

# Experimentally induced hyperoxaluria in MCP-1 null mice

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**Abstract** Experimental animal model studies suggest that calcium oxalate (CaOx) crystal deposition in the kidneys is associated with the development of oxidative stress, epithelial injury and inflammation. There is increased production of inflammatory molecules including osteopontin (OPN), monocyte chemoattractant protein-1 (MCP-1) and various subunits of inter-alpha-inhibitor such as bikunin. What does the increased production of such molecules suggest? Is it a cause or consequence of crystal deposition? We hypothesized that over-expression and increased production of MCP-1 is a result of the interaction between renal epithelial cells and CaOx crystals after their deposition in the renal tubules. We induced hyperoxaluria in MCP-1 null as well as wild type mice and examined pathological changes in their kidneys and urine. Both wild type and MCP-1 null male mice became hyperoxaluric and demonstrated CaOx crystalluria. Neither of them developed crystal deposits in their kidneys. Both showed some morphological changes in their renal proximal tubules. Significant pathological changes such as cell death and increased urinary excretion of LDH were not seen. Results suggest that at least in mice (1) Increase in oxalate and decrease in citrate excretion can lead to CaOx crystalluria but not CaOx nephrolithiasis; (2) MCP-1 does not play a role in crystal retention within the kidneys; (3) Expression of OPN and MCP-1 is not increased in the kidneys in the absence of crystal deposition; (4) Crystal deposition is necessary for significant pathological changes

and movement of monocytes and macrophages into the interstitium.

**Keywords** Calcium oxalate · Nephrolithiasis · MCP-1 · Inflammation

## Introduction

Animal model and tissue culture studies have provided evidence for an association between calcium oxalate (CaOx) crystal deposition in the kidneys and renal tubular injury [4, 6, 22]. Results indicate that experimentally induced hyperoxaluria leads to CaOx crystal formation in the renal tubules. Crystals either settle in the kidneys or are excreted in the urine. Many of these retained crystals migrate into the renal interstitium, where they are seen surrounded by the inflammatory leukocytes, the monocytes, macrophages as well as multinucleated giant cells [3, 11]. Interaction between renal epithelial cells and high oxalate and/or CaOx crystals leads to the production of a variety of macromolecules including osteopontin (OPN), monocyte chemoattractant protein-1 (MCP-1), inter-alpha-inhibitor (I-alpha-I), bikunin, alpha-1 microglobulin, urinary prothrombin fragment-1 (UPTF-1), Tamm-Horsfall protein (THP) [15]. Many of the upregulated macromolecules such as osteopontin, urinary prothrombin fragment-1, bikunin have well known roles in crystallization processes [9]. But what may be the explanation for the other molecules such as MCP-1, with no known role in crystallization, being produced by cells exposed to high oxalate and/or crystals [19]? MCP-1 is a well-characterized member of CC chemokine family and plays key role in inflammation recruiting monocytes in response to various extracellular stimuli. Production of MCP-1 by renal tubular epithelial cells challenged with

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oxalate and/or crystals is significantly increased. Crystal-induced upregulation of MCP-1 was also seen in the renal fibroblasts [21]. Other upregulated molecules, such as OPN, UPTF-1, THP, and bikunin, are also part of inflammatory cascade. Furthermore, increased expression of MCP-1 may also be associated with oxidative stress and low grade inflammation seen in kidneys of the stone patients [1]. Does inflammation and expression of inflammatory chemokines such as MCP-1 play a role in crystallization and crystal deposition in the kidneys? Is MCP-1 a promoter or inhibitor of crystal deposition? To answer these questions and better appreciate the involvement of MCP-1 in CaOx nephrolithiasis, we induced hyperoxaluria in male and female MCP-1 null mice and examined their urine and kidneys for pathological changes including CaOx crystal deposition.

