material, enabling minimal costs and a large number of duplicates. Therefore, providing encouragement to reduce drug costs through cost-effective testing methods and giving a more direct assessment towards extract or compound performance than standard in vivo research and thereby delivering significant insights into basic biological effects mechanisms. It also avoids or decreases the need for laboratory personnel experienced in animal handling and there is no need to submit permission by the institutional animal ethics committee.\[3\]

For research on the earliest stages of stone formation, many surgically treated kidneys are removed because of infection or persistent blockage. The creation of a trustworthy animal model for studying the pathophysiology of urinary stone disease is urgently needed as a community of physicians and fundamental scientists work to unravel the secrets of biomineralization.\[4\] Looking back over the past 50 years, there haven't been many significant advancements in stone prevention. Innovations in pharmaceutical prophylaxis have been constrained by the absence of a valid animal model. To try and identify risk factors for future stone formation, the majority of urologists do a metabolic evaluation that includes laboratorial testing such as targeted 24-hour urine collections and blood chemistries.
Although the metabolic assessment hasn't changed in nearly 50 years, the incidence and recurrence of stones have both increased. It is necessary to construct an animal model to more fully comprehend the mechanisms by which stones form in order to reduce future stone formation and give a roadmap for the development of new prophylactic regimens.\cite{5}

Although no single animal model system can yet be considered to be perfect, examining the animals that have already been used can provide important new information. The anatomical parallels and differences between each animal and humans must be emphasised. The techniques used by researchers to induce stone formation are based on the benefits and drawbacks of each model. The numerous in vitro and in vivo techniques for examining antiurolithiatic activity are the main topic of this review paper. Even though in vitro models are superior to animal models in many ways, both methods are required to complete a study on urolithiasis.\cite{6}

**In vitro models for screening antiurolithiatic activity**

1. **Determination of effect on CaC\(_2\)O\(_4\) crystallization**

The CaC\(_2\)O\(_4\) Crystallization was determined by the time course measurement of turbidity change due to the crystal formation and aggregation in the metastable solutions of Ca\(^{2+}\) and oxalate.

The procedure followed is: Stock solutions of CaCl\(_2\) (8.5 Mm) and Na\(_2\)C\(_2\)O\(_4\) (1.5 Mm), containing 200 mM NaCl and 10 mM sodium acetate were adjusted to pH 5.7. The CaCl\(_2\) solution (0.5 ml) was stirred constantly both in the absence and presence of different concentrations of the test material or reference drug at 37\(^0\)C. After obtaining a stable baseline, crystallization was induced by the addition of Na\(_2\)C\(_2\)O\(_4\) solution (0.5 ml) to obtain the final concentration of Ca\(^{2+}\) as 4.25 Mm and oxalate as 0.75 Mm. The time course measurement of turbidity was simultaneously started on a chart, moving at the speed of 30 mm/h and continued for 15 min with constant stirring of the solutions. Optical density was measured at 620nm. All experiments were run in triplicate. Slope of nucleation (SN) and aggregation (SA) phases were calculated using linear regression analysis.

Using the slopes, the percentage inhibition was calculated as \[ \frac{(1 - Sm)}{Sc} \times 100 \]

Where Sm is slope in the presence of modifier
Sc is slope of the control experiment.

To determine the effect of incubation with the test material on CaC\(_2\)O\(_4\) crystal formation, stock solutions of CaCl\(_2\) and Na\(_2\)C\(_2\)O\(_4\) having composition similar to those in the kinetic
study were used. CaCl₂ solutions, containing different concentrations of the test material or reference drug, were aliquoted (0.5 ml) to the flat bottomed tubes in a 24 well plate. To each of these tubes Na₂C₂O₄ solution (0.5 ml) was added to obtain the final concentration of Ca²⁺ as 4.25 and oxalate as 0.75 mM. Each concentration of the test material was prepared in triplicate. The plates were then incubated in a shaking water bath at 90 oscillations/min at a temperature of 37°C for 45 min. Each tube then observed under an inverted microscope for crystal morphology and count in five randomly selected fields (200x).[7]

