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An animal model of calcium oxalate urolithiasis based on a cyclooxygenase 2 selective inhibitor

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Abstracts Our aim was to develop a stone-forming animal model involving renal tubular injury using a cyclooxygenase 2 selective inhibitor. Male Sprague-Dawley rats fed chow containing 3% sodium oxalate with or without 100 mg/kg celecoxib were compared to animals fed normal chow. Rats were killed after 2 or 4 weeks and the kidneys were harvested for morphological examination. Collections of 24-h urine were made before kidney harvest. After 2 weeks only a few crystals were observed in rats that received oxalate and celecoxib, but after 4 weeks more crystals were observed at the renal papilla than in rats that received only oxalate. Few crystals were found in rats fed normal chow with or without celecoxib. The urinary activities of gamma-glutamyl transpeptidase (GGT) were increased by celecoxib administration whereas creatinine clearance rates were unchanged. In rats fed oxalate, urinary oxalate excretion increased, but calcium excretion decreased. This model using a cyclooxygenase 2 selective inhibitor is a useful stone forming animal model involving mild renal tubular injury together with mild hyperoxaluria.

Keywords Celecoxib · Oxalate · Urolithiasis · Animal model · COX-2

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Introduction

A number of experimental models based on oxalate infusion or feeding a pyridoxine deficient diet, and the administration of oxalate precursors like ethylene glycol, hydroxyproline, glycolic acid, and glyoxylic acid have been developed to investigate the mechanisms of calcium oxalate urolithiasis [1]. However, these models have disadvantages such as a high incidence of nephrotoxicity, generalized metabolic acidosis, severe hyperoxaluria, unusual crystal locations, and a late onset of crystal and stone development.

Kumar et al. [2] developed a new model that overcame these disadvantages by using a treatment that combined moderate hyperoxaluria with mild tubular dysfunction/injury using gentamicin. This model was useful because it produced a higher frequency of stone formation, reduced the time required for stone formation, and caused less harm to kidneys than the other available models. In addition, this study demonstrated that renal tubular injury augments crystal formation and growth.

However, recently, *Oxalobacter formigenes*, *Lactobacillus*, and nanobacteria were suggested as causative factors of nephrolithiasis [3, 4, 5, 6], but the animal model of Kumar et al. cannot be used for experiments using bacteria due to the bactericidal effect of gentamicin. Thus, we investigated the use of a non-steroid anti-inflammatory drug (NSAID), as a new nephrotoxic replacement for gentamicin. However, NSAIDs have G-I toxic side effects that could have adversely affected our experiment and sometimes have led to rat mortality. Thus we chose the selective cyclooxygenase-2 inhibitor,celecoxib, which is known to have few G-I side effects, as the basis for the establishment of a model that allows the study of bacterial involvement of nephrolithiasis.

Materials and methods

All experiments were performed according to the rules governing animal experimentation and the Guidelines

for the Care and Use of Laboratory Animals of the Clinical Research Institute of Seoul National University Hospital.

Male Sprague-Dawley rats, weighing 200–220 g were divided into four groups: (1) control, (2) celecoxib, (3) oxalate, (4) celecoxib + oxalate. Group 1 was maintained on standard rat chow for the whole study. Group 2 received standard rat chow and was given 1 ml of normal saline mixed with celecoxib (100 mg/kg) (Pfizer, New York, USA) by gavage for the first 8 days. The amount of celecoxib administered was decided from the results of pilot studies based on various doses. Group 3 was fed standard rat chow supplemented with 3% ammonium oxalate, and Group 4 rats were maintained on standard rat chow supplemented with 3% ammonium oxalate, and for the first 8 days each rat also received 1 ml of celecoxib (100 mg/kg) (Fig. 1). On days 14 and 28 of the experiment, rats were transferred to metabolic cages for 24-h urine collection and then sacrificed.

