Urol Res (2008) 36:139–147
DOI 10.1007/s00240-008-0143-7

ORIGINAL PAPER

Glyoxylate induces renal tubular cell injury and microstructural changes in experimental mouse

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Received: 28 December 2007 / Accepted: 21 May 2008 / Published online: 10 June 2008

Abstract Crystal formation in mice could not be induced either by the administration of ethylene glycol or by glycolate. To clarify the reasons for the difference among these oxalate precursors in mice, we studied renal tubular epithelial injury by immunohistochemical staining of oxidative stress and observing microstructures. Daily intra-abdominal injection of saline solution [10 ml/(kg day)], ethylene glycol[(48.3 mmol/(kg day)], glycolate [1.31 mmol/(kg day)], and glyoxylate [1.35 mmol/(kg day)] into C57BL/6 male mice (8 weeks) was performed for 7 days. Immunohistochemical staining of superoxide dismutase (SOD) and malondialdehyde (MDA), and transmission electron microscopy (TEM) of renal tubular epithelial cells were performed to observe oxidative stress and morphological changes, respectively. Decreased SOD and increased MDA were shown only in glyoxylate-treated mouse kidneys. The TEM study with glyoxylate-treated mouse kidneys demonstrated that the internal structure of mitochondria in renal tubular cells underwent destruction and vacuolization, and microvilli density decreased. These changes in renal tubular cells were located in the crystal-forming area. However, such changes were not detected in the other groups. Each precursor of oxalate induces different changes in renal epithelial cells regarding oxidative stress and the microstructural changes. It is suggested that calcium oxalate crystal formation requires cell injury and morphological changes of renal epithelial tubular cells induced by glyoxylate administration in the mouse kidney.

Keywords Cell injury · Oxidative stress · Crystal formation · Renal tubules · Oxalate precursors

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Introduction

Renal stones are produced by various environmental and genetic factors. The pathogenesis of renal stone formation can be examined by various methods such as using renal tubule cultured cells or laboratory animals. In the recent studies, rats were used as a renal stone model [1, 2]. To investigate the function of the objective gene, a transgenic mouse study, including a knockout mouse, is very useful. Several studies indicated the function of stone-related protein using a knockout mouse. Previously, renal stone formation was induced by ethylene glycol administration in osteopontin knockout mice and Tamm–Horsfall glycoprotein knockout mice, but not normal mice [3, 4]. Recently, we established renal calcium oxalate formation in mice by the administration of glyoxylate, a precursor of oxalate [5]. In rats, renal calcium oxalate crystals were formed easily by oral treatment of ethylene glycol and glycolate, other oxalate precursors [6–8]. However, our previous study showed that calcium oxalate crystals were not formed by oral treatment or intraperitoneal injection with ethylene glycol and glycolate in mice [5].

Renal epithelial cell injury is regarded as a major risk factor for crystallization and crystal formation in the kidney

[9–12]. It has been reported that glyoxylate caused more severe cell injury than ethylene glycol in vitro [13]. Also, the administration of an antioxidant (citric acid, vitamin E, etc.) inhibited cell injury and renal stone formation [14–18]. Sublethal injury provides sites for crystal attachment to injured cell membranes [10, 19]. Many current models of calcium oxalate stone disease suggest that the generation of reactive oxygen species (ROS) and subsequent lipid peroxidation is an integral part of the process [20–22]. We thought that the difference in calcium oxalate formation between these oxalate precursors depended on cell injury due to oxidative stress.

In this study, we examined epithelial cell injury and oxidative stress in mice with calcium oxalate crystal formation induced via the administration of glyoxylate, and non-calcium oxalate crystal formation with the administration of ethylene glycol and glycolate, using immunohistochemical staining and TEM.

Materials and methods

Animals

All animal studies followed the recommendations of the NIH Guide for the care and use of laboratory animals. The experimental animals (purchased from Charles River Japan, Yokohama, Japan) were C57BL/6 mice weighing 18–22 g (8 weeks old). The mice were placed in metabolic cages for 3 days for acclimation before beginning the experiment. The ambient temperature was maintained at 23°C ± 1 on a 12-h light/dark cycle. All animals had free access to standard chow (including calcium: 1.12 g, phosphorus: 0.9 g, magnesium: 0.26 g, and sodium: 0.21 g/100 g; Oriental Yeast, Tokyo, Japan) and water.