## Materials and methods

4–6-week-old, male and female wild type C57/BL6 and MCP-1 null mice weighing between 18 and 30 g were used for investigation. MCP-1 null mice were obtained from Jax labs, while wild type B6 mice were from Charles River. It is challenging to induce CaOx nephrolithiasis in mice. Therefore, we decided to utilize two different methods to induce hyperoxaluria. In one investigation, protocol 1, mice were given a modified AIN-76A diet mixed with 1.5% potassium oxalate. In the other experiment, protocol 2, mice received normal rodent chow (Tekland Global 2018S) and 1.5% ethylene glycol in the drinking water. Mice were allowed to adjust to their food for 7 days. Food, water intake and weight were regularly recorded. Pooled 24-h urine samples were collected weekly using mouse metabolic cages. Urinary pH, blood and protein were determined by dipsticks. Urinary lactate dehydrogenase (LDH), calcium (Ca), oxalate

(Ox), creatinine (Cr) and citrate (Cit) levels were determined on 0, 7, 14, 21, 28, 35, 42, 49, and 56 days using methods described previously [8]. Urine was also examined for the presence of crystals by light microscopy. Selected samples were filtered and examined by scanning electron microscopy as previously described. At the end of the experiment, mice were killed by CO<sub>2</sub>. Kidneys were harvested and processed for light microscopic examination as previously described [8]. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) and examined using polarizing optics. In addition, immunohistochemical staining was performed to determine the expression of osteopontin, MCP-1, and ED-1.

## Results

Male wild type as well as MCP-1 null mice were bigger and weighed more than the female mice. Male as well as female mice maintained or gained weight on both hyperoxaluria inducing protocols. On protocol 1, where oxalate was added to the AIN-76A diet, weight of male wild type mice increased from  $22.2 \pm 2.49$  g on day 0 to  $25.4 \pm 3.13$  g on day 56 while that of female changed from  $18.4 \pm 0.89$  to  $21.2 \pm 1.10$  g. Male MCP-1 null mice weight changed from  $22.6 \pm 0.55$  to  $27 \pm 1.22$  g while that of female changed from  $17.6 \pm 0.55$  to  $20.8 \pm 1.10$  g. On protocol 2, where hyperoxaluria was induced by administration of 1.5% ethylene glycol in drinking water (Table 1), weight of male wild type mice increased somewhat, from  $27.33 \pm 0.58$  to  $28.67 \pm 1.15$  g. Weight of female wild type mice changed from  $21.25 \pm 0.96$  to  $24.00 \pm 0.00$  g. MCP-1 null male mice increased their weight from  $27.00 \pm 1.15$  to  $29.75 \pm 1.50$  g while female mice increased in weight from  $21.67 \pm 0.58$  to  $25.67 \pm 0.58$  g.

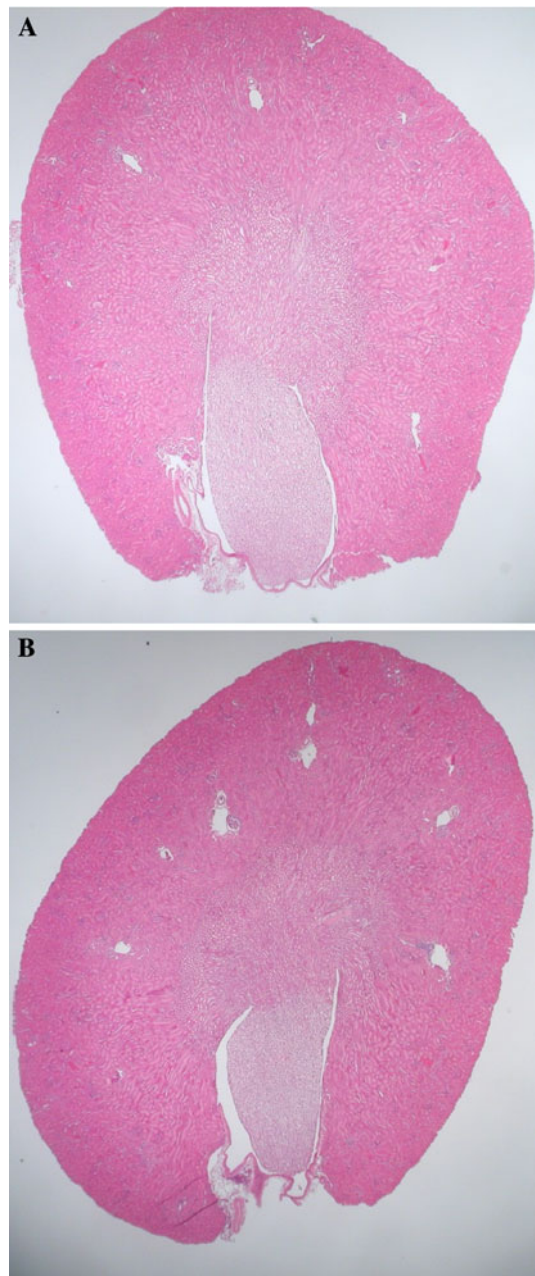
**Table 1** Urinary chemistry from day 0 to 56 of the male and female MCP-1 null mice who drank 1.5% ethylene glycol solution

