Published in final edited form as:

J Urol. 2010 September ; 184(3): 1189–1196. doi:10.1016/j.juro.2010.04.065.

Of Mice and Men: Experimental Induction of Calcium Oxalate Nephrolithiasis in Mice

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Abstract

Purpose—Availability of various transgenic and knockout mice provides an excellent opportunity to better understand the pathophysiology of calcium oxalate (CaOx) stone disease. However attempts to produce CaOx nephrolithiasis in mice have not been very successful. We have hypothesized that CaOx nephrolithiasis in mice requires increasing the urinary excretion of calcium as well as oxalate and that experimentally induced hyperoxaluria alone is not sufficient. To provide evidence we induced hyperoxaluria by administering hyperoxaluria inducing agents to normocalciuric as well as hypercalciuric mice and investigated various aspects of nephrolithiasis.

Materials and Methods—Ethylene glycol (EG), glyoxylate (GOx) or hydroxyl proline (HLP) were administered through diet to male and female normocalciuric B6 mice as well as hypercalciuric Npt2a $-/-$ mice for 4 weeks. 24 hour urine samples were collected on 0,3,7,14,21 and 28 days and analyzed for pH, creatinine, lactate dehydrogenase (LDH) calcium and oxalate. Kidneys were examined using light microscopy. Urine was examined for crystals using both light and scanning electron microscopy.

Results—Hypercalciuric mice on HLP did not tolerate the treatment and had to be sacrificed before 28 days. All mice receiving EG, GOx or HLP became hyperoxaluric and demonstrated CaOx crystalluria. None of the female mice, normo or hypercalciuric developed renal CaOx crystal deposits. All mice on GOx and some on EG developed CaOx nephrolithiasis. Kidneys of all mice showed epithelial injury. Male mice particularly on GOx showed more renal injury and migration of inflammatory cells into the interstitium around the crystal deposits.

Conclusions—Results confirm that induction of hyperoxaluria alone is not sufficient for CaOx nephrolithiasis in mice. Hypercalciuria is also required. Kidneys of male mice are more prone to injury than those of female mice and are susceptible to CaOx crystal deposition. Perhaps epithelial injury promotes crystal retention. Thus CaOx nephrolithiasis in mice is gender dependent and requires both hypercalciuria and hyperoxaluria.

Keywords

Calcium Oxalate; Calcium Phosphate; Nephrolithiasis; Kidney Stones; Hypercalciuria; Hyperoxaluria; Npt2a Knock Out Mice

INTRODUCTION

Idiopathic calcium oxalate (CaOx) stone disease is a common urological disorder affecting 6-10% of the US population and costing over 2 billion dollars/year¹. The incidence of kidney stones is on the rise and in the United States of America rose by 37% between 1976–1980 and 1988–1994 in both genders². A number of animal models, specifically rat models have been utilized to study CaOx nephrolithiasis, the deposition of CaOx crystals in the renal tubules^{3,4}. Most commonly acute or chronic hyperoxaluria is induced in male rats by the administration of hyperoxaluria inducing agents (HIA). Intraperitoneal administration of the HIA such as sodium oxalate leads to rapid crystal deposition⁵. Administration of ethylene glycol (EG) or hydroxyl-L-proline (HLP) through food or water, on the other hand, produces chronic hyperoxaluria⁶. Hyperoxaluria is followed by and associated with CaOx crystalluria and eventually CaOx nephrolithiasis. These models have made significant contributions to our understanding of the mechanisms involved in CaOx nephrolithiasis and are also used to determine the efficacy of various therapies. Despite years of sustained research, however, the pathophysiology of idiopathic CaOx stone disease is still poorly understood. The availability of various genetically engineered transgenic and knockout mice (KO) provides an excellent opportunity to advance our knowledge in this regard. It has however proven difficult to consistently induce deposition of CaOx crystals in the mice kidneys by inducing hyperoxaluria alone^{7,8}. Recently daily intrabdominal injections of glyoxylate have been shown to produce CaOx nephrolithiasis in mice. However, renal crystal deposition increased in the first week and then started to decrease, crystals disappearing from the kidneys after two weeks^{9,10}. We have hypothesized that in mice, experimental induction of hyperoxaluria alone is not sufficient to produce CaOx nephrolithiasis. It requires an increase in the urinary excretion of both calcium and oxalate¹¹. To investigate it further and develop a dependable mice model, we decided to test various HIA proven successful in the rats to induce CaOx nephrolithiasis in the normocalciuric as well as hypercalciuric mice.