Experimental protocols

The animals were divided into four groups: control group (isotonic sodium chloride solution-treated group: $N = 7$), ethylene glycol (Wako, Tokyo, Japan)-treated group ($N = 7$), glycolate (Wako, Tokyo)-treated group ($N = 7$) and, glyoxylate (Wako, Tokyo)-treated group ($N = 7$). All prepared oxalate precursors and isotonic sodium chloride solution were kept at 4°C until administration. Intra-abdominal injection was performed according to the weight of each mouse with a clean 27-gauge needle every day. Mice were administered each type of oxalate precursor and isotonic sodium chloride solution with a daily intra-abdominal injection for 7 days, as described in the previous study [5] [0.9% isotonic sodium chloride solution: 10 ml/(kg day), ethylene glycol: 3,000 mg/(kg day) (48.3 mmol/(kg day)), glycolate: 100 mg/(kg day) (1.31 mmol/(kg day)), and glyoxylate: 100 mg/(kg day) (1.35 mmol/(kg day))]. Twenty-four-hour urine was collected on day 7 after the administration of oxalate precursors, and kidneys were extracted.

Detection of renal calcium oxalate crystal formation

The extracted kidneys were fixed in 4% paraformaldehyde, and embedded in paraffin. Four-micrometer-thick cross-sections were stained using the previously described Pizzolato staining method [23] to detect oxalate-containing crystals. Briefly, paraffin sections were dewaxed and washed in distilled water following the usual method. A mixture of 1 ml each of 30% hydrogen peroxide and 5% silver nitrate (pH 6.0) was made, and poured onto the slide with tissue sections. The slide was exposed to light from a 60-W incandescent lamp at a distance of 15 cm (6 in.) for 15–30 min. Numerous gas bubbles developed, and it was necessary to pour off the mixture and add a fresh solution. The slide was then washed thoroughly with distilled water and counterstained with safranin, and then dehydrated in the usual manner. For the quantification of calcium oxalate crystal deposition area, Pizzolato positive-staining regions were measured and expressed as percentages of the total tissue area of cross-sections of the kidney with soft NIH image 1.61 (Scion Inc., USA). The percentage of calcium oxalate crystal deposition area is shown as the mean ± SD.

Urine biochemistry

Twenty-four-hour urine was sampled in two ways: normal urine collection and acidified urine collection with the addition of 20 µl of 1 M hydrochloric acid. The collected urine was stored at −4°C until analysis. Twenty-four-hour oxalate excretion was measured in acidified urine by capillary electrophoresis (SRL Inc., Tokyo, Japan). The excretion of citrate, calcium, phosphorus, magnesium, creatinine for 24 h and urinary volume of 24 h were measured in normal urine (SRL Inc.).

Immunohistochemical staining

For the estimation of oxidative stress, immunohistochemical staining of superoxide dismutase (SOD) was performed. For the estimation of renal tubular epithelial cell injury, immunohistochemical staining of malondialdehyde (MDA) was conducted. Sections were treated with microwave processing for 15 min, blocked with 0.5% H_2O_2 in methanol for 30 min, and washed several times in 0.01 M phosphate-buffered saline (PBS). PBS, with added skimmed milk, was reacted with sections at room temperature for 1 h.

For SOD detection, these slides were incubated with anti-SOD-1(C-17) goat polyclonal antibody (Santa Cruz Biotechnology Inc., CA, USA) at 4°C overnight. The

reacted antibody was detected using anti-goat IgG VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's instructions.

To detect MDA, these slides were incubated with anti-malondialdehyde monoclonal antibody (NOF Co., Tokyo, Japan) at 4°C overnight, and the reacted antibody was detected using an anti-mouse IgG M.O.M.™ Kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions.

Observation of microstructure

The microstructure was observed with TEM [24, 25]. Perfusion fixation was performed with 20 ml of 0.1 M phosphoric acid buffer, and 20 ml of 2.5% glutaraldehyde, and kidney tissue was extracted. Kidney tissue was washed with phosphoric acid buffer, followed by fixation with 2% osmium liquid for 2 h. Dehydration was performed with a series of ethanol from 50 to 100%. Renal tissue was embedded in epoxy resin. Polymerism was performed at 60°C for 48 h. After making a super slice (99 nm), double staining with uranium and lead was performed and observed by JEM-1011 (JEOL Ltd, Tokyo, Japan).

Statistical analysis

The urine biochemistry examination shows the mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test with SPSS 10.0 for Windows statistical software package (SPSS Inc., Chicago, IL, USA). A probability of 0.05 was regarded as significant.

Results

Confirmation of renal calcium oxalate crystal formation

Calcium oxalate crystal formation was detected in renal distal tubular cells in glyoxylate-administrated mice. In the glyoxylate-treated group, the lumen of the renal tubules were extended, the renal tubular cells became flat, and partly collapsed. Calcium oxalate crystal formation and these changes were not detected in the control, ethylene glycol-treated, or glycolate-treated groups (Fig. 1; Table 1). These results support those of our previous study [5].