Observation of crystal formation in kidneys

Rats were anesthetized with an intramuscular injection of 0.8 cc of an 8:2 mixture of ketamine (50 mg/ml) (Yuhan, Seoul, Korea) and rompun (23 mg/ml) (Bayer, Leverkusen, Germany). A midline laparotomy was performed and the abdominal aorta cannulated using a 20-gauge angiocath. Kidneys were flushed retrograde with 10 ml of cold (0°C) Krebs ringer bicarbonate (KRB) buffer (pH 7.4). After the kidneys were excised, they were longitudinally bisected and placed in a beaker of cold (0°C) KRB. The bisected kidneys were then examined under a dissecting microscope (Olympus, Japan) for crystal numbers and distribution. Kidneys were processed for conventional light microscopy. Each kidney was scored according to the method described by Kumar et al. [2]: 0 = no crystals, 1 = few crystals (one or two per field), 2 = moderate number of crystals (10–20 per field), 3 = frequent crystals (>20 per field) and 4 = abundant crystals (>100 per field).

Examination of urine chemistry and enzymes

Urine pH was determined using freshly voided urine. Creatinine clearance was assessed using 24-h urine collections to evaluate kidney function. Urinary stone-related factors such as oxalate, calcium, and citrate were measured in the urine. To determine the presence of renal tubular epithelial cell injury, gamma glutamyl transpeptidase (GGT) and n-acetyl glucosaminidase (NAG) were quantified by the methods of Szasz et al. [7] and Stirling [8]. Enzymatic determination was used to assay urine oxalate and citrate [9, 10]. Urine calcium level was determined by the procedure of Cohen and Sideman [11]. Creatinine was determined by the method of Jaffe [12].

Statistical analysis

Statistical analysis was done using the SPSS 10.0. The Mann-Whitney U-test was used to perform simple comparisons between experimental groups. Differences were considered significant for $P < 0.05$.

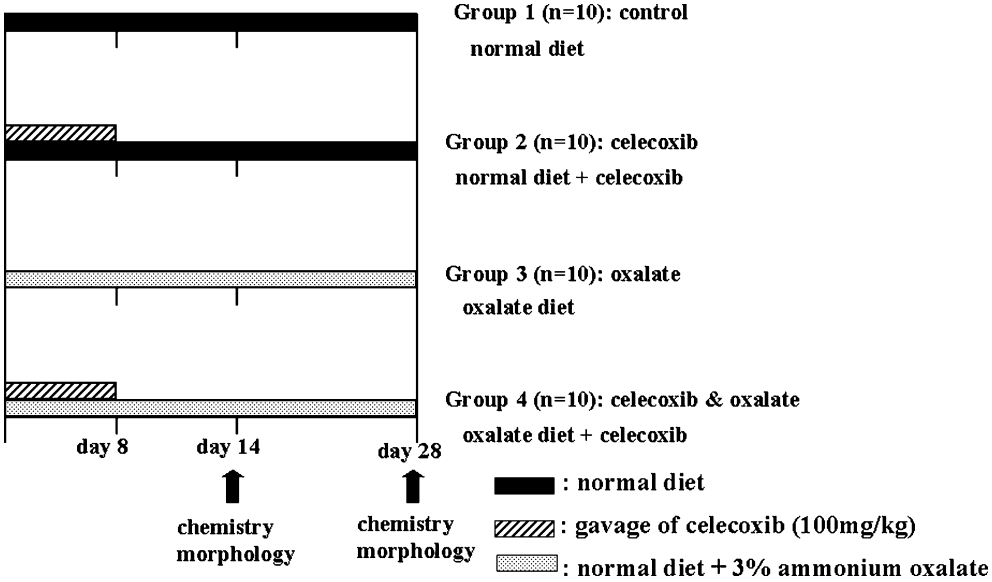

Fig. 1 Experimental paradigm. Normal Sprague-Dawley rats were divided into four groups: (1) control, (2) celecoxib, (3) oxalate, (4) celecoxib + oxalate. Group 1 was maintained on standard rat chow for the whole study. Group 2 received standard rat chow and 1 ml of normal saline mixed with celecoxib (100 mg/kg) by gavage for the first 8 days. Group 3 was fed standard rat chow supplemented with 3% ammonium oxalate. Group 4 rats were maintained on standard rat chow supplemented with 3% ammonium oxalate and each rat was administered celecoxib for the first 8 days. On days 14 and 28 of the experiment 24-h urine samples were collected and animals were sacrificed

Results

No statistical differences were found in terms of body weights or the amount of water drunk by rats in the four groups during the study period.