MATERIALS AND METHODS

Literature search shows that most studies have utilized male rats and used EG as an agent to induce hyperoxaluria¹². HLP, GOX or glycolate have also been successfully utilized. We decided to use EG, HLP, and GOX to induce hyperoxaluria. We utilized the normocalciuric B-6 (C57BL/6J) mice and hypercalciuric Npt2a $-/-$ mice. Latter were utilized to investigate the role of hypercalciuria in CaOx nephrolithiasis in the presence of hyperoxaluria. B6 is the most commonly used strain. It is generally used as background strain for the generation of congenics carrying the spontaneous and induced mutations. It is also the background strain used to generate Npt2a $-/-$ mice.

Four to six month old male and female B6 wild type (WT) and Npt2a KO mice weighing 18-30 gm were housed in separate cages and were allowed to adjust to their environment for three days. Mice belonging to Npt2a KO and B6 WT were divided into male and females. Each gender group was divided into 4 subgroups according to dietary supplement. Control group include 4 mice. There were at least 4 mice in each subgroup, control and the three receiving HLP, EG, or Gox. Control mice were given regular powdered sterile rodent chow moistened with water and formed into small balls. Treated mice received the chow mixed with 1.5% Gox, 5% HLP or 1.25% EG. Food was prepared and replaced daily because additives can break down and spoil the food. Water and food consumption as well as mice weights were monitored regularly. A drop of urine was examined daily to check for the presence of crystals. Mice were housed in mouse metabolic cages for urine collection. 24-hour urine samples were collected on days 0, 3, 7, 14, 21 and 28 and their pH measured.

Urine was analyzed for calcium and oxalate. Mice were sacrificed on day 28, using carbon dioxide. Methods are described in detail in our earlier publications ^{11, 13}.

RESULTS

Normocalciuric B6 Mice (urinary chemistry results shown for male mice only)

The male mice weighed 32 to 33 g, significantly heavier, than the female mice, which weighed 22-25 g at the beginning of the experiment. The mice ate their food and drank water during 4 weeks of the experiment. There was no significant change in weight of the control mice, male (Figure 1A) as well as female, during the next 4 weeks. Weight of the male as well as female mice on EG containing HI diet also did not change significantly. However, weight of the male mice on both the GOx and HLP diets started to decrease by day 7 and was significantly reduced by 28th day when experiment was terminated. Some male mice on HLP showed dramatic weight loss and appeared lethargic. As a result two male mice had to be sacrificed after 10 and 23 days only, much earlier than planned.

With regard to the urinary volume, females produced significantly less urine than the male mice (day 0, male 4.6 vs female 2.25 ml). Within each gender and treatment, differences in volume on various days were not significant except in the case of HLP treated animals. There was a gradual decrease in their urinary volume and on day 28 it was significantly lower than on day 7. Urinary pH of male B6 mice ranged between 6.5-7 and was generally not affected by various hyperoxaluria inducing diets (Figure 1B). However there was a drop in urinary pH of EG receiving male mice on day 7. There was no significant change in urinary pH of the female mice except that control mice had higher pH on days 14, 21 and 27 compared to the day 0.

Normal female B6 mice excreted significantly less creatinine in their urine than the normal male mice. There was gradual decline in urinary excretion of creatinine by the male mice on the HLP treatments (Figure 1C). Hyperoxaluria treatments led to gradual and significant increase in urinary LDH levels of both the male (Figure 1D) and female mice. Glyoxylate treatment produced the most LDH increase in both the genders.

There was no significant difference between normal male and female mice with regards to the urinary excretion of calcium and oxalate. By day 7 of the hyperoxaluric diet, however, urinary excretion of oxalate increased significantly in both the male (Figure 1E) and female mice. In the male mice, highest increase in urinary oxalate was seen in those mice administered EG. On the other hand, there were no significant differences in urinary oxalate excretion by the female mice on various treatments. Urinary excretion of calcium increased significantly in mice treated with the HLP in both the male (Figure 1F) and female mice after 14, 21 and 28 days of the treatments. On the other hand male mice receiving EG or Gox had lower calcium than the controls on 21 and 28 days.

Urine of normal mice contained a few struvite crystals (Figure 2A). By day 3 of the hyperoxaluria inducing treatments struvite crystals mostly disappeared from the urine and were replaced with the CaOx crystals. Both plate like and twinned CaOx monohydrate and pyramidal CaOx dihydrate crystals were easily identifiable (Figure 2B-D).

There were no identifiable crystal deposits in the kidneys of any mice, male or female, on hyperoxaluric or regular diet (Figure 3). However after 28 days of hyperoxaluric diet, when animals were sacrificed, kidneys showed signs of injury and inflammation (Figure 3). Vacuolar degeneration of the tubules was common and inflammatory cells were visible in the cortical interstitium. Male mice showed more injury (Figure 3B) than the female mice (Figure 3A).