Urine biochemistry

Urinary parameters on day 7 are shown in Table 1. Urinary oxalate excretion in the ethylene glycol-treated group increased to about two times in comparison with the control group, respectively. Urinary oxalate excretion in the glycolate- and glyoxylate-treated groups was similar to that in the control group. Urinary citrate excretion in the glyoxylate-treated group was tendency to decrease, but not significantly. Urinary citrate excretion in the ethylene glycol- and

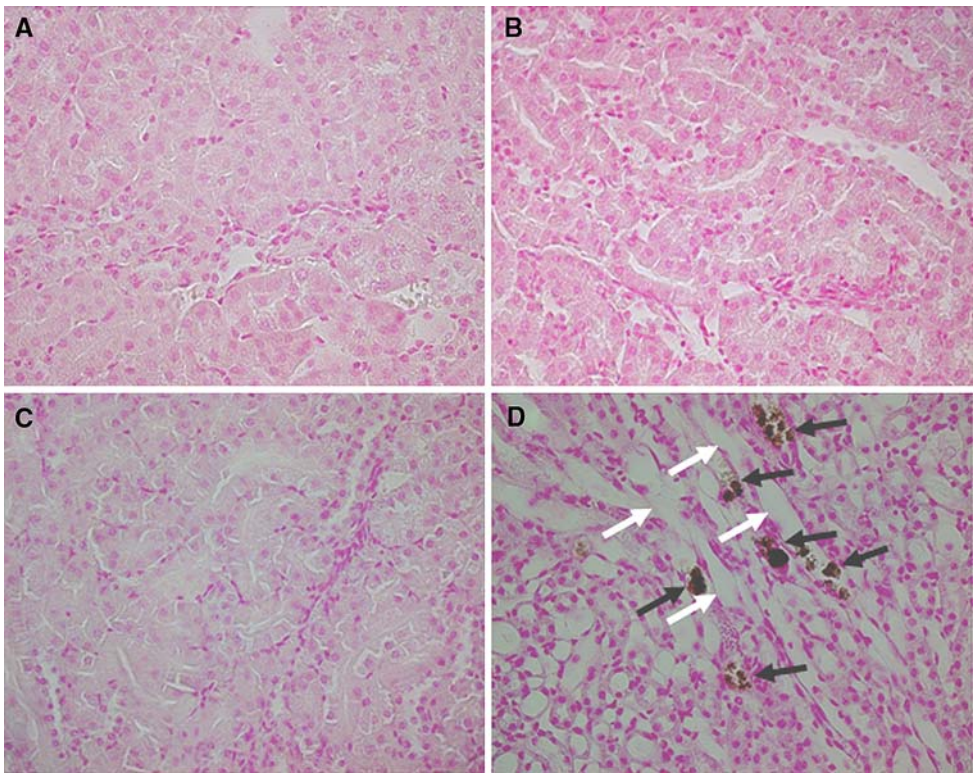

Fig. 1 Detection of renal calcium oxalate crystal formation by Pizzolato staining. **a** Control group, **b** ethylene glycol-treated group, **c** glycolate-treated group, **d** glyoxylate-treated group. Calcium oxalate crystal formation (*black arrows*) was detected in distal tubular cells only in the glyoxylate-treated group (**d**). And, the lumen of the renal tubules were extended, the renal tubular cells became flat, and partly collapsed (**d**), (*white arrows*). Crystal formation and these changes were not detected in the control (**a**), ethylene glycol-treated (**b**), nor glycolate-treated (**c**) groups (magnification ×400)

Table 1 Urine Biochemistry

	Control ($n = 7$)	Ethyleneglycol ($n = 7$)	Glycolate ($n = 7$)	Glyoxylate ($n = 7$)	P value
24-h urine biochemistry (mg/24 h)					
Oxalate	15.45 ± 4.92	32.98 ± 4.71	9.93 ± 1.02	15.38 ± 1.80	0.033*
Citrate	80.07 ± 15.90	122.04 ± 73.68	69.06 ± 20.01	42.98 ± 37.03	0.293
Calcium	3.23 ± 1.21	8.57 ± 4.79	2.89 ± 2.56	3.46 ± 1.71	0.248
Magnesium	2.34 ± 3.34	18.76 ± 13.71	0.26 ± 0.09	0.25 ± 0.08	0.070
Phosphorus	97.96 ± 28.88	150.51 ± 70.39	80.52 ± 16.33	102.31 ± 14.18	0.392
Creatinine	18.25 ± 3.26	24.01 ± 11.83	14.29 ± 3.91	15.27 ± 4.46	0.546
Urine volume (ml)	0.84 ± 0.18	2.15 ± 0.87	0.84 ± 0.18	1.51 ± 0.31	0.038*
Calcium oxalate Crystal deposition area (%)	None	None	None	0.59 ± 0.08	0.001*