Morphological study of crystal formation

The crystals proved to be calcium oxalate by examination under polarized light. We defined a kidney with a score of ≥2 as a crystal-forming kidney and estimated the percentage crystal-forming kidneys in each group.

Few crystals were observed in group 4 after 14 days of experiment. After 28 days of experiment animals that received standard chow alone or in combination with celecoxib had no kidney crystals. The median crystal score of group 3 fed with 3% oxalate alone was 1 (range, 1–3), and 35% were crystal-containing. On the other hand, group 4, which received both celecoxib and oxalate, had a median crystal score of 2 (range, 1–4) and 65% of kidneys were crystal containing (Table 1).

24-h urine chemistry and enzyme results

Volumes of 24-h urine ranged from 20 to 30 ml across the experimental groups and was not significantly different between groups. Table 2 shows creatinine clearance results as a functional index, urine pH, and urinary excretion of oxalate, calcium, and citrate as stone forming parameters, and the activities of GGT and NAG as an index of renal tubular epithelial cell injury in the different groups after 28 days.

Animals in all groups retained a relatively normal renal function as judged with significant difference in creatinine clearance levels between the four groups. The urinary pH level was lower in groups 3 and 4, which were fed chows containing 3% ammonium oxalate, than groups fed standard chow, but this was not significantly different. In group 4 treated with celecoxib + oxalate, urinary activities of GGT and NAG were significantly elevated versus the control, which indicated the presence of renal tubular epithelial cell injury.

Groups administered ammonium oxalate alone or in combination with celecoxib showed an expected increase in the urinary excretion of oxalate, and a simultaneously marked decrease in calcium excretion, perhaps as a result of calcium oxalate crystal formation. The urinary excretion of citrate, a known inhibitor of stone formation, decreased in groups 2, 3, and 4 compared to group 1(control), but this was not statistically significant.

The values of parameters, indicating the severity of nephrotoxicity such as creatinine clearance and urinary activities of NAG and GGT in each experimental group were divided by the value of the parameters in each control group, and the present 14-day and the 28-day study compared with Kumar et al. (Table 3). There was no significant difference in creatinine clearance between the experimental groups in all the three studies. Urinary activity of GGT increased in groups administered with nephrotoxic agents compared with the control in Kumar et al. and the 28-day study, but was not significantly different between each experimental group in the present 14-day study. Urinary NAG activity increased in groups fed with oxalate in all three studies. While gentamicin administration in the Kumar et al. study increased greatly, the activity of urinary NAG, celecoxib administration in both of the present 14-day and 28-day studies did not show a significant increase in urinary NAG.

Microscopic examination of kidneys

Crystals were found almost exclusively within the papilla of kidneys of animals that were administered celecoxib and oxalate after 28 days (Fig. 2). A histological examination of the kidneys of animals treated celecoxib and oxalate showed crystals in the lumens of collecting tubules (Fig. 3).

Table 1 Crystal abundance within kidneys and the numbers of kidneys containing crystals after 28 days. Data are median and range (parentheses). Scoring system according to Kumar et al. [2]: 0 indicates no crystal, 1 few crystals (one or two per field), 2 moderate number of crystals (10–20 per field), 3 frequent crystals (≥20 per field), and 4 abundant crystals (> 100 per field)

Groups	Average crystal abundance	No. of crystal forming kidneys/No. of total kidneys (%)
Control	0 (0–1)	0/20 (0)
Celecoxib	0 (0–1)	0/20 (0)
Oxalate	1 (0–3)	7/20 (35)
Celecoxib + oxalate	2 (1–4)*	13/20 (65)*

*$P < 0.05$ relative to the oxalate group

Discussion

Urinary oxalate concentration is an important factor in calcium oxalate stone formation because changes in oxalic acid levels are known to affect crystallization more than the calcium level. Accordingly, many animal models of nephrolithiasis inducing hyperoxaluria have been developed [1, 13, 14].