1.1. Nucleation and Aggregation assay of CaOx crystals
The spectrophotometric assay method is used to determine the inhibitory activity of the test compound in the nucleation and aggregation of CaOx crystals. Calcium chloride dihydrate aqueous solution (10 mM) and sodium oxalate solution (1.0 mM), are diluted by a buffer of sodium chloride (200 mM) and sodium acetate trihydrate (10 mM) at pH 5.7 maintaining temperature at 37°C with circulating water bath. For crystallization experiments, Cystone (reference drug) or test solutions (1 mL) at 0.5 or 1.0 mg/mL in water are added to the stirred sodium oxalate solution (25 mL) at 37°C followed by the addition of calcium chloride solution (25 mL). The absorbance is recorded at 620 nm by spectrophotometer using the control with no test solution. All the experiments are performed in triplicate. The percentage inhibition is calculated as follows: Percentage inhibition (%): \[1 − (Tsi /Tsc)\] × 100, Where Tsc = turbidity slope of the control Tsi = turbidity slope in the presence of the inhibitor

1.2. Growth assay
The inhibitory activity of the test material on the CaOx crystals growth is determined by a spectrophotometric assay. Calcium chloride solution (4 mM, 20 ml) and 4 mM sodium oxalate (4 mM, 20 ml) are added to a buffer solution (30 ml) of sodium chloride (90 mM) and Tris HCl (10 mM) at pH 7.2 followed by the addition of calcium oxalate monohydrate (COM) crystal slurry (600 μl) prepared in acetate buffer (1.5 mg/ml). Consumption of oxalate commences immediately after the addition of the COM slurry, which is monitored at 214 nm for 600 s absorbance disappearance. Further, cystone or test solution (1 mL) at 0.5 or 1.0 mg/mL in water is added separately into the above solution and depletion of free oxalate ions is calculated by spectrophotometer as inhibition in calcium oxalate crystals growth using the following equation:

Inhibitory of crystal growth (%) = \([(C – S) /C]\) × 100
Where C = reduction of free oxalate without any inhibitor  
S = reduction of free oxalate with inhibitor  
All the experiments are performed in triplicate \[8\].

2. Simultaneous static flow model

The inhibition of CaOx and CaP mineralization is evaluated by Simultaneous Static Flow Model (S.S.M). Different concentrations of test, Calcium acetate (0.1 M) and Na\(_2\)C\(_2\)O\(_4\) -0.1 M (for calcium oxalate) or sodium phosphate (for calcium phosphate) are filled (25 ml) in three separate burettes and are allowed to fall simultaneously into an empty clean beaker with a slow and steady pace.

For 10 min, the mixture is kept in hot water bath and then cool to room temperature. Supernatant is discarded and the precipitate formed is collected into a preweighed Petri plate. Dry the precipitate collected in plates in a hot air oven at 120°C, cool and weigh \[9\]. Weight of the dry precipitate is recorded. Then percentage inhibition is calculated by following formula:

\[
\text{% Inhibition of CaOx/CaP} = \left(\frac{\text{Weight of ppt. in blank set} - \text{Weight of ppt. in experimental set}}{\text{Weight of ppt. in blank set}}\right) \times 100
\]

3. Calcium phosphate (CaP) assay:

In vitro calcium phosphate (CaP) assay is studied on in vitro homogeneous system of initial mineral phase formation for CaP, its subsequent growth and demineralization by employing 5.0 ml system which is prepared by adding 0.5 ml of KH\(_2\)PO\(_4\) (50 mM), 0.5 ml of CaCl\(_2\) (50 mM), 2.5 ml of Tris buffer (210 mM NaCl + 0.1 mM tris HCl) and increasing volume of the test ranging from 0.2 ml to 1.5 ml. This system is centrifuged at 4500 rpm and precipitate so obtained is dissolved in 5 ml of 0.1 N HCl. This 5 ml system for mineralization, is used to study the extent of in vitro mineral phase formation in the absence of any matrix.

For the growth, firstly 5 ml system is prepared using standard protocols then again 5 ml systems are re-grown on the same tubes with the additions of increasing volumes of the test solution. Calcium and phosphate are then estimated on the precipitates obtained and dissolved in 0.1 N HCl. In case of control no extract was added. To check the demineralization, again 5 ml system is prepared having no extract added to that and precipitate is obtained. To these precipitates, 2.5 ml of Tris buffer (210 mM NaCl + 0.1 mM Tris HCl) and increased volumes of test solutions ranging from 0.2 ml to 1.5 ml is added and then centrifuged at 4500 rpm for 15 min. Calcium and phosphate are then estimated in.
supernatant obtained after centrifugation. The Ca$^{2+}$ and HPO$_4^{2-}$ ions are estimated by the methods of Trinder and Gomori, respectively. Percent inhibition of mineral phase in the presence of test is calculated as:

\[
\text{% inhibition} = \left( \frac{(C - T)}{C} \right) \times 100
\]

where

- T is the concentration of Ca$^{2+}$ or HPO$_4^{2-}$ ion of the precipitate formed in test
- C is the concentration of Ca$^{2+}$ or HPO$_4^{2-}$ ion of the precipitate formed in control system which had distilled water and no extract.$^{[10]}$

