Hyperoxalemia Leads to Oxidative Stress in Endothelial Cells and Mice with Chronic Kidney Disease

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Keywords

End-stage renal disease · Hyperoxalemia · Vascular calcification · Oxidative stress

Abstract

Introduction: Cardiovascular disease is the most common cause of morbidity and mortality in patients with ESRD. In addition to phosphate overload, oxalate, a common uremic toxin, is also involved in vascular calcification in patients with ESRD. The present study investigated the role and mechanism of hyperoxalemia in vascular calcification in mice with uremia. **Methods:** A uremic atherosclerosis (UA) model was established by left renal excision and right renal electrocoagulation in apoE^{-/-} mice to investigate the relationship between oxalate loading and vascular calcification. After 12 weeks, serum and vascular levels of oxalate, vascular calcification, inflammatory factors (TNF- α and IL-6), oxidative stress markers (malondialdehyde [MDA], and advanced oxidation protein products [AOPP]) were assessed in UA mice. The oral oxalate-degrading microbe *Oxalobacter formigenes* (*O. formigenes*) was used to evaluate the effect of a reduction in oxalate levels on vascular calcification. The mechanism underlying the effect of oxalate loading on vascular calcification was assessed in cultured human aortic en-

dothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs). **Results:** Serum oxalate levels were significantly increased in UA mice. Compared to the control mice, UA mice developed more areas of aortic calcification and showed significant increases in aortic oxalate levels and serum levels of oxidative stress markers and inflammatory factors. The correlation analysis showed that serum oxalate levels were positively correlated with the vascular oxalate levels and serum MDA, AOPP, and TNF- α levels, and negatively correlated with superoxide dismutase activity. The *O. formigenes* intervention decreased serum and vascular oxalate levels, while did not improve vascular calcification significantly. In addition, systemic inflammation and oxidative stress were also improved in the *O. formigenes* group. In vitro, high concentrations of oxalate dose-dependently increased oxidative stress and inflammatory factor expression in HAECs, but not in HASMCs. **Conclusions:** Our results indicated that hyperoxalemia led to the systemic inflammation and the activation of oxidative stress. The reduction in oxalate levels by *O. formigenes* might be a promising treatment for the prevention of oxalate deposition in calcified areas of patients with ESRD.

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Introduction

Cardiovascular disease (CVD) is the most common cause of morbidity and mortality in patients with CKD and ESRD [1]. Vascular calcification induced by hyperphosphatemia has been suggested to be a crucial factor in the pathophysiology of CVD [2]. However, it does not fully explain the extremely high rate of vascular calcification in patients with ESRD, and other factors that might be involved [3, 4]. Uremic toxins produced by disturbances in the intestinal flora, including sulfuric acid, cresol, indole, and oxalate, were indicated to be associated with a high CVD incidence rate and high mortality rate in patients with ESRD [5, 6].

Oxalate is one of the small-molecule toxins retained in patients with ESRD. A positive linear correlation was observed between serum oxalate and creatinine levels in patients with ESRD, and oxalate is difficult to remove sufficiently by conventional hemodialysis [7, 8]. The normal range of serum oxalate levels is approximately 1–5 $\mu\text{mol/L}$, while serum oxalate levels in patients with ESRD are 4–78 times the normal limits, even as high as 200 $\mu\text{mol/L}$ [9]. Gulhan et al. [10] reported that serum oxalate levels in hemodialysis patients are positively correlated with the pulse wave conduction velocity, central artery systolic blood pressure, and diastolic blood pressure, which were independent predictors of increased cardiovascular morbidity and mortality [10]. Furthermore, oxalate deposits were observed in atherosclerotic plaques during autopsies of patients with atherosclerosis [11]. In vivo, a uremic mouse model was established in C57BL/6 mice by providing a high oxalate diet for 3 weeks. The arterial pressure was significantly increased, accompanied by significant cardiac fibrosis [12]. In vitro, high oxalate levels increase the intracellular calcium concentration in endothelial cells [13] and inhibit the proliferation and remigration of endothelial cells [14], thus affecting the re-endothelialization of injured blood vessels and promoting the progression of arteriosclerosis. Based on these studies, oxalate overload might play an important role in vascular calcification in patients with ESRD.