| Days | Male             |               |             |             |                 | Female           |               |              |             |                 |
|------|------------------|---------------|-------------|-------------|-----------------|------------------|---------------|--------------|-------------|-----------------|
|      | Wt (g)           | Cit (mg/24 h) | Ca (mmol/L) | Ox (mmol/L) | Creat (mg/24 h) | Wt (g)           | Cit (mg/24 h) | Cal (mmol/L) | Ox (mmol/L) | Creat (mg/24 h) |
| 0    | $27.0 \pm 1.15$  | 1.47          | 0.36        | 0.64        | 1.66            | $21.67 \pm 0.58$ | 0.84          | 0.30         | 1.10        | 0.79            |
| 7    | $28.75 \pm 1.71$ | 1.14          | 0.33        | 1.71        | 2.53            | $22.67 \pm 0.58$ | 0.62          | 0.40         | 0.80        | 1.61            |
| 14   | $27.75 \pm 0.96$ | 1.25          | 0.27        | 1.32        | 2.7             | $23.33 \pm 0.58$ | 0.58          | 0.36         | 0.98        | 0.97            |
| 21   | $28.5 \pm 2.65$  | 1.26          | 0.18        | 1.1         | 2.12            | $23.67 \pm 0.58$ | 0.65          | 0.42         | 1.02        | 1.21            |
| 28   | $28.5 \pm 1.29$  | 1.47          | 0.24        | 1.32        | 1.95            | $23.33 \pm 0.57$ | 0.88          | 0.48         | 0.47        | 1.1             |
| 35   | $28.75 \pm 1.71$ | 1.21          | 0.24        | 1.76        | 1.84            | $24.67 \pm 1.15$ | 0.62          | 0.33         | 0.69        | 1.05            |
| 42   | $29.75 \pm 1.50$ | 0.60          | 0.27        | 1.84        | 1.88            | $24.33 \pm 0.56$ | 0.58          | 0.42         | 0.65        | 1.09            |
| 49   | $29.25 \pm 0.96$ | 0.73          | 0.21        | 1.16        | 1.96            | $25.67 \pm 0.58$ | 0.22          | 0.63         | 0.33        | 1.4             |
| 56   | $29.75 \pm 1.50$ | 0.48          | 0.27        | 1.77        | 1.7             | $25.67 \pm 0.58$ | 0.44          | 0.42         | 0.77        | 1.1             |