4. Oxalate depletion assay

The effects of the test on the crystal growth are determined by oxalate depletion assay. Different dilutions of test and standard are prepared in distilled water. CaOx slurry (3g/2ml) is prepared in 0.05 M sodium acetate buffer at pH 5.7. 1 ml of calcium chloride and 1 ml of sodium oxalate (0.004 M each) solutions are mixed with 1.5 ml of Tris-HCl (0.01 M) and NaCl (0.09 M) at pH 7.4. CaOx slurry (30 μl) is added to the reaction mixture and the growth of crystals is observed by the addition of 1 ml of each dilution of the test and standard to this reaction mixture. The rate of CaOx depletion is determined by measuring the optical density (absorbance) at 214 nm for 10 min. The difference in optical density is calculated and the percentage inhibition of the crystal growth is determined by the formula as described for the nucleation assay.$^{[11]}$

5. Titrimetry

The experimental kidney stones of calcium oxalate (CaOx) are prepared in the laboratory by taking equimolar solution of calcium chloride dihydrate in distilled water and sodium oxalate in 10 ml of 2N H$_2$SO$_4$. Both are allowed to react in sufficient quantity of distilled water in a beaker, the resulting precipitate is calcium oxalate. The precipitate is freed from traces of sulphuric acid by ammonia solution, washed with distilled water and dry at 60$^\circ$C.

The dissolution percentage of calcium oxalate is evaluated by taking exactly 1 mg of calcium oxalate and 10 mg of the extract, pack it together in semi permeable membrane of egg. This is allowed to suspend in a conical flask containing 100 ml of 0.1M Tris buffer. First group served as blank containing only 1 mg of calcium oxalate. The second group served as positive control containing 1 mg of calcium oxalate and along with the 10mg standard drug. The 3rd group along with 1 mg of calcium oxalate contain test. The conical flasks of all groups are kept in an incubator preheat to 37$^\circ$C for 2h. Remove the contents of semi permeable membranes from each group into separate test tubes, add 2 ml of 1N sulphuric acid to each
test tube and titrate with 0.9494 N KMnO₄ till a light pink colour end point obtained. The amount of remaining undissolved calcium oxalate is substracted from the total quantity used in the experiment in the beginning to know the total quantity of dissolved calcium oxalate by test.[¹²]

6. Cell culture
Normal rat epithelial derived renal tubular epithelial (NRK 52E) cells are used. The cells are maintained as monolayers in Dulbecco’s Modified Eagle’s Medium (DMEM) with 2.0 mM L-glutamine adjusted to contain 3.7 g/L sodium bicarbonate, 4.5 g/L glucose. Media is supplemented with 1% Penicillin (100 units/mL)-Streptomycin (10,000 µg/mL) and 10% fetal bovine serum. Cells are cultured in 25 cm² tissue-culture treated flasks at 37°C and 5% CO₂ in humidified chambers.

6.1. Oxalate-induced cell injury
NRK 52E cells are incubated in DMEM containing 1 mM sodium oxalate in the presence of different concentrations of the test sample (10 µg/mL, 25 µg/mL and 50 µg/mL) for 72 hours. Cell injury is assessed by measuring the cell viability through trypan blue and monitoring the lactate dehydrogenase (LDH) leakage into the medium.

6.2. Cytotoxicity assessment - Trypan Blue Assay
The cytotoxicity of the test is assessed by cell viability using trypan blue exclusion method. For the determination of cell viability, cells are plated at the density of 4 × 10⁴ cells/well and cultured for 72 h. The medium is replaced with serum-free medium and the cells are treated with various concentrations of the test for a further 72 h. The percentage viability for the cells is calculated as (live cells/total cells) x 100.