Earlier, Randall revealed that the attachment of crystals to renal papillae is closely associated with tissue injury [15], and several investigators have demonstrated the association between injury to the tubular epithelium and deposition of crystals in renal tubules [16, 17]. In addition, many results have helped to establish the hypothesis that alterations in tubular function may play an important role in the pathogenesis of urolithiasis [18, 19, 20]. In particular, Kumar et al. showed that a treatment paradigm involving a combination of dietary

Table 2 Results of 24-h urine chemistry and enzymes after 28 days. Creatinine clearance: ml/min, calcium excretion: mg/24-h, oxalate and citrate: μmol/24-h, GGT and NAG: unit/mmole creatinine. Data are means ± SD

Parameters	Control	Celecoxib	Oxalate	Celecoxib + oxalate
Urinary pH	7.9 ± 0.58	7.8 ± 0.58	7.1 ± 0.85	7.0 ± 1.08
Creatine clearance	1.56 ± 0.40	1.9 ± 0.11	1.76 ± 0.38	1.55 ± 0.26
Calcium excretion	0.89 ± 0.29	1.66 ± 0.49	0.073 ± 0.036*	0.085 ± 0.019*
Oxalate excretion	17.8 ± 3.8	18.3 ± 2.5	53.8 ± 11.7*	49.0 ± 9.0*
Citrate excretion	17.5 ± 7.5	11.5 ± 7.6	12.5 ± 9.8	13 ± 6.3
GGT excretion	179.6 ± 39.3	301.7 ± 112.4*	194.2 ± 54.7	295.2 ± 42.5*
NAG excretion	2.31 ± 0.38	2.82 ± 0.90	4.52 ± 1.47*	4.47 ± 0.91*

* $P < 0.05$ relative to control

Table 3 The urinary nephrotoxic parameters of each group relative to control in the Kumar et al. study [2] and the present study. Each value is divided by the value of control. † Gentamycin was used in Kumar's study and celecoxib was used in this study as a nephrotoxic agent

Parameters	Study	Control	Nephrotoxic agent†	Oxalate	Nephrotoxic agent + oxalate
Creatine clearance (ratio to control)	Kumar et al. (14 days)	1	0.8	0.8	0.8
	Present study (14 days)	1	1.1	0.7	1.3
	Present study (28 days)	1	1.2	1.1	1
GGT excretion (ratio to control)	Kumar et al. (14 days)	1	3.8*	1.1	1.8*
	Present study (14 days)	1	1.1	0.8	1.1
	Present study (28 days)	1	1.7*	1.1	1.6*
NAG excretion (ratio to control)	Kumar et al. (14 days)	1	3.7*	2*	3.9*
	Present study (14 days)	1	1.5	2.6*	2.4*
	Present study (28 days)	1	1.2	2*	1.9*

* $P < 0.05$ relative to control

oxalate and gentamicin induced tubular injury in association with a higher frequency of stones in a shorter period than and without the significant renal toxicity of other treatment paradigms [2]. Accordingly, they developed an animal model of nephrolithiasis involving renal tubular injury.

The intestinal anaerobe *O. formigenes* is attracting attention because of its possible role in the regulation of the intestinal absorption of oxalate in humans. We reported an inverse correlation between intestinal *O. formigenes* and urinary oxalate levels in patients with calcium oxalate urolithiasis, and Kleinschmidt et al. reported that the colony forming units of *O. formigenes* per gram of feces are inversely correlated with the frequency of episodes of kidney stone formation. They also reported a complete absence of this bacterium in patients with four or more stone episodes [3, 4]. Campieri et al. revealed that the feeding of a mixture of freeze-dried lactic acid bacteria led to a significant reduction in the urinary excretion of oxalate in a group of patients with idiopathic calcium-oxalate urolithiasis and mild hyperoxaluria, suggesting that lactic acid bacteria may have an oxalate degrading activity [5]. Kajander et al. recently discovered a very small bacteria, nanobacteria, in human and bovine blood and in commercial blood products, and identified it as a possible infectious cause of kidney stones [6].