* $P < 0.05$, (P values indicate the significance of changes in urine parameters measured in the control and each agent—administrated groups)

glycolate-treated groups showed no change. Urinary calcium, phosphorus, magnesium and creatinine excretion in all treated groups showed no significant change. Urinary volume in the glyoxylate-treated group increased about two times in comparison with the control group. In the ethylene glycol-treated group, urinary volume was tendency to increase, and glycolate-treated group showed no change.

Cell injury, oxidative stress

In immunohistochemical staining of SOD, in the control group, we recognized SOD expression of proximal and distal renal tubular epithelial cells in the renal medulla, and weak expression by proximal and distal renal tubular epithelial cells in the renal cortical region. However, in the glyoxylate-treated group, SOD expression of both proximal and distal renal tubular epithelial cells in the medulla was not detected, but a weak expression was noted by both the proximal and distal renal tubular epithelial cells in the border of the medulla to the cortical region. In the ethylene glycol- and glycolate-treated groups, SOD expression of both the proximal and distal renal tubular epithelial cells in the renal medulla and cortical region showed no change in comparison with the control group (Fig. 2).

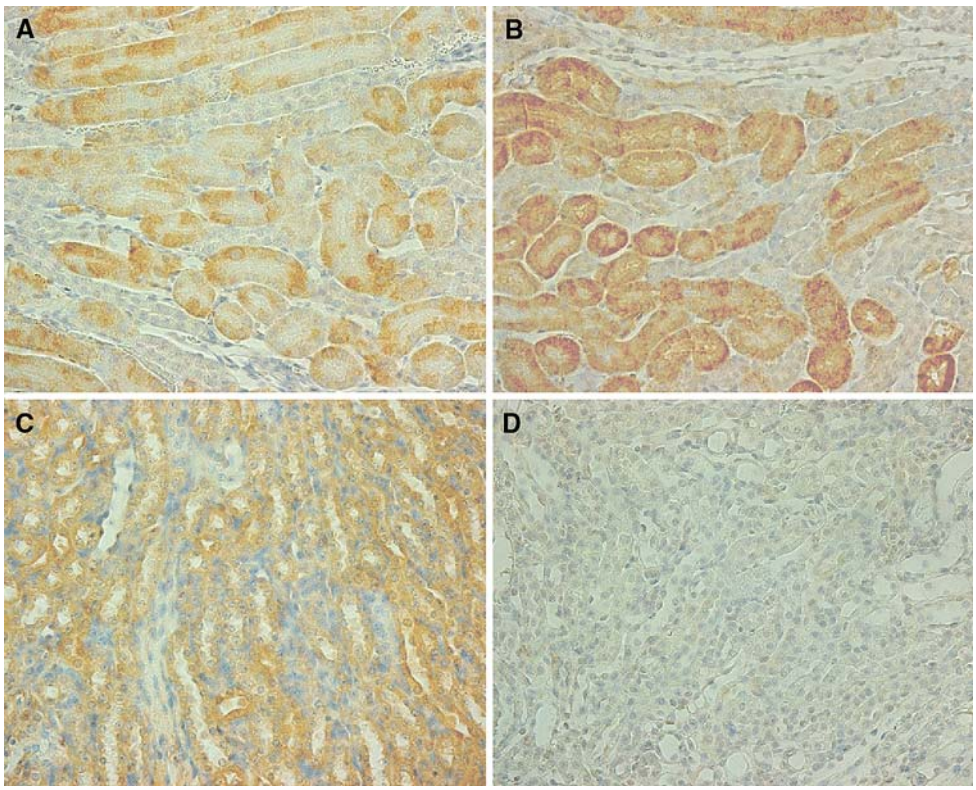

Fig. 2 Immunohistochemical staining of SOD. **a** Control group, **b** ethylene glycol-treated group, **c** glycolate-treated group, **d** glyoxylate-treated group. In the control group, SOD expression was recognized in both the proximal and distal renal tubular epithelial cells (**a**). SOD expression in both the proximal and distal renal tubular epithelial cells decreased in the glyoxylate-treated group in comparison with the control group (**d**). In the ethylene glycol-treated (**b**) and glycolate-treated (**c**) groups, SOD expression in both the proximal and distal renal tubular epithelial cells showed no change in comparison with the control group (magnification ×400)

In immunohistochemical staining of MDA, in the control group, MDA expression was not seen anywhere in the kidney. In the glyoxylate-treated group, MDA expression was observed with the expanded and disordered renal tubular epithelial cells in the renal medulla. The expanded and disordered renal tubular epithelial cells were not identified as to whether they were proximal or distal. In the ethylene glycol- and glycolate-treated groups, MDA expression was not noted, the same as in the control group (Fig. 3).