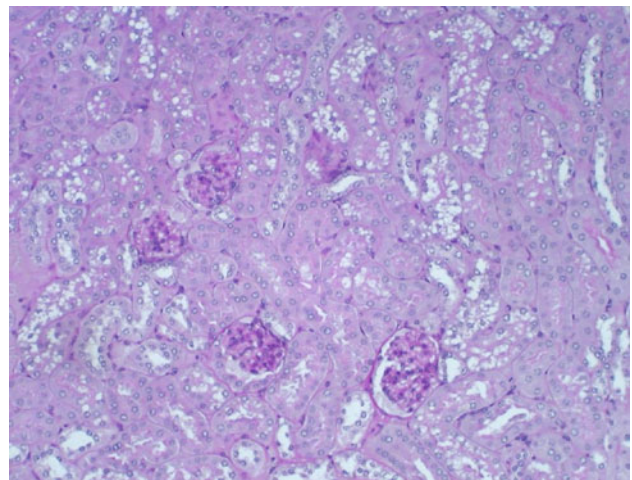
In the males, urinary oxalate increased while citrate decreased with no significant change in urinary excretion of calcium or creatinine  
Wt weight, Cit citrate, Ca calcium, Ox oxalate, Creat creatinine

There were no CaOx crystals in kidneys of any mice, male or female, wild type or MCP-1 null on either of the two hyperoxaluria inducing protocols (Fig. 1). Kidneys appeared normal except for some vacuolar changes in the proximal tubular epithelial cells of mostly the male mice (Fig. 2) receiving EG in their drinking water. There was no difference in expression of MCP-1, OPN and ED-1 between the wild type and MCP-1 null mice.

Both protocols 1 and 2 produced hyperoxaluria. Urinary excretion of calcium and creatinine did not change noticeably from day 0 to day 56 by both the male and female mice



**Fig. 1** Absence of crystal deposition in the kidneys of hyperoxaluric mice. **a** Female B6 and **b** female MCP-1 null

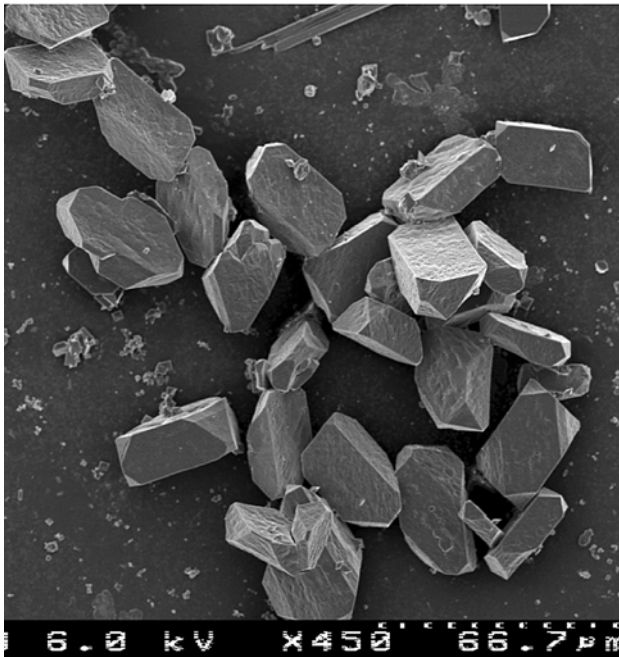


**Fig. 2** Periodic acid Schiff stained section of kidneys from a male MCP-1 null mice showing vacuole in the tubular epithelial cells