Oxalobacter formigenes (*O. formigenes*) is an anaerobic bacterium that reduces serum and urinary oxalate levels by degrading oxalate in the intestinal tract [15–17]. Gulhan et al. [10] found that the lack of *O. formigenes* in hemodialysis patients was related to the increased serum oxalate level. Thus, increased colonization of *O. formigenes* might potentially decrease serum oxalate levels and improve vascular calcification in patients with ESRD. In this study, we investigated the role and mechanism of hy-

peroxalemia in the development of vascular calcification in models of ESRD and the potential therapeutic effect of *O. formigenes*.

Materials and Methods

Mouse Model of Uremic Atherosclerosis

All animal studies were approved by the Ethics Committee of Naval Medical University. The mice were housed in polycarbonate cages under pathogen-free conditions on a 12-h light-dark cycle at the Animal Center of Naval Medical University (Shanghai, China) and provided free access to water and a high-fat diet for 3 months. The components of the high-fat diet as listed by the manufacturer (Jiangsu Synergetic Pharmaceutical Bioengineering Co., Ltd.) were a standard diet, 15% fat, 1% cholesterol, and 0.35% cholic acid.

Eight-week-old male apoE^{-/-} mice (Kavens Experimental Animal Co., Ltd. Changzhou, China) were divided into 3 groups ($n = 15$ mice per group): the control group, uremic atherosclerosis (UA) group, and UA group treated with *O. formigenes* intervention (UA + OxF). We used a 2-step procedure to create a UA model. In brief, we applied cortical electrocautery to the right kidney through a 2-cm flank incision and performed left total nephrectomy through a similar incision 2 weeks later, as described by Gagnon et al. [18]. Penicillin was administered for 3 days after the operation to prevent infection. In the control group, both kidneys were exposed during the 2 operations, and no surgical intervention was performed.

Isolation and Purification of *O. Formigenes* from Fresh Human Feces

First, the anaerobic environment was optimized, which would not hinder the growth of *O. formigenes* (the ratio of N₂, CO₂, and H₂ in the anaerobic tank was 85:10:5). Second, a sterile cotton swab was dipped in fresh feces and diluted with normal saline to 10⁻³ and prepared the OxD agar solid medium and liquid OxB medium, as previously described [19, 20]. Then, 100 μL of diluent was added to the plate, and 5 mL of OxD agar solid medium was added at a temperature of 45°C. The culture medium was quickly spread on the bottom of the plate with the coating rod. After the medium had cooled and solidified, the plate was cultured in an anaerobic tank at 37°C. When some colonies on the plate were visible as 0.1- to 0.3-mm transparent dots or fusiform colonies, they were separated and purified. An inoculating loop was used to pick clear colonies on the OxD medium, and the bacteria were added to the liquid OxB medium and cultured on a shaker at 37°C. After 2 days of culture, the presence of the following phenomena indicated that the bacteria grew well and produced gas: the culture medium changed from yellowish and clear to turbid with white granular suspended solids, white particles were observed on the inner wall of the vial, and the sealing pressure of the silicone rubber stopper increased. One milliliter of bacterial solution was used to measure the bacterial concentration, and 1 mL of blank OxB medium was used as a control. The concentration of the *O. formigenes* bacterial solution after 48 h of culture was 10⁹ cfu/mL. The prepared strains were stored in a -80°C freezer until further experiments. In the UA + OxF group, each UA mouse was gavaged with 0.1 mL of *O. formigenes* suspension per day for 12 weeks. The

Table 1. Primer sequences for RT-PCR

Gene	Primers (5'–3')	
	forward	reverse
TNF- α	CTTTGGGATCATTGCCCTGTG	CGAAGTGGTGGTCTTGTGCT
IL-6	CGAATCTCCGACCACCACTAC	TGCACATAAGCCTCGTTATCC
CRP	AAGCCCAGGGTGAGGAAGAGT	CCCGCCAAGATAGATGGTGTTA
GAPDH	TTCTTGCCTCTTGTCTCTT	ATTTCTTCCATTCTGTCTT

other groups were gavaged with 0.1 mL of normal saline per day for 12 weeks as a control.