Observation of microstructure

In the control group, the internal structure of mitochondria was regular. The membranes of mitochondria also showed a clear continuity and a double membrane structure. The internal mitochondrial structure was changed 7 days after glyoxylate administration. The double membranes of mitochondria became indistinctive and discontinuous, and partly collapsed. The shape of mitochondria changed, resembling fat droplets. However, in the ethylene glycol- and glycolate-treated groups, these particular changes in the internal structure of mitochondria in renal tubule cells were not recognized (Fig. 4). Microvilli of renal tubule cells in the control group were tall and regular, the cells were thick, and the nucleus was circular. There were not recognized anything in the lumen of the renal tubules. In the glyoxylate-treated group, the microvilli of renal tubule cells decreased, the renal tubular cells became flat, and the nucleus was not a perfect circle, but these changes did not occur in the ethylene glycol- and glycolate-treated groups (Fig. 5).

Discussion

For decades, investigations into the pathogenesis of renal stone formation have been performed using the hyperoxaluric rat model, in which free drinking of ethylene glycol by rats is effective for renal stone formation. However, such a method cannot improve consecutive transgenic animal investigations including knockout mice, for the reason that gene recombinant techniques have not been established in rats.

Recently, we performed a preliminary examination using mice administered several types of oxalate precursor, and clarified that an intraabdominal injection of glyoxylate was effective for renal crystal formation, but the administration of ethylene glycol and glycolate could not produce the calcium oxalate crystal formation [5]. In SD rats, Khan detected a calcium oxalate crystal on the oral administration of ethylene glycol for 8 weeks, but, in C57BL/6 mice, I could not observe a calcium oxalate crystal by either the oral or intra-abdominal administration of ethylene glycol for 4 or 8 weeks [5]. We performed the intra-abdominal administration of ethylene glycol for 8 weeks in C57BL/6 mice again, but no calcium oxalate crystal was detected.