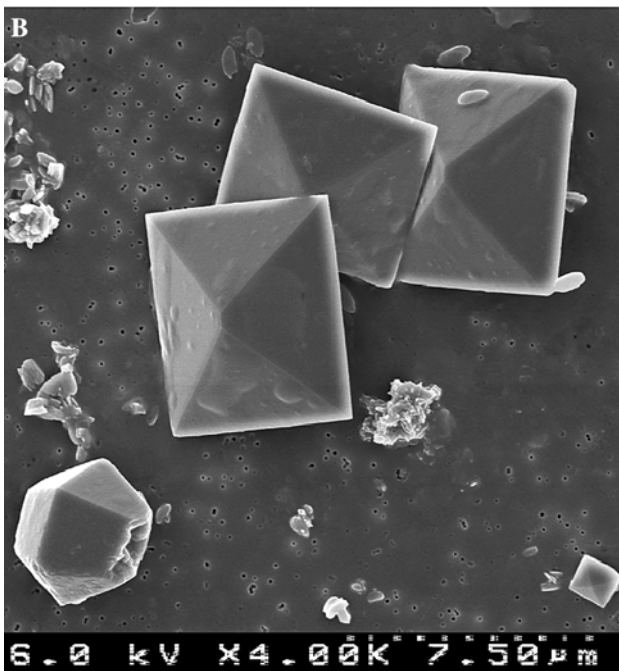
on the hyperoxaluric diets (results of only MCP-1 null mice on protocol 2 shown, Table 1). On the other hand, there was considerable increase in the urinary excretion of oxalate by the male mice receiving either oxalate in the food or EG in the drinking water. Male MCP-1 null mice on EG increased their urinary oxalate from 0.64 mmol/L on day 0 to 1.77 mmol/L on day 7. Excretion of oxalate in the urine by the female mice did not appear to change. Urinary excretion of citrate by both the male and female mice decreased considerably over time, from 1.47 mg/24 h on day 0 to 0.48 mg/24 h on day 56 by the MCP-1 null male mice and from 0.84 mg/24 h on day 0 to 0.44 mg/24 h on day 56 by the MCP-1 null female mice. Wild type mice also decreased excretion of urinary citrate, from 1.7 mg/24 h on day 0 to 0.43 mg/24 h on day 56 by males and from 1.20 mg/24 h on day 0 to 0.48 mg/24 h on day 56 by females. Urinary excretion of LDH did not change with the administration of hyperoxaluria inducing ethylene glycol (results not shown).

No blood was detected in any urinary sample. Urinary pH of both male and female MCP-1 null and wild type mice ranged between 6.0 and 6.5. Urine of all mice, male or female, wild type as well as MCP-1 null mice contained some struvite crystals (Fig. 3a) on day 0 and continued to have them to the end. In addition, mice on hyperoxaluria protocol 1 started showing occasional presence of calcium oxalate monohydrate (COM) and/or calcium oxalate dehydrate (COD) crystals (Fig. 3b) by day 14 and continued to do so till the end on day 56. When hyperoxaluria was induced by EG treatment, COM and COD crystals started appearing in the urine by day 7, and were identified in all urine samples from both the wild type as well as MCP-1 null mice of both genders. Number of crystals was higher in urine of mice on protocols 2 than in the urine of protocol 1 mice. Interestingly, male mice produced both COM and

**A** Struvite crystals in the urine of normal rat before induction of hyperoxaluria.



**B** Calcium oxalate dihydrate crystals in the urine of hyperoxaluric mice.



**Fig. 3** Urinary crystals. **a** Struvite crystal on day 0 and **b** calcium oxalate dehydrate crystals

COD crystals while female mice produced only COD crystals in the first 21 days of the EG consumption. After that both male and female mice produced both forms of CaOx crystals.

## Discussion

Mice are generally resistant to crystal deposition in their kidneys because of the high crystallization inhibitory capacity of their primary urine or the tubular epithelial tolerance for high oxalate. Renal CaP deposits are seen in only two of more than a dozen mice models of monogenic hypercalciuria [13]. The Npt2a knock out (KO) mice are born with CaP deposits in their kidneys [2]. Caveolin-1 KO mice produce bladder stones. Recent studies have shown that mutant NHERF-1 null mice also produce CaP deposits in their kidneys [23]. Studies of calcification in mice deficient in OPN and/or THP have shown that 10% of the mice lacking OPN and 14.3% of mice lacking THP spontaneously form renal deposits of CaP [12].