6.3. LDH Leakage Assay
6.6 mM NADH and 30 mM sodium pyruvate are prepared in Tris (0.2M, pH 7.3). Reaction is initiated with the addition of 50 µL of the test sample and the disappearance of NADH is monitored at 340 nm, for 5 min at an interval of 1 min. The percentage of LDH release is calculated by dividing the activity of LDH in the supernatant by the LDH activity measured after complete cell lysis achieved by sonication.[¹³]
Animal models to study urolithiasis

1. Rat model
Since the 19th century, scientists have used rats as a reliable, reasonably affordable model. Studies using the rat for urinary stone disease have mostly concentrated on duplicating both hypercalciuria and hyperoxaluria, two of the most frequent pathophysiologic alterations connected with urinary stone disease. The largest of these differences is that rat kidneys are unipapillary in contrast to human kidneys, which are multi-papillary, according to anatomical and physiological comparisons between rats and humans. According to Khan, rat kidneys typically weigh 0.75–1.2 g, measure 1.6×1.0×0.9 cm, have fewer urine tubules, and have 30,000 nephrons on average, compared to human kidneys, which weigh around 170 g, measure 11×6×2 cm, and have over 1,000,000 nephrons. Despite these glaring discrepancies rat’s cortex to medulla ratio (2:1) is comparable to humans (2:1).[14]

1.1. Hypercalciuria
One of the most prevalent risk factors for the development of urinary stone disease has been identified as hypercalciuria. In order to produce hypercalcemic offspring, scientists have developed a strain of multi-generational inbred Sprague-Dawley rats. Multiple studies have shown that in these genetically hypercalciuric stone-forming (GHS) rats, increased calcium absorption is caused by an increase in the number of Vitamin D receptors in the GI tract, kidneys, and bone. This increased calcium absorption was initially believed to be secondary to increased calcium absorption in the gut. It has been demonstrated that these GHS rats excrete noticeably more calcium than controls in each subsequent generation. Male GHS rats have been shown to excrete more oxalate on a daily basis than their female counterparts, despite the fact that the majority of these research used female rats.[15]

1.2. Hyperoxaluria
Exogenous injection of lithogenic substances such sodium oxalate, glycolic acid, ethylene glycol (EG), and hydroxy-L-proline has been used in a number of models for hyperoxaluria in rats (HLP). These medications can be administered to rats in a variety of ways, including subcutaneous implantation of oxalate-containing osmotic mini-pumps, enriched chow, gavage instillation, intraperitoneal injection, and drinking water modification. This section reviews how each lithogenic agent and procedures has been studied in the rat and shown to culminate in stone induction.[16]
Fig. 1: Crystal deposition in the renal tubule of a rat following intraperitoneal injection of sodium oxalate. (A) Three hours after the injection, CaOx crystals were seen in all segments of the renal tubules. Here, plate-like CaOx monohydrate crystals are seen in the loop of Henle. (B) Brush border of the proximal tubule showing sign of injury. There is focal ballooning of the microvilli. (C) Just 15 minutes after the injection, a CaOx dehydrate crystal is lodged in the proximal tubule. (D) Cross-fractured proximal tubule shows a rosette of plate-like CaOx monohydrate crystals.\textsuperscript{[17]}

1.2.1. Sodium oxalate
Depending on the amount of time that has passed since the intraperitoneal injection of sodium oxalate, different regions of the nephron exhibit predictable calcium oxalate crystal development. Male Sprague-Dawley rats were given sodium oxalate in a range of doses (3, 5, 7, 9, and 10 mg/kg), and these levels resulted in persistent hyperoxaluria and crystals. Rats receiving 10 mg/kg had almost 500% more oxalate excreted than controls, with crystals that persisted for up to 7 days following injection. Within 15 minutes of injection, CaOx crystals could be seen, and by 6 hours, there was clear crystal aggregation in the ducts of Bellini.\textsuperscript{[18]}

1.2.2. Glycolic acid
The male Wistar-strain rats create large levels of 24-hour urine oxalate and subsequently calcium oxalate calculi when powdered 3% glycolic acid is dissolved in drinking water. Intriguingly, this study also shown that despite relatively high levels of urine oxalate excretion, adding magnesium salts to a high glycolic acid diet raised urinary citrate levels.\textsuperscript{[19]}

1.2.3. Ethylene glycol
It has been demonstrated that administering ethylene glycol (EG) by drinking water consistently induces hyperoxaluria, crystalluria, and calcium oxalate nephrolithiasis. Male rats receiving only 0.75% EG over the course of 12 days had persistent crystalluria, and three
weeks later, kidney crystal deposits. EG is frequently coupled with other substances to increase the formation of crystals, such as ammonium chloride (AC) to lower urine pH, calcium chloride or vitamin D to induce hypercalcemia and hypercalciuria. The time for crystalluria decreased due to this lithogenic combination from 12 to 3 days, while the period for detectable calcium oxalate nephrolithiasis dropped from 3 weeks to 1 week. However, numerous investigations have demonstrated that EG is a hazardous substance that can lead to multi-organ failure. Rats exposed to EG and AC suffer from reduced body weights, poor renal function, and elevated levels of urine N-acetyl-b-D-glucosaminidase (NAG), a sign of renal toxicity. Lipid peroxidation, elevated free radical levels, and metabolic acidosis have all been discovered in other studies.[20]