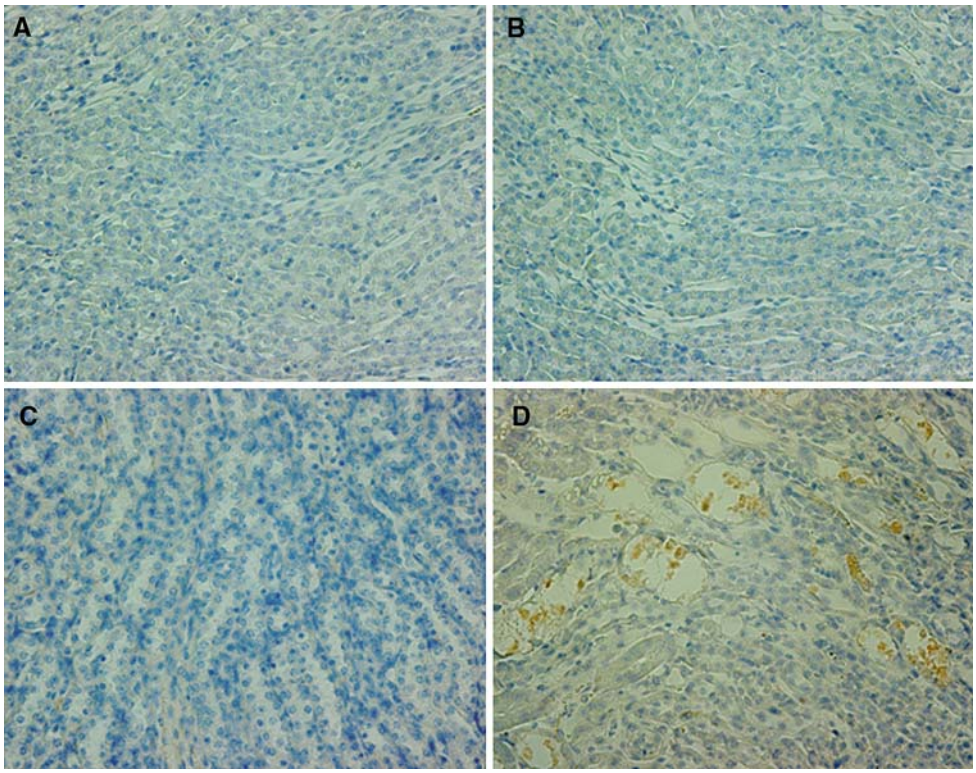

Fig. 3 Immunohistochemical staining of MDA. **a** Control group, **b** ethylene glycol-treated group, **c** glycolate-treated group, **d** glyoxylate-treated group. In the control group, no MDA expression was seen in the kidney (**a**). In the glyoxylate-treated group, MDA expression was observed in expanded and disordered renal tubular epithelial cells in the renal medulla (**d**), but not in the ethylene glycol-treated (**b**), nor glycolate-treated (**c**) groups. The expanded and disordered renal tubular epithelial cells were not identified as to whether they were proximal or distal (magnification ×400)

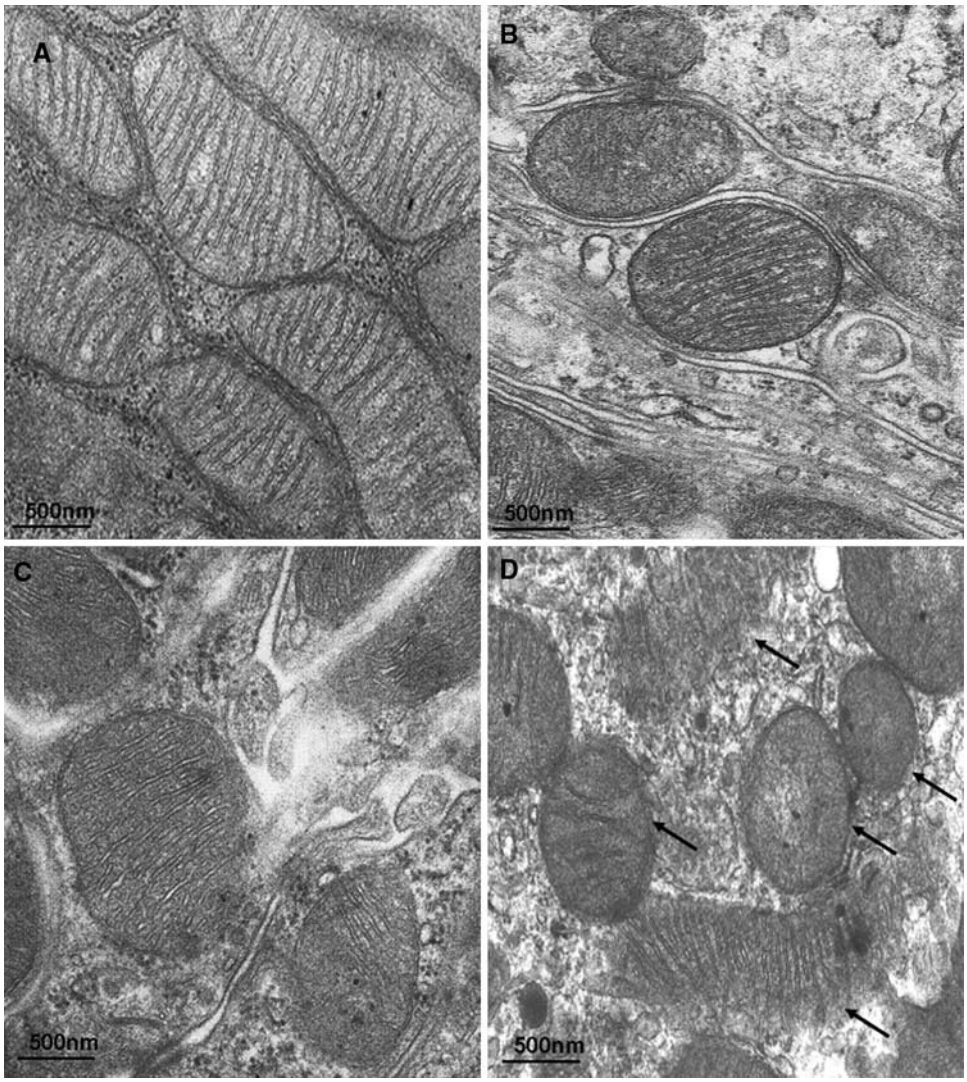

Fig. 4 Microstructure of mitochondria. **a** Control group, **b** ethylene glycol-treated group, **c** glycolate-treated group, **d** glyoxylate-treated group. In the control group, the internal structure of mitochondria was regular. The membranes of mitochondria showed clear continuity, with a double membrane structure (**a**). In the glyoxylate-treated group (**d**), the arrangement of the internal structure of mitochondria was disordered, and the double membranes became indistinct, partly disrupted, lost continuity, and collapsed (*black arrows*). The other groups did not show collapsed mitochondria (magnification ×15,000)