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. Even genetically modified mice with severe hyperoxaluria do not develop renal deposits of CaOx crystals. Alanine glyoxylate aminotransferase KO mice excrete high levels of oxalate in their urine, but very few CaOx crystals deposit in their kidneys [14]. Mice lacking anion transporter Slc26a6 also develop severe hyperoxaluria [5, 7], but only one of the two strains develops CaOx crystal deposits [5]. We were able to produce CaOx crystal deposits in kidneys of hypercalciuric Npt2a<sup>-/-</sup> mice [8]. Results of our study presented here confirm that hyperoxaluria alone is not enough for CaOx crystal deposition in the kidneys. Normal wild type mice given a high dose of EG, quickly became hyperoxaluric, produced CaOx crystalluria, but did not deposit CaOx crystals in their kidneys. MCP-1 null mice similarly became hyperoxaluric, but did not deposit CaOx crystals in their kidneys.

Both wild type as well as MCP-1 null mice showed a drop in urinary excretion of citrate. Citrate is a well-known inhibitor of CaOx crystallization [16]. Reduced excretion of citrate in urine along with increase in urinary oxalate may have been responsible for CaOx crystallization and crystalluria. Apparently citrate has no effect on crystal retention within the kidneys.

Results of a number of recent animal model studies indicate an association between renal epithelial injury, inflammation and CaOx crystal deposition in the kidneys [6]. Crystals deposited in the tubules are surrounded by ED-1 positive leukocytes and have also been seen inside the giant cells. Since renin-angiotensin system plays a significant role in the development of renal tubulointerstitial fibrosis, Toblli et al. [18] investigated its involvement in hyperoxaluria-induced TI lesion. They induced hyperoxaluria in male Sprague-Dawley rats by administering ethylene glycol and evaluated whether angiotensin II type 1 receptor blockade

prevents the development of CaOx deposits in the kidneys [18]. Despite similar oxalate levels, hyperoxaluric rats receiving losartan, an angiotensin II type 1 receptor blocker showed fewer CaOx crystal deposits in the kidneys and fewer ED-1 positive cells in the interstitium. It was concluded that beneficial effect of losartan is a result of combination of factors including reduction in crystal formation and control of inflammation. Toblli et al. [17] obtained similar results when an angiotensin converting enzyme (ACE) inhibitor, enalapril, was given to the hyperoxaluric rats.

We induced hyperoxaluria in 10-week-old male Sprague-Dawley rats and treated them with AT1 receptor blocker, candesartan. Kidneys were examined for crystal deposits, ED1 positive cells, and expression of osteopontin mRNA. PCR was used to quantify OPN, renin and ACE mRNA in kidneys. Radioimmunoassay was used to determine renal, plasma, and urinary OPN, plasma renin, Ang II and ACE and renal Ang II. mRNA for OPN, renin and ACE was significantly elevated in hyperoxaluric rats. OPN synthesis and production increased with hyperoxaluria but to a lesser extent in candesartan-treated hyperoxaluric rats. Despite similar urinary calcium and oxalate levels, kidneys of hyperoxaluric rats on candesartan had fewer CaOx crystals, fewer ED1 positive cells, reduced OPN expression than the control hyperoxaluric rats [20]. Osteopontin is a monocyte chemoattractant, specifically for the renal interstitium and upregulation of osteopontin precedes interstitial monocyte infiltration [10]. Ethylene glycol administration to OPN knock out mice resulted in intratubular deposition of CaOx while there was no deposition in the wild type mice given the same treatment [24]. This study was performed to determine whether the absence of MCP-1 would promote CaOx nephrolithiasis in hyperoxaluric conditions. Our results suggest that at least in mice the absence of MCP-1 does not support CaOx crystal deposition even in the hyperoxaluric conditions sufficient to produce CaOx crystalluria. Results also suggest that at least in mice, (1) Increase in oxalate and decrease in citrate excretion can lead to CaOx crystalluria but not CaOx nephrolithiasis; (2) Expression of OPN and MCP-1 is not increased in the kidneys in the absence of crystal deposition; (3) Crystal deposition is necessary for significant pathological changes and movement of monocytes and macrophages into the interstitium.

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