Biochemical Analysis

Under short-term anesthesia with diethyl ether (Meilunbio, China), blood samples were obtained from the inner canthal orbital vein. The samples were centrifuged at 4,000 rpm for 10 min at 4°C, and serum was stored at –80°C before further processing. Twenty-four-hour urine samples were collected in a tube containing 1 mL of mineral oil to prevent evaporation and 50 μ L of 2% sodium azide to prevent bacterial growth. Serum levels of urea nitrogen, high-density lipoprotein, low-density lipoprotein, total cholesterol, triglycerides, calcium, and phosphate were assessed using a Hitachi 917 autoanalyzer (Roche, France). Superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels were measured according to the manufacturer's instructions (BioTNT, China). Serum levels of advanced oxidation protein products (AOPP) were measured using an OxiSelectAOPP Assay Kit (Cellbio, South Korea). TNF- α and IL-6 levels were measured using ELISA (Anogen, China) according to the manufacturer's instructions. Serum and urine oxalate levels were detected using ion chromatography (Dionex IonPacTMS14, 4 $\times$ 250 mm).

Qualitative and Quantitative Evaluation of Aortic Calcification

Qualitative Evaluation of Aortic Calcification

The aortic arch and descending aorta were thoroughly cleaned of adventitial fat and cut open longitudinally. Vascular calcifications were evaluated by performing Von Kossa staining in cryosections of the aortic tissue. In brief, the cryosections were placed in a 1% silver nitrate solution (Sigma Aldrich) for 30 min in the dark. Then, they were fixed with a 5% sodium thiosulfate solution for 1 min and stained with basic fuchsin. Calcium deposits appeared black on the bright red-colored surrounding tissue. Data are presented as the relative proportion of the calcified area to the total surface area of calcification lesions, as described previously [21].

Quantitative Evaluation of Vascular Oxalate and Phosphate Levels

Aortic oxalate and phosphate levels were quantitatively detected using ion chromatography (Dionex IonPacTMS14, 4 $\times$ 250 mm). The blood vessels were shredded and placed in a centrifuge tube. After adding 5% HCl, the samples were extracted with ultrasound for 30 min and then centrifuged at 15,000 rpm for 10 min at 4°C. The same volume of chloroform was added to the supernatant, and the mixture was centrifuged at 15,000 rpm for 5 min at

4°C. Linear dilutions of oxalate and phosphate solutions were prepared according to the instructions, and the contents of oxalate and phosphate in the vascular wall were calculated from a standard curve.

Histology

Paraffin-embedded mouse kidney sections (3 mm thickness) were prepared using a routine procedure. The sections were stained with periodic acid-Schiff staining reagents using a standard protocol described in a previous study [22].

Cell Culture

Primary human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs) were purchased from Science Cell Research Laboratories (Carlsbad, CA, USA) and cultured as previously described [23]. In brief, HAECs were grown in endothelial culture medium (No. 1001, Science Cell) containing 5% fetal bovine serum (No. 0025), 1% endothelial cell growth supplement (No. 1052), and 1% penicillin/streptomycin solution (No. 0503) at 37°C with 5% CO₂. All experiments were performed on HAECs between passages 2 and 5. HASMCs were cultured in smooth muscle cell medium (Cat. #1101) supplemented with 2% fetal bovine serum, 1% SMCGS, and 1% penicillin and streptomycin. Experiments were performed using cells from passages 3–6. HAECs and HASMCs were treated with normal medium, 100 μ mol/L oxalate, 200 μ mol/L oxalate, or and 500 μ mol/L oxalate for 6 h or 24 h. All other reagents were obtained from Sigma.

MDA Levels and SOD Activity Assays

Confluent HAECs and HASMCs cultured in 6-well plates were incubated with different doses of oxalate (0, 50, 100, 200, or 500 μ mol) for 24 h. Then, the cell supernatant was collected. SOD activity and MDA levels were measured according to the manufacturer's instructions (Bio TNT, China).

Measurement of Intracellular Reactive Oxygen Species Levels

For measurement of reactive oxygen species (ROS) production, HAECs and HASMCs cultured in 6-well plates were labeled for 30 min with 10 μ M of 6-carboxy-H2DCF-DA-di-AM. Cells were washed with PBS and treated for 6 h with sodium oxalate at different concentrations. After 6 h, the production of ROS was measured by flow cytometry. The production of intracellular ROS was detected by measuring the fluorescence of 6-carboxy-H2DCF-DA-di-AM by the SpectraMax i3x Fluorescence multi-well plate reader (USA) at excitation and emission wavelengths of 485 and 525 nm, respectively, and normalized to cellular protein concentrations.