We confirmed that the administration of ethylene glycol to C57BL/6 mice does not lead to the formation of calcium oxalate crystals. This experimental renal calcium oxalate crystal mouse model has some interesting characteristics. There were differences in renal stone formation between administrations of glyoxylate and other agents (ethylene glycol and glycolate). Though ethylene glycol induced hyperoxaluria, ethylene glycol administration did not lead to crystal formation. The tendency for urinary citrate and magnesium levels to decrease in the glyoxylate-administrated mouse might cause crystal formation by decreasing the ability to inhibit it. We hypothesized that the reason depended on renal tubular epithelial cell injury of oxalate precursors, not only on urinary oxalate excretion.

We investigated the differences of SOD and MDA expression in the mouse kidney among the oxalate precursors administered. SOD is a free radical scavenger with activity to convert O_2^- to H_2O_2 to weaken the toxicity of free radicals [26, 27]. MDA is the terminal product of the arachidonate cascade, which is activated by chemical or physical stimulation of the cell membrane [16, 28]. MDA does not participate directly in crystal formation. Decreased SOD and increased MDA are often used as indexes of cell viability on oxidative stress and cell damage [16, 26–29]. In this study, decreased SOD and increased MDA were observed in glyoxylate-administrated mouse renal tissue, but not in ethylene glycol- and glycolate-administrated mouse renal tissue. In addition, SOD and MDA expression in the mouse kidney following the intra-abdominal injection of ethylene glycol for 8 weeks did not show any changes in comparison with the control group (data not shown). This study suggested that renal epithelial cell injury is essential for calcium oxalate crystal formation.

The present study using TEM showed mitochondrial changes in renal tubular epithelial cells in glyoxylate-administrated mice. Mitochondria, as sites of aerobic metabolism, are likely to be a major source of intracellular ROS [30, 31], and cellular injury or stress often increases