1.2.4. Hydroxy-L-proline Hydroxy-L-proline (HLP)

Hydroxy-L-proline is a component of collagen and is generated from the amino acid proline and is metabolised, principally in the renal proximal tubule and hepatocyte mitochondria, to both pyruvate and glyoxalate.[19] It has been demonstrated to be less harmful than other lithogenic substances and is a popular element in Western diets. Calcium oxalate crystals were seen in the rat kidney after intraperitoneal administration of HLP. Scanning electron microscopy revealed that a high dose of 4-HLP (2.5 g/kg) generated both calcium oxalate dihydrate (Weddellite) and calcium oxalate monohydrate (Whewellite) crystals. Male Sprague-Dawley rats were given 5% HLP (weight/weight HLP/chow) by Khan et al., and treated rats were contrasted with controls at 4, 6, and 9 weeks. The majority of the CaOx crystals were identified in the tubular lumens of the distal tubules and collecting ducts in all treated rats at 4 weeks after treatment. By nine weeks, the renal papillae's tips were where most of these crystals were seen. Trans-4- HLP was observed to change the type and excretion of urine calcium in GHS rats at concentrations of 1%, 3%, and 5%, respectively. Rats receiving 5% HLP had decreased calcium excretion in their urine and calculi with a constant calcium oxalate composition.[21]

1.2.5. Dietary manipulation

In an effort to develop a different crystal induction technique from HLP, Wiessner et al. discovered that calcium oxalate crystals could only be produced in male Dahl salt-sensitive and Brown Norway rats at a concentration of 5% potassium oxalate. Meanwhile, calcium phosphate (apatite) stone formation was increased in hyperoxaluric mice who were denied access to dietary Mg. In order to increase hyperoxaluria, hypocitraturia, and consequent
calcium oxalate crystal formation in rats, intentional vitamin B6 shortage can also be used. Researchers found that giving these pyridoxine-deficient rats more Mg might effectively prevent calcium oxalate crystals and counteract hypocitraturia.[22]

1.2.6. Intestinal resection
Since Smith et al studies small bowel resection has been linked to a higher risk of nephrolithiasis. Connor et al. performed a distal 40–45 cm resection of the terminal ileum in rats and contrasted the results with sham controls, which received a distal ileum transection, re-anastomosis, and no intestine removal. Following surgery, these rats were either given a normal diet or one that had a high oxalate (1% sodium oxalate)/low calcium (0.02%)/high lipids (18%) diet before being euthanized at 4, 5, 6, and 7 months. On the high oxalate/low calcium diet, resected rats had hyperoxaluria, hypocitraturia, and crystals throughout the cortex, medulla, and renal papilla, according to 24-hour urine samples. These crystals were made up of a combination of calcium carbonate, calcium phosphate, and calcium oxalate. Interestingly, none of the sham animals—not even those on a high-oxalate diet subsequently formed crystals, emphasising the significance of bowel resection in crystal formation.[23]

1.2.7. Gastric bypass surgery
According to epidemiologic research, patients who have had Roux-en-Y gastric bypass (RYGB) have a higher risk of developing stones, with a 2-fold increase in those who have never had urinary stones and a 4-fold increase in those who have previously formed stones. Numerous investigations have demonstrated that these post-RYGB patients have substantial hypocitraturia and hyperoxaluria. The pathophysiologic cause of this elevated urinary oxalate level is most likely due to a saponification process in which more fatty acids and bile salts bind to calcium, releasing extra unbound oxalate that can then be more readily absorbed by the enteric system. Canales et al. used a diet-induced obesity (DIO) paradigm in male Sprague-Dawley rats, followed by a randomised intervention to either sham surgery (controls) or RYGB, to study the mechanism by which post-RYGB hyperoxaluria occurs. This study compared the faecal lipid content, 24-hour urine volume, pH, oxalate, and calcium levels in stool and urine.[24]

In comparison to controls, post-RYGB rats on a high (40%) fat diet with added potassium oxalate absorbed considerably less dietary fat and expelled an 8-fold greater amount of faecal fat. Additionally, post-RYGB rats receiving additional oxalate saw a 5-fold increase in urinary oxalate excretion, compared to animals receiving no extra oxalate, who experienced a
2-fold increase in urine calcium excretion. In addition, regardless of the amount of dietary oxalate or fat, all RYGB rats experienced a 250% increase in water consumption and a 2-fold increase in urine volume output. It indicates a notable difference in comparison to findings from other human studies that have showed large decreases in urine volume post-RYGB, even if it is hypothesised to be because of changed thirst mechanisms. Future research is required to clarify the relationship between RYGB and urine volume production.²⁵