Table 2. Serum and urinary biochemical parameters at week 12

	Control group (N = 15)	UA group ^a (N = 13)	UA + OxF ^b (N = 13)
Body weight, g	28.09±0.68	25.84±0.57**	25.81±0.94
Serum urea, mmol/L	6.53±1.14	22.66±6.34**	21.32±4.05
TC, mmol/L	21.11±4.25	30.1±1.72**	30.21±3.62
TG, mmol/L	1.21±0.34	1.38±0.74	1.25±0.46
HDL, mmol/L	2.97±0.40	2.68±0.69	2.92±0.52
LDL, mmol/L	19.32±4.93	26.2±4.44**	24.20±4.60
Serum calcium, mmol/L	2.32±0.14	2.33±0.13	2.30±1.09
Serum phosphate, mmol/L	2.21±0.34	3.80±0.95**	3.46±1.09
Serum oxalate, μmol/L	7.22±1.54	26.9±5.46**	20.39±1.76*
Urinary oxalate excretion, μmol/24 h	0.42±0.07	0.23±0.06*	0.16±0.04*
Urinary calcium, mg/24 h	7.39±0.92	13.89±1.62*	15.86±1.12*

UA, uremic atherosclerosis. ^a Compared to the control group. ^b Compared to the UA group. * $p < 0.05$. ** $p < 0.001$.

Real-Time Quantitative PCR

Total RNA was extracted from HAECs and HASMCs by using RNAiso Plus (Takara). cDNA was synthesized by reverse transcriptase M-MLV (Takara). RT-qPCR was performed by using FastStart Universal SYBR Master (Roche) in an Applied Biosystems ViiA7 system (Thermo Fisher Scientific). RT-PCR primer sequences are listed in Table 1. GAPDH was used as the endogenous control. The relative amount of mRNA compared to the internal control was calculated using the $2^{-\Delta\Delta CT}$ method [24].

Western Blot Analysis

Protein samples were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (80 g of total protein per lane) and transferred onto a polyvinylidene fluoride membrane (Merck Millipore, Danver, MA, USA). In addition, gels stained with bromophenol blue were used to confirm the equal amounts of protein loaded on each lane. The membranes were incubated overnight at 4°C with rabbit anti-p-JAK2 (1:1,000, CST), rabbit anti-JAK2 (1:1,000, CST), rabbit anti-p-STAT3 (1:1,000, CST), or mouse anti-Stat3: mouse (1:1,000, CST). The membranes were extensively applied with PBS Tween and incubated for 2 h with horseradish peroxidase-conjugated secondary antibody (1:2,000; Rockland)) at room temperature. The immune complexes were detected using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). The density of the specific bands was analyzed using Image Lab 4 software (Bio-Rad, Hercules, CA, USA), and the expression levels of these proteins were normalized to GAPDH (1:10,000, Abcam).

Statistical Analysis

All data are presented as means ± SD. Significant differences between means were analyzed using ANOVA followed by the Student-Newman-Keuls test. The Pearson correlation coefficient was calculated for a single factor correlation analysis, and r was the correlation coefficient. A p value < 0.05 was considered statistically significant.

Results

Oxalate Levels in Serum and Aortic Calcification Plaques Were Significantly Increased in the Mouse Model of UA

Mice were divided into the following 3 groups to determine the role of oxalate in the progression of UA: control, UA, and UA + OxF groups. Table 2 shows the findings of serum and urinary biochemical analyses conducted after 12 weeks. Urinary oxalate excretion in the UA group was significantly decreased compared with the control group (see online suppl. Fig. 1a; see www.karger.com/doi/10.1159/000516013 for all online suppl. material), and urinary calcium levels were increased in the UA groups (online suppl. Fig. 1b). In addition, significant increases in serum urea, total cholesterol, low-density lipoprotein, oxalate, and phosphate levels were observed in the UA group compared to the control group.