These ongoing studies require in vivo studies in a rat model of nephrolithiasis. However, the recent animal model of renal tubular injury based on gentamicin as developed by Kumar et al. cannot be applied to such studies because of the antimicrobial effect of gentamicin. This is why we developed this animal model using NSAIDs.

NSAIDs have an anti-inflammatory effect because they inhibit the cyclo-oxygenase (COX) pathway, by which various inflammatory mediators, prostaglandins, thromboxanes, and leucotriens are derived from phospholipids in cell membranes. On the other hand, prostaglandins are involved in many physiological actions other than inflammation, such as in the maintenance of gastric mucosal integrity and in the modulation of renal microvascular hemodynamics, renin release, and tubular salt and water reabsorption [21, 22, 23, 24]. Hence gastric toxic effects and nephrotoxicity are mandatory. In the 1990s it was recognized that two enzymes, COX-1 and COX-2, are involved in the cyclo-oxygenase pathway, and that the inhibition of COX-1 is related to the gastric side effects of NSAIDs [25, 26]. Thus, COX-2 selective inhibitors, which have few G-I toxic effects, were developed.

In this study, we selected celecoxib, which has few gastrointestinal side effects, as a substitute for gentamicin. No information is available on the simultaneous administration of celecoxib and oxalate in rats. In particular, few studies have been conducted on the dose-dependent nephrotoxicity of celecoxib in rats. One study on gastrointestinal damage induced by celecoxib, however, did report that the administration of a very high dose of celecoxib (200 mg/kg) for 5 days by gavage in