1.2.8. Zinc disc induced model
Anesthesia was administered to rats using sodium pentobarbitone (40 mg/kg, ip). The urine bladder was made visible through a suprabic incision. At the bladder's top, a tiny cut was made. After that, the urine was aspirated aseptically into a sterile vial for bacterial analysis and pH measurement (using narrow range pH paper BDH). The rats were given a week to recover after having previously weighted sterile zinc discs placed into their bladders. The wound was then closed with a single stitch made of absorbable 4-0 chronic catgut (Ethicon). The formation of urinary stones and enlargement of the organ's smooth musculature were caused by the implantation of zinc foreign bodies into the urinary bladder and were greater in males than in females after 4 and 8 weeks of surgery, respectively.

1.2.9. Xenoplantation model
One male patient with kidney stones underwent PCNL (percutaneous lithotomy) to remove the stone fragments. Before use, the chosen stone is divided into portions with a diameter of 2-3 mm, weighted, and kept in a sanitary environment. Male eight-week-old rats weighing between 250 and 300 g were chosen and randomly divided into the control, standard, and test groups. A suprapubic incision was made to expose the bladder after administering sodium pentobarbital (50 mg/kg body weight) intraperitoneally to the rats. Each rat was then given one prepared stone particle through a 4-5 mm incision made at the top of the bladder, which was then closed together with the suprapubic incision. Since the first postoperative day (day 2), ethanol was added to drinking water at a final concentration of 1% for a total of four weeks.

A graded ethanol series was used to dry the kidneys, and they were then imbedded in paraffin, after the kidney and urine bladder were separated after four weeks. Von Kossa histochemical staining was used to evaluate the development of renal stones. The best portion pane was chosen by transversally sectioning the bladder stone with a diamond wire saw after it had been extracted, weighed, and kept in 75% ethanol for 24 hours. When the core could be
seen clearly under a transmitted light microscope, sectioned blocks were next placed on a glass slide using thermoplastic glue and polished repeatedly using 1,200 grit sandpaper and a combination of alumina polishing compounds (3, 1 and 0.3 m) with a tiny amount of water.\[26\]

2. Mouse model
Since both genomes are roughly 3.1 billion base pairs in size and, on average, 85% identical, with some genomes being more than 95% identical, mice and human genomes are similar. Mice typically weigh 25–35 g, making them 1/2500 the size of an adult person. The average mouse kidney weighs 0.8% of the animal's body weight, which is considerably denser than the average human kidney, which weighs 0.2% of an individual's body weight. The maximum urine concentration is also higher in mice (4000 mOsm/kg H2O vs. 1200 mOsm/kg H2O, respectively) than in humans. Similar to rats, the fundamental distinction between mouse and human kidneys is that the latter have unipapillary kidneys. A glomerular unit, tubule structures, and other microscopic features demonstrate a comparable cortical and medullary composition.\[27\]

2.1. Hyperoxaluria
Researchers have tried delivering lithogenic substances including EG, HLP, and glyoxylate to mice in an effort to induce hyperoxaluria in a manner similar to that seen in rats. Exogenous delivery of these substances alone has shown just a brief duration of relatively few CaOx crystals in mice, despite successfully producing hyperoxaluria. Therefore, studies have paired transgenic mice with lithogenic substances to aid in the induction of stone formation. Researchers have demonstrated the important function of these macromolecules as inhibitors of stone formation by selectively knocking out (KO) osteopontin (OPN) and Tamm-Horsfall protein (THP). By administering 1% ethylene glycol (EG), Wesson et al. demonstrated that an in vivo model of OPN KO mice could create CaOx crystals by inducing hyperoxaluria in both wild-type and KO mice.