The histological evaluation of the aorta using Von Kossa staining revealed that aortic calcification was present in all 3 groups. The area of aortic calcification plaques was significantly increased in the UA group compared to the control group (Fig. 1). We further examined the oxalate level in aortic calcification plaques by performing ion chromatography to investigate the role of hyperoxalemia in aortic calcification (online suppl. Fig. 2). The vascular oxalate level in the UA group was significantly higher than that in the control group ($24.96 \pm 7.59 \mu\text{g/g}$ vs. $2.64 \pm 0.54 \mu\text{g/g}$, Fig. 2a), reflecting the relatively high oxalate burden in the aortic plaques from the UA group. A positive correlation

Fig. 1. Effects of oxalate on aortic calcification. **A–C** Representative photomicrographs of the aorta stained by the Von Kossa method in the control group (**A**), UA group (**B**), and UA + OxF group (magnification $\times 40$) (**C**); the area of aortic calcification in the 3 groups (**D**). UA, uremic atherosclerosis; UA + OxF group, UA group treated with *O. formigenes* intervention.

Fig. 2. Serum and vascular oxalate in 3 groups. **A** Vascular oxalate and phosphate in 3 groups. The unit of $\mu\text{g/g}$ represents the level of oxalate/phosphate per gram of blood vessels. **B** The correlation between serum oxalate and vascular oxalate levels ($r = 0.78$, $p < 0.001$). All results were expressed as “means $\pm$ SD.” $N = 10$ in each group. * $p < 0.05$; ** $p < 0.01$. UA, uremic atherosclerosis; UA + OxF group, UA group treated with *O. formigenes* intervention.

was identified between the vascular oxalate level and serum oxalate level (Fig. 2b).

After intervention with *O. formigenes* for 12 weeks, serum, urinary, and vascular oxalate levels all decreased significantly compared to the UA group (Table 2; Fig. 2a). *O. formigenes* was maintained at a concentration of 10^6

cfu/g in feces from the UA + OxF group, while no *O. formigenes* was detected in the UA and control groups (online suppl. Fig. 1c), indicating the considerable colonization of *O. formigenes* in the UA + OxF group. However, the vascular calcification area was not decreased significantly after the *O. formigenes* intervention (Fig. 1).

Fig. 3. Effects of hyperoxalemia on the oxidative stress markers and inflammatory factors expression. **A, B** Level of serum MDA, IL-6, AOPP, TNF-α (**A**), and SOD activity (**B**) among 3 groups. Results were expressed as “means $\pm$ SD.” $n = 10$ in each group. **C–F** Correlation between serum oxalate and serum MDA levels ($r = 0.70$, $p < 0.001$) (**C**), AOPP level ($r = 0.52$, $p = 0.004$) (**D**), TNF-α level ($r = 0.71$, $p < 0.001$) (**E**), and SOD activity (**F**) ($r = -0.54$, $p = 0.04$). UA, uremic atherosclerosis; MDA, malondialdehyde; AOPP, advanced oxidation protein products; SOD, superoxide dismutase; UA + OxF group, UA group treated with *O. formigenes* intervention.

Hyperoxalemia Induced Oxidative Stress and Inflammatory Gene Expression in the Mouse UA Model

Serum MDA, IL-6, AOPP, TNF-α, and SOD levels were measured to further investigate the level of oxidative stress and the expression of inflammatory factors induced by hyperoxalemia. Compared to the control group, serum MDA, IL-6, AOPP, and TNF-α levels were significantly increased in the UA group, while SOD activity was sig-

nificantly decreased ($p < 0.001$, Fig. 3a, b). In addition, serum oxalate levels positively correlated with serum MDA, AOPP, and TNF-α levels and negatively correlated with SOD activity ($p < 0.05$, Fig. 3c–f). The administration of 10^8 cfu of *O. formigenes* significantly reduced serum MDA, AOPP, and TNF-α levels and increased serum SOD activity ($p < 0.05$, Fig. 3a, b). However, significant differences in IL-6 levels were not observed between the UA group and UA + OxF group.

Fig. 4. High oxalate-induced oxidative stress and inflammatory factors expression in HAECs via the JAK2/STAT3 signaling pathway. **A–C** Expression of oxidative stress markers including ROS (**A**), MDA (**B**), and SOD (**C**) in HAECs and HASMCs stimulated by different levels of sodium oxalate. **D** mRNA expressions of inflammatory factors including TNF- α , CRP, and IL-6 in HAECs. **E–G** High oxalate-induced JAK2 and STAT3 phosphorylation and

activation in HAECs. Representative Western blots (**E**) and quantitative data (**F**, **G**) of phosphorylated JAK2 and STAT3 in HAECs stimulated by different levels of sodium oxalate. All results were expressed as “means $\pm$ SD.” $N = 3$ in each group. * $p < 0.05$; ** $p < 0.01$. HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cells; MDA, malondialdehyde; SOD, superoxide dismutase; ROS, reactive oxygen species.