Hypercalciuric Mice (urinary chemistry results shown for male mice only)

Male mice ranged in weight from 30.5g to 36g while females ranged in weight from 25g to 28g on day 0, the beginning of the experiment. During the next 28 days, there was no significant change in the weight of the control male mice (Figure 4A) while female control mice showed significant increase on days 21 and 28 from the baseline. Male as well as female mice on the hyperoxaluria inducing diets either maintained or lost weight. Both male and female mice on HLP and Gox lost significant weight compared to their respective controls as well as the baseline. Some of them became lethargic and died or had to be sacrificed before planned ending of the experiment after 28 days. Male mice receiving 5%HLP started dying as early as 5th day of the experiment and none of them survived the entire duration.

Urinary pH of various mice within the groups did not differ significantly (Figure 4B). There were no significant changes in urinary pH of both the male and female control mice during experimental period. Male mice showed slight but significant drop in pH on days 14, 21 and 28. There were significant differences in urinary volume of male and female mice between controls and experimental animals on the HI diets (Results not shown). Female mice produced significantly lower amounts of urine. Male mice on EG and Gox diets produced significantly more urine than their controls. Female mice also produced more urine than their controls significantly so on days 21 and 28 only.

Urinary creatinine levels per 24 hours were also lower in the females compared to male mice. Differences between the male mice on EG or Gox and their controls were not significant except on 21st and 28th day (Figure 4C). There were no significant differences in urinary creatinine excretion between the controls and female mice on HI diets. Levels of LDH in the urine were however, significantly higher in urine of both male (Figure 4D) and female mice on the hyperoxaluria inducing diets than their controls on various days as well as from the day 0.

There was a significant increase in urinary oxalate excretion by both the male (Figure 4E) and female mice on hyperoxaluria inducing diets compared to the baseline as well as their respective controls. However urinary oxalate excretion by female mice receiving HLP was lower on days 21 and 28 compared to those receiving EG or Gox. There were no significant differences in urinary excretion of calcium by male (Figure 4F) or female mice from different groups receiving different HI diets except the mice on HLP.

Urine of the control KO mice generally appeared devoid of any identifiable crystals, when viewed under a light microscope. Scanning electron microscopic examination however revealed amorphous structures as well as brushite crystals which we were later able to identify even using light microscopy (Figure 5A). On the other hand mice consuming various hyperoxaluria inducing agents, excreted CaOx crystals in their urine, by day 3 of the treatment (Figure 5B-D). Both twinned tabular CaOx monohydrates and dipyramidal CaOx dihydrate crystals were identified.

The kidneys of all Npt2a KO male mice who received GOx as HI agent developed CaOx deposits (Figure 6B). Two of four male KO mice that survived 28 days of EG treatment developed CaOx crystals in their kidneys (Figure 6A). HLP treatment did not lead to CaOx crystal deposits in the male mice. However, none of them lasted the entire 28 day duration of the experiment. Female mice eating food supplemented with Gox or EG, did not develop CaOx crystal deposits in the kidneys.

Kidneys of all the KO mice, males as well as females, had calcium phosphate crystals (Figure 7A). Most were located in the renal cortex. Almost all kidneys also contained a few

crystal deposits in the renal papillae. The crystals were mostly intratubular. But interstitial deposits of CaP crystals were also identified.

Kidneys of hyperoxaluric mice showed vacuolar degeneration and inflammation. As in the case of normocalciuric B6 mice, Npt2a KO males also had significantly more vacuolar degeneration than the females. In addition interstitial inflammation was observed around the calcium oxalate crystal containing tubules. Crystal containing tubules themselves showed signs of necrosis.

Most CaOx crystals were intratubular, located in the renal cortex and outer medulla, often at the corticomedullary junction (Figure 6A). The CaOx crystals did not appear to deposit on the calcium phosphate crystals (Figure 7A). Higher magnifications often revealed CaOx crystals in the cells as well as the renal interstitium (Figure 7). In a few kidneys, CaOx crystals were seen in the urinary space attached to the papillary surface epithelium.

DISCUSSION

It has proven difficult to induce deposition of CaOx and/or CaP crystals in the mice kidneys. Urinary CaP deposits are seen in only two of more than a dozen mice models of monogenic hypercalciuria¹⁴. The Npt2a knock out (KO) mice develop deposits in their kidneys¹⁵. Caveolin-1 KO mice produce bladder stones. Recent studies have shown that mutant NHERF-1 null mice also produce CaP deposits in their kidneys¹⁶. Studies of calcification in mice deficient in OPN and/or THP has shown that 10% of the mice lacking OPN and 14.3% of mice lacking THP spontaneously form renal deposits of CaP⁷.