As 1% EG and 4 IU/ml Vitamin D3 were added to drinking water for wild type and THP KO mice, Mo et al. found that 76% of the THP KO mice produced calcium crystals while none were found in the wild type mice. These experiments showed that there was a compensatory increase in the expression of the remaining macromolecule (OPN or THP) following the administration of a hyperoxaluric load. Although this reaction was insufficient to stop
crystallisation, it did point to a potential synergistic interaction. This idea was further supported by a later investigation, which found that more double-null mice (around 39%) spontaneously acquired renal papillary calcium deposits than THP-null animals (14%), and OPN-null mice (9%). In a recent study, mice with metabolic syndrome and Leptin gene defects (Ob/Ob) were compared to wild type (lean) mice to examine the effects of a high-fat diet and hyperoxaluria on crystal formation. Ob/Ob animals given both 1% EG and a high-fat diet (62%) also exhibited extensive CaOx renal crystal deposits in the intratubular regions of the renal cortex-medulla in addition to hypercalciuria and hyperoxaluria.\[28\]

2.2. Transporter knockout model

Researchers have also performed solely transporter knockout in the mouse to induce hyperoxaluria, hypercalciuria, hyperuricosuria, and cystinuria.

2.2.1. Oxalate transporter

Both Slc26a1 and Slc26a6, two anion exchangers that are found on the apical membrane of renal epithelial cells and assist in mediating oxalate exchange, are known as sulphate anion transporter-1 (Sat1). Oxalate homeostasis in Sat1-null and Slc26a6-null mice has been found to be aberrant. Knockout animals had oxalate concentrations in their urine and plasma that were respectively 2-3.5 times and 1.5-2 times greater than those of wild-type mice. In comparison, 26% and 88% of Slc26a1 and Slc26a6 knockout mice developed calcium oxalate crystal deposits in the renal tubules and collecting ducts, as well as visually identifiable bladder stones.\[29\]

2.2.2. Na⁺ phosphate transporter

The Na⁺ phosphate co-transporter 2a is one of the renal tubular transporters that the sodium-hydrogen exchanger regulator factor-1 (NHERF-1) interacts to (Npt2a). Increased excretion of calcium, phosphate, and uric acid in the urine by NHERF-1 mutant mice led to tubulo-interstitial crystal deposits in the kidney. Npt2a knockout mice displayed hypercalciuria and renal crystal deposits in the kidney, just like NHEFR-1 mice. The kidneys of NHERF-1 and Npt2a mutant mice both contained calcium phosphate crystals.\[30\]

2.2.3. Cystine transporter

Cystinuria, an autosomal-recessive disorder of proximal tubular reabsorption of cystine and dibasic amino acids that results in cystine stones. It has been studied using a knockout transporter model. Based on the heterozygosity of cystine and dibasic amino aciduria, there
are currently 2 cystinuria phenotypes described. The term "type I cystinuria" alludes to the first cystinuria gene, SLC3A1, which codes for the rBAT transporter in both intestine and renal epithelial cells on chromosome 2 (2p21). A second cystinuria gene, SLC7A9, which encodes the light chain b0,+AT protein and is located on chromosome 19 (19q13), is involved in non-type I. Researchers have shown that cystinuria, cystine crystalluria, and cystine urolithiasis were present in Slc7a9 knockout mice.\cite{31}

![Fig. 2: Cystine stones of urinary tract in Slc7a9 knockout mice.](A) Comparison between urinary tract anatomy of a normal mouse (Slc7a9+/+) versus non-type I knockout (Slc7a9−/−) mouse. (B) Radiograph view of the same mice urinary systems-wild type (+/+, left) and homozygous (−/−, right). Arrowheads, kidney stones; arrows, bladder stones.\cite{31}

3. Fly model

Comparing Drosophila melanogaster to other vertebrate animal models, there are many benefits. The fruit fly does not require a formal animal experimentation evaluation as it is an invertebrate in order to obtain and carry out a study protocol. The ability of female flies to produce hundreds of progeny in a short period of time allows them to produce vast numbers of specimens very quickly. Additionally, due of its brief lifespan in a laboratory setting (usually 60 days), researchers can track an animal's progression of a disease. Last but not least, starting and running a fly lab is significantly less expensive due to the lab's comparatively minimal space and dietary requirements.

Genetically speaking, Drosophila only have 4 pairs of chromosomes as opposed to 23 in humans. Nearly 80% of human renal transporters are identified in the fly Malpighian tubule, and approximately 75% of human illness genes have Drosophila melanogaster correlations. Targeted animal gene manipulation has become considerably more doable because of a fully sequenced genome and a free, well-established database called FlyBase (http://flybase.org)
that is dedicated to identifying gene similarity between human and fly genes. In order to examine urinary stone illness, researchers have begun using this potent translational model.