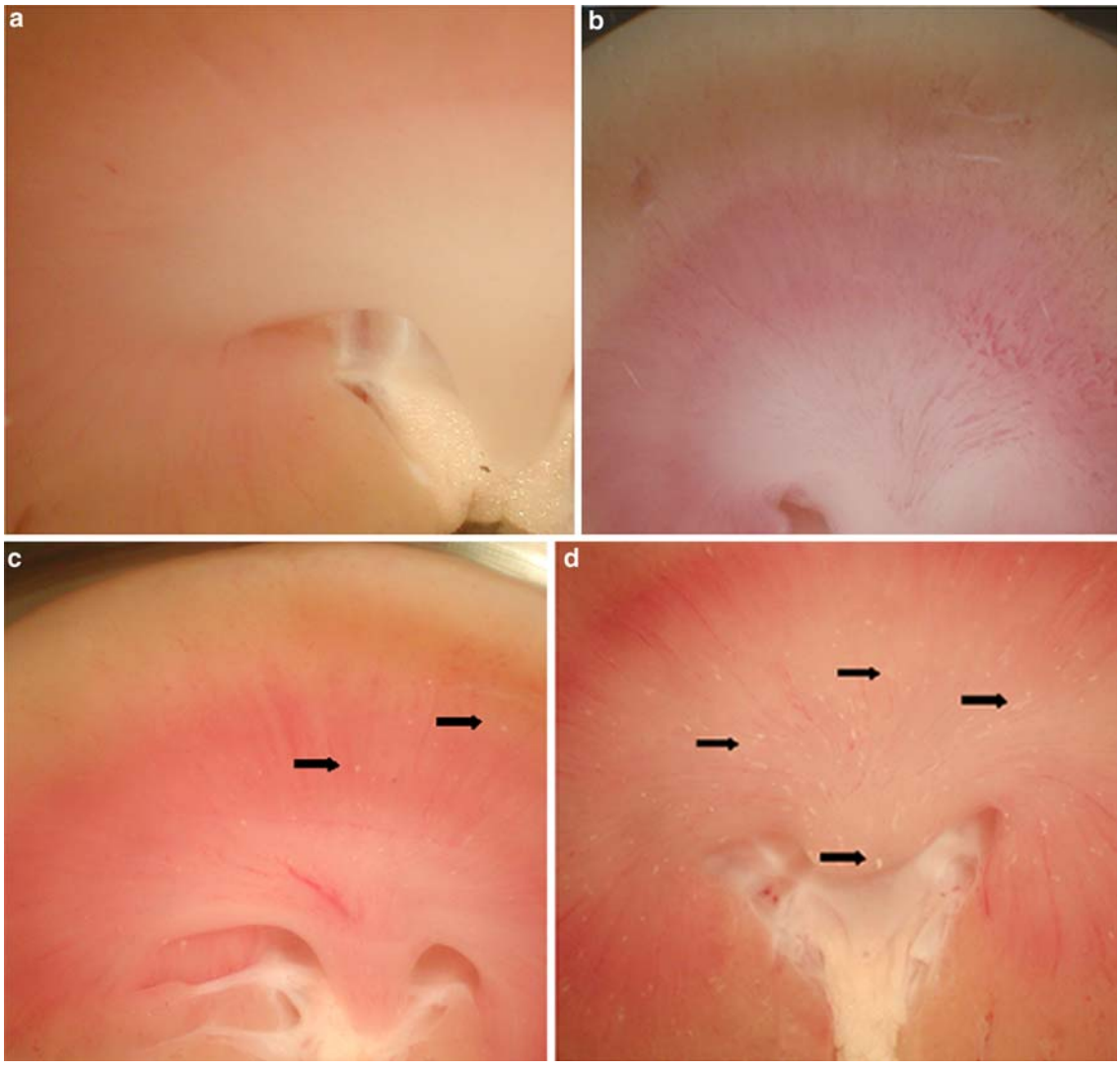

Fig. 2 Crystals were identified under the dissecting microscope after kidney bisection at 28 days. **A**, **B** Control and celecoxib groups, respectively. No crystals were found in the kidneys. **C** The oxalate group; several crystals were observed. **D** The celecoxib + oxalate group; more crystals were observed at the medulla in the kidneys compared to other groups. *Arrows* indicate crystals

rats did not lead to any ulcers in the stomach or intestine [27].

Celecoxib administration increased the excretion of the urinary enzymes GGT (brush border enzyme) and NAG (a lysosomal enzyme), but had no effect on oxalate or citrate excretion. The present model, based on the administration of celecoxib and oxalate, shows elevated 24-h urine oxalate and decreased calcium, suggesting hyperoxaluria due to oxalate feeding and simultaneous hypocalciuria due to calcium oxalate crystal formation.

The present model is similar to that of Kumar et al. in as much as both are based on the hypothesis that renal tubular injury promotes urinary stone formation. In fact, we observed renal tubular injury as evidenced by elevated urinary GGT and NAG excretion in our model. In addition, the patterns of creatinine clearance, urine pH, and the urinary excretion of oxalate, calcium, and citrate are similar for the two models. But the extent of hypocalciuria in the gentamicin model is less severe than in the present model because gentamicin induces hypercalciuria [28].

Our study has some limitations because, first, the mechanism of a COX-2 inhibitor induced renal injury is little known. Secondly, Lieske and colleagues suggest that renal prostaglandins may inhibit crystal attachment to renal epithelial cells [29]. Thus, it is possible that COX-2 inhibition has an inhibitory effect on prostaglandin production, provoking crystal attachment to renal epithelial cells. In addition to the nephrotoxic effect, the other effects of COX-2 inhibition still require study. Last, although we chose the COX-2 inhibitor in order to avoid the bactericidal effect of gentamicin in our stone forming animal model involving renal tubular injury, COX-2 inhibitor might affect the behavior of *Oxalobacter*, *Lactobacillus*, and nanobacteria. Thus, it should be considered as to whether COX-2 inhibitor has an impact on the bacteria species. We have already performed a pilot study on the probiotic effects of