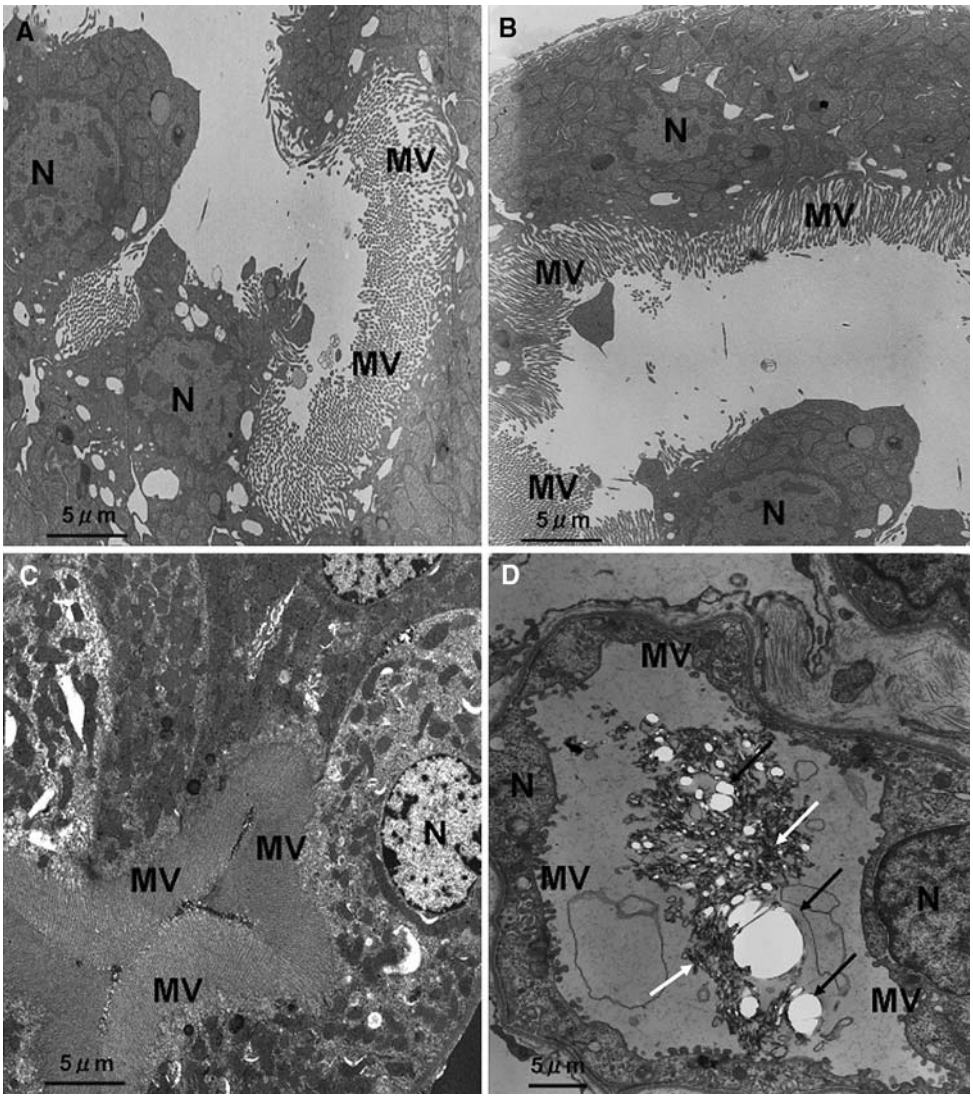

Fig. 5 Microstructure of renal tubular cells. **a** Control group, **b** ethylene glycol-treated group, **c** glycolate-treated group, **d** glyoxylate-treated group. In the control group, microvilli of renal tubule cells were tall and regular, renal tubule cells were thick, and the nucleus was circular. Materials were not recognized in the lumen of renal tubules (**a**). In the glyoxylate-treated group, microvilli of renal tubule cells decreased, the renal tubular cells became flat, and the nucleus was not a perfect circle. Around the area which seemed to be a calcium oxalate crystal in the renal tubule lumen, some mitochondria (*white arrow*) were shown. The mitochondria had a disrupted internal structure. Also, the double membranes of mitochondria became indistinct (**d**). However, these changes were not detected in the ethylene glycol-treated (**b**) nor glycolate-treated (**c**) groups. *Black arrow* shows that calcium oxalate crystal deposition may have been present, but fell out at dislodged from 99 nm slices (magnification ×6,000). *N* nucleus, *MV* microvilli

ROS production by mitochondria [32, 33]. Indeed, mitochondria appear to be a major source of the superoxide produced in MDCK cells following exposure to oxalate in its soluble or crystalline form, since inhibitors of the mitochondrial proton gradient also inhibited oxalate-induced superoxide generation [34]. Mitochondria seemed to be injured or changed as a result of ROS generated by glyoxylate. TEM observation showed the destruction of microvilli, transformation of intracellular organelles, and intra-tubular aggregation of the collapsed structure from the destruction of mitochondria and microvilli of renal tubular cells. In the calcium oxalate crystal formation rat model, Khan et al. [35, 36] recognized the collapse of microvilli and the proximal urinary duct. We considered these disintegrated materials in the tubular lumens as cores and starting points of renal stone formation in the mouse kidney.

Our study suggested that the cause of differences in calcium oxalate crystal formation with oxalate precursors depends on the differences in cell injury. The reason for cell injury due to glyoxylate administration seems to be that glyoxylate is more toxic than ethylene glycol and glycolate. Poldelski et al. [13] showed that glyoxylate and glycoaldehyde in oxalate precursors induce severe cell injury, whereas ethylene glycol and glycolate do not. It has also been reported that glyoxylate impairs ATP generation within isolated mitochondria through selective blockade within the citric acid cycle in cell toxicity [13, 37]. The renal tubular epithelial cell changes observed by TEM in a calcium oxalate crystal model rat administered ethylene glycol [35, 36] were similar to the changes noted in this study in mice administered glyoxylate. It is suggested that calcium oxalate crystal formation requires renal tubular epithelial cell injury [14–18]. In the rat, the oral administration of ethylene glycol led to calcium oxalate crystals, but in the mouse, the oral and intra-abdominal administration of ethylene glycol did not lead to the formation of calcium oxalate crystals. A possible reason may be that the metabolic pathway for oxalate precursors in mice is different

from that in rats, and the mouse may have a defense mechanism against cell injury.

In conclusion, calcium oxalate crystal formation was detected on glyoxylate administration, but not on ethylene glycol and glycolate administration. This study suggests that not only hyperoxaluria but also cell injury is important in the formation of calcium oxalate crystals.

Acknowledgments We would like to thank Ms. N. Kasuga, Ms. A. Hayashi and Ms. Y. Kobayashi for their expert management of mice and secretarial assistance. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Science and Technology (No. 17591691, No. 18209050, No. 18791132).

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