Even though most rats with experimentally induced hyperoxaluria develop CaOx renal deposits, mice with hyperoxaluria alone either do not produce any deposits or only a few CaOx crystals in their kidneys. Alanine glyoxylate aminotransferase KO mice develop severe hyperoxaluria but develop only a few CaOx crystal deposits in their kidneys⁹. Mice lacking anion transporter Slc26a6 also develop severe hyperoxaluria^{3,5}, but only those with higher urinary calcium excretion develop CaOx crystal deposits³. Thus, hypercalciuria or hyperoxaluria alone are not sufficient to produce CaP and/or CaOx crystal deposits in mice kidneys. Additional factors appear to be involved.

Our results indicate that CaOx crystal deposition in mice kidneys requires increased urinary excretion of both the oxalate and calcium. However hyperoxaluria produced crystal deposits in only male hypercalciuric mice. Female mice even with increased urinary excretion of calcium and oxalate did not produce renal CaOx deposits. In the case of humans too, idiopathic CaOx nephrolithiasis is more common in males than in the females^{17,18}. Gender differences in development of kidney stones are well known. Even female rats appear resistant to CaOx nephrolithiasis¹³. It has been suggested that male sex hormone testosterone promotes while female sex hormone estrogen inhibit CaOx crystal formation and retention within the kidneys^{19,20}.

Even though men outnumber women in CaOx stone formation, there are no significant differences between the two in urinary excretion of oxalate expressed as either the mmol/24h or $\mu\text{mol}/\text{mmol}$ urine creatinine²¹. Results of our study presented here also show the absence of significant differences in the amounts of urinary oxalate between various groups of mice, male or female; normocalciuric or hypercalciuric on day 0. However, on day 7 of the HI diets, male mice in every group excreted more oxalate in their urine than the female mice in the similar group, some highly significantly. This trend continued throughout the experimental period, when urine was tested for oxalate on days 14, 21 and 28. Thus, with regard to oxalate excretion, male and female mice, similar to rats, responded differently to

various HI inducing protocols. Apparently the two genders process oxalate precursors differently.

Renal epithelial injury and excretion of membrane fragments in the urine are also suggested to play a role in crystallization and crystal retention within the kidneys²²⁻²⁴. Dead cells and sloughed microvilli shed into the tubular lumen promote crystal nucleation and their retention within the tubules. CaOx crystal deposition in kidneys of male C57BL/6 mice achieved by daily intra-abdominal (IB) injections of glyoxylate^{9, 10} has also been suggested to be the result of cell injury caused by IB glyoxylate. Intra-abdominal injections of ethylene glycol or glycolate did not cause severe renal injury and as a result produce CaOx crystal deposits. In our study, none of the B6 mice, male or female, that received oxalate precursors orally, produced CaOx crystal deposits. Even the male mice that showed vacuolar degeneration of the renal tubular cells did not develop renal CaOx crystal deposits. However, vacuolar degeneration is generally reversible and does not lead to membrane shedding. The absence of crystal deposits in the kidneys of hyperoxaluric B6 males may therefore be a result of lack of membranous cellular degradation products as nucleation and aggregation promoters in the urine.

In addition to significant increases in the urinary excretion of Ox, all mice on HI diets also showed significantly increased LDH in the urine compared to the controls as well as day 0. Significant increase in urinary excretion of LDH in the hyperoxaluric mice including those without CaOx crystal deposits, indicates the toxic nature of the oxalate. That oxalate is injurious to renal epithelial cells by itself is also indicated by the histological changes in the kidneys. Interestingly degenerative changes were more common in Gox treated mice and least in those receiving EG in the diet. Gox treated mice also excreted more LDH than the other treated mice.

A number of animal models of hypercalciuria and hyperoxaluria, the two major risk factors, have been developed to understand the pathophysiology of stone disease. All require high doses of lithogens because most animals used have innate capacities to withstand various challenges necessary for induction of crystal deposition. Different animals appear to have different levels of tolerance to high oxalate as well as threshold for crystallization. In some animals threshold for crystallization is reached before upper limit of tolerance. Rats are less resistant to crystallization than mice²⁵. In normal humans crystals form before oxalate levels reach high enough to be toxic while in mice it appears to be the other way around. Formation of small urinary crystals that can be easily excreted may be advantageous because it reduces available oxalate to interact with the cells.

Acknowledgments

Research supported by National Institutes of Health Grant RO1 DK 065658 and University of Florida Center for the Study of Lithiasis.