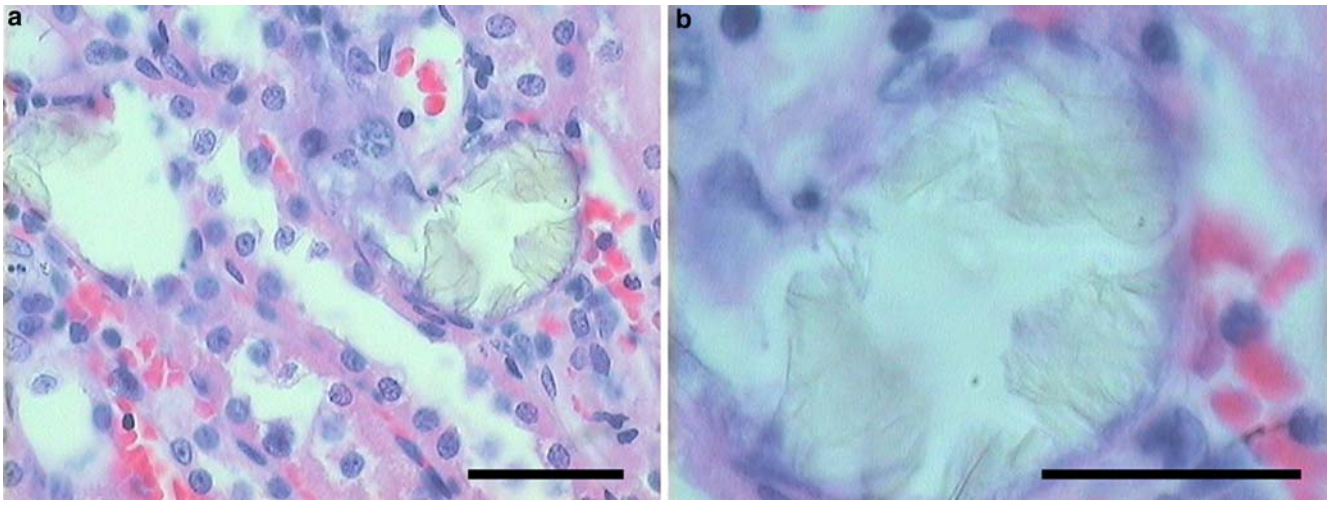

Fig. 3 Photomicrograph of a hematoxylin and eosin stained kidney of the celecoxib + oxalate group after kidney bisection at 28 days. **A** Crystals obstructing the lumen of collecting ducts at low power (250×). Tubules appear normal. **B** Calcium oxalate crystals at high power (400×). The size of each *bar* is 10 μm

Lactobacillus using this model. In this study, *Lactobacillus* was well colonized in the stool of rats administered with COX-2 inhibitor and oxalate.

When the kidneys of rats administered celecoxib and oxalate were bisected and observed after 2 weeks of treatment, few crystals were seen. This suggests that the time required for crystal formation in the present model is greater than 2 weeks. However, Kumar et al. model produced crystals in only 2 weeks. The comparison of urinary nephrotoxic parameters between Kumar et al. (14 days), the present 14-day, and the present 28-day study suggest that gentamicin is more nephrotoxic than celecoxib and that the time to nephrotoxicity induced by celecoxib is longer time than for gentamicin.

Some studies have reported that urine NAG activity is a measure of the altered function in the renal tubules and not simply an indicator of damage [30]. In the Kumar et al. study, both urinary GGT and NAG activity increased with gentamicin administration, but in the present study mainly urinary GGT activity increased with celecoxib, implying that celecoxib is less likely to alter renal tubular function compared with gentamicin. These results suggest that gentamicin and celecoxib have different nephrotoxic mechanisms and nephrotoxicities.

The site of crystal formation and the existence of papillary plaques are critical points for stone-forming animal models. Our model is similar to that of Kumar et al. in terms of both the location of crystal formation and the abundance of papillary plaques.

Irrespective of its precise mode of action, it is significant that combining an oxalate diet with celecoxib treatment produced more crystals than an oxalate diet alone. In addition, the present model had relatively little deleterious effect on the growth rate or normal renal function in experimental animals.

The present study indicates that the administration of oxalate and the induction of renal tubular damage by celecoxib forms crystals in kidneys more abundantly than oxalate feeding alone. In addition, our data suggest that this model may be useful for studying bacteria as a causative factor in renal stone formation.

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