They have an open circulatory system with a hemocoel chamber filled with fluid that transports hemolymph similarly to how blood vessels in humans do. Agglomerular renal system and two sets of free-floating Malpighian renal tubules are both present in this cavity. The Malpighian tubule, homologous to human renal tubules, filters the fly hemolymph in a manner similar to that of human tubules. Recent research has clarified that the striking parallels between the fly and human systems imply an evolutionary connection. The nephrocytes and Malpighian tubules, which make up the renal system in Drosophila, are two anatomically distinct organs. Similar to podocytes in the human glomerulus, nephrocytes filter the hemolymph and eliminate waste. The Malpighian tubule’s anterior and posterior pairs, however, are function similarly to a human nephron.[32]

3.1. Diet induced model
With the administration of Ethylene glycol, hyperoxaluria in the fly can be caused, just like in rodent models. In as little as 6 hours, adult Drosophila treated with EG generated CaOx concretions. CaOx microliths formed in 2 days after Drosophila larvae were fed sodium oxalate that had been dissolved in common development media. The distinctive birefringence of these CaOx crystals is visible when polarised light is used to illuminate dissected Malpighian tubules. Additionally, these CaOx crystals can be seen clearly on micro-computed tomography.[33]

![Fig. 3: Comparison of Malpighian tubules of D. melanogaster fed high oxalate versus control low oxalate diet. Polarized light demonstrates birefringence of calcium oxalate concretions in high oxalate tubules.][34]
3.2. Genetic models of stone disease
With the use of the previously described GAL4 driver/UAS (upstream activation sequence) transgenic system, scientists can specifically suppress the genes thought to be involved in the creation of stones in Drosophila. Investigators can easily instruct their intended driver to express or mute particular gene end products using the resources provided for Drosophila. Genetic manipulation in the mouse model requires a minimum of 3-6 months, but it only takes 2-3 weeks in the fly model.[35]

4. Porcine model
Theoretically, the porcine model would be a good one because human kidneys are anatomically and physiologically similar to those of pigs. In order to test new endourologic surgical techniques, some researchers have implanted human kidney stones inside of pig kidneys. An undivided renal cortex, several medullary pyramids that each form a separate papilla, or sporadically a complex papilla, particularly in the higher pole, are anatomical similarities between porcine and human kidneys. Humans typically have 4-18 papillae, but pigs often have 8-12. Moreover as Kirkman showed, porcine renal physiology parallels that of humans with respect to maximal urine concentration (porcine 1080 vs human 1160 mOsm/L), glomerular filtration rate (porcine 130 vs human 126-175 mL/min per 70 kg), and total renal blood flow (porcine 4.0 vs human 3.0-4.4 mL/min.g).[36]

4.1. Hyperoxaluria
Porcine models to far have concentrated on simulating a hyperoxaluric state by giving hydroxyproline (HP). As demonstrated in young pigs by Mandel et al., all HP-fed pigs had a maximal increase in urinary oxalate content by day 6 after receiving 10% weight per weight of trans-4-hydroxy-L-proline/food. An intriguing discovery was that increasing the HP/food concentration up to 20% did not cause a higher level of hyperoxaluria, despite the fact that the urinary oxalate levels in these young pigs did not decrease until HP was withdrawn from their diet. In either a standard or acidified diet, gravid sows experienced hyperoxaluria and hyperglycoluria when fed 10% HP, as shown by Kaplon et al. Using 24-hour urine samples, hyperoxaluria and hyperglycoluria peaked on the first day of feeding and then started to diminish even though HP was still being administered.[37]
CONCLUSION
In a supersaturated solution, dissolved compounds spontaneously crystallise in a phase-change thermodynamic process known as CaOx nucleation. The nucleation experiment, which may mimic the biological process, showed a similar phase change and the creation of CaOx crystals. The most frequent occurrence of COM, a CaOx polymorph, in urolithiasis, which is more stable and has more aggregatory and sticky properties produces substantial crystal aggregates that lodge deeply into the renal epithelial tissue and cause damage. Various assays and other in vitro models can be used to evaluate the CaOx inhibiting capacity of a test substance.

Both humans and rats can develop calcium oxalate urolithiasis as a result of hyperoxaluria. Rats and humans are thought to have nearly identical oxalate metabolisms. Thus, a rat model of calcium oxalate urolithiasis can be utilised to study the mechanisms underlying the development of kidney stones in humans and to test potential antiurolithiatic drugs. Understanding the pace of the production of urinary stones requires the use of animal models. In order for outcomes to be complementary and similar, it would ultimately be ideal if the urology and nephrology communities could agree on a few animal models.

ACKNOWLEDGEMENT
The author is grateful to the Head of Department of Pharmacology and other faculties, moreover the Principal of Pushpagiri College of Pharmacy, Thiruvalla (Kerala university of health sciences) for the continuous support and encouragement.

REFERENCES