Key of Definitions for Abbreviations

CaOx	Calcium Oxalate
CaP	Calcium Phosphate
HIA	Hyperoxaluria inducing agent
EG	Ethylene glycol
GOx	Glyoxylate
HLP	Hydroxyl-L-proline

LDH	Lactate dehydrogenase
KO	Knockout
Npt2a	Sodium phosphate co-transporter type 2 a

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A

αP < 0.05 Treated vs Treated within the time period

B

*P < 0.05 Control vs Treated within the time period

C

*P<0.05 Control vs Treated within the time period

xP<0.05 Treated vs Treated within the time period

D

*P < 0.05 Control vs Treated within the time period

xP < 0.05 Treated vs Treated within the time period

E

*P<0.05 Control vs Treated within the time periods

¤P<0.05 Treated vs Treated within the time periods

F

*P<0.05 Control vs Treated within the time period

¤P<0.05 Treated vs Treated within the time period

Figure 1.

Urinary chemistry of Male B6 Mice.

A. Weight. There was no significant change in weight of the control mice as well as those on the EG, over the 28 day experimental period. However the weight of mice on Gox or HLP was significantly lower than the control as well as EG treated mice.

B. Urinary pH. Mice on HLP diet had significantly lower pH except on 28th day of the experiment.

C. Urinary creatinine. No significant differences in urinary creatinine excretion were observed between the controls and mice on various HI dietary treatments. However there was a gradual decline in urinary creatinine of mice on HLP from day 14 onward.

D. Urinary LDH. No change in urinary LDH of control animals over 28 days. All HI treatments produced significant increases in urinary LDH compared to the controls.

E. Urinary Oxalate. There was a significant increase in urinary excretion of oxalate by the mice receiving HI treatments. Ethylene glycol produced biggest increase and significantly more than Gox and HLP treatments on days 21 and 28.

F. Urinary Calcium. There were no significant changes in urinary calcium by EG or Gox treatments except on days 21 and 28 when it was significantly lower than the controls. However, urinary excretion of calcium increased significantly after 14, 21 and 28 days of the treatments in mice treated with the HLP.

Figure 2.
Crystals in B6 mice urine. **A.** Control, **B.** Ethylene glycol treated, **C.** Hydroxy L Proline treated, **D.** Glyoxylate treated

Figure 3. Kidneys of B6 mice on Glyoxylate showing renal tubular vacuolar degeneration **A.** Female Hyperoxaluric Mice, original magnification X40. **B.** Male Hyperoxaluric Mice. Reduced from X45.

A

* P<0.05 Control vs Treated within the time period

x P<0.05 Treated vs Treated within the time period

B

*P<0.05 Control vs Treated within the time period

C

D

E

* $P < 0.05$ Control vs Treated within the time period

$P < 0.05$ Treated vs Treated within the time period

F

$P < 0.05$ Treated vs Treated within the time period

Figure 4.

Urinary Chemistry of Male Npt2a KO Mice. A. Weight. There was a significant decrease in weight of the mice on HI diets over the 28 day period compared to the control as well as the baseline. Mice on Gox and HLP had the greatest loss. Actually none of the HLP mice survived the treatment.

B. Urinary pH. Urinary pH of mice on HI diets was significantly lower than control as well as baseline on days 14, 21 and 28.

C. Urinary Creatinine. Differences between the male mice on EG or Gox and their controls were not significant except on 28th day.

D. Urinary LDH. Levels of LDH in the urine were significantly higher in urine of male mice on the HI diets than their controls on various days as well as from the day 0.

E. Urinary Oxalate. Mice on HI inducing diets had significantly increased urinary excretion of oxalate compared to the controls and baseline. EG produced significantly more urinary oxalate than GOx.

F. Urinary Calcium. Male Mice. Significant differences were seen on day 21.

Figure 5. Crystal in Npt2aKO mice urine. **A.** Control, **B.** Ethylene glycol treated, **C.** Hydroxy L Proline treated, **D.** Glyoxylate treated

Figure 6. Calcium Oxalate Nephrolithiasis in the Npt2aKO mice. Birefringent crystals are visible in the kidneys of male mice receiving, **A.** Ethylene glycol or **B.** Glyoxylate as hyperoxaluria inducing agents. Reduced from X1.25.

Figure 7. Calcium Oxalate Nephrolithiasis in the Npt2aKO mice on glyoxylate. **A.** Both CaOx (black arrows) and CaP (white arrow) deposits are visible. Calcium phosphate appears interstitial while CaOx crystals are both, intratubular as well as intracellular and interstitial. **B.** Inflammation is seen around the tubules with the birefringent CaOx crystals. Reduced from X45.