Oxalate Overload Induced Oxidative Stress and Inflammatory Factor Expression in HAECs via the JAK2/STAT3 Signaling Pathway

An in vitro experiment using cultured HAECs and HASMCs was performed to determine the target cells involved in oxalate-induced vascular calcification. Intracellular ROS generation, as measured by the average fluorescence intensity, was significantly increased in HAECs after 6 h of stimulation with 200 and 500 μ M sodium oxalate, while no change was observed in HASMCs (Fig. 4a). Similarly, oxalate caused a dose-dependent increase in the mRNA levels of MDA, IL-6, and CRP but led to a decrease in SOD activity in HAECs after 24 h of stimulation (Fig. 4b–d). The oxalate load did not alter the expression of MDA, IL-6, TNF- α , or CRP in HASMCs (online suppl. Table 1).

HAECs were incubated with different concentrations of oxalate for 24 h to investigate the potential role of JAK2/STAT3 signaling in the activation of oxidative stress and inflammatory factor expression by hyperoxalemia in HAECs. Levels of the phosphorylated JAK2 and STAT3 proteins were analyzed using Western blotting. As shown in Fig. 4e, p-JAK2 and p-STAT3 levels were significantly upregulated in a concentration-dependent manner, suggesting the activation of the JAK2/STAT3 signaling pathway in response to high oxalate-induced HAEC injury.

Discussion

CKD has become a global health problem because of its high incidence and mortality rates. CVD is the most common cause of death in patients with ESRD, especially in patients on dialysis [25]. Evidence has revealed the harmful effects of phosphate overload on the cardiovascular system in patients with ESRD. However, phosphate binders are unable to fully reverse vascular calcification in patients with ESRD. The role of oxalate in the formation of calcium oxalate stones has been widely studied [26, 27]. As an exogenous uremic toxin, serum oxalate was shown to positively correlate with the serum creatinine level, pulse wave conduction velocity, central artery systolic blood pressure, and diastolic blood pressure [18, 28]. In the present study, we investigated the role of oxalate in the formation of vascular calcification in uremia models.

Serum oxalate levels were significantly increased and urinary oxalate levels were significantly decreased in the UA group as kidney function deteriorated. In addition, the present study illustrated that oxalate was deposited on

calcified vessels (in both the UA group and control group), and serum oxalate levels positively correlated with vascular oxalate levels. These findings are consistent with the results from the study by Nishizawa et al. [29] who showed that calcium oxalate might be one of the components of coronary artery calcification in dialysis patients. Furthermore, a significantly higher ratio of aortic oxalate to phosphate levels was observed in the UA group than in the control group, indicating that oxalate might play an important role in the development of vascular calcification in individuals with ESRD.

High oxalate concentrations have been shown to increase the production of reactive oxygen species in renal tubular epithelial cells, leading to endothelial injury and apoptosis, which plays an important role in the formation of kidney crystals [14, 30]. Previous studies found that oxalate treatment increased the levels of LDH, H₂O₂, MDA, and ROS in HK-2 cells, which indicated the activation of oxidative stress [31–33]. Consistent with the findings of urolithiasis studies, we found that oxidative stress was activated in uremic atherosclerotic mice with hyperoxalemia as well. Serum oxalate levels positively correlated with serum MDA, AOPP, and TNF- α levels and negatively correlated with SOD activity. In vitro, oxalate caused a dose-dependent increase in the secretion of inflammatory factors, including MDA, IL-6, TNF- α , and CRP, in HAECs but not in HASMCs, indicating that vascular endothelial cells might be the target cells of oxalate-induced vascular calcification. The JAK2/STAT3 signaling pathway plays an important role in regulating the secretion of inflammatory factors [34, 35]. Our study indicated a potential role for JAK2/STAT3 signaling in increasing the inflammatory factors and activating oxidative stress by high oxalate concentrations as p-JAK2 and p-STAT3 levels were significantly upregulated in HAECs. In studies of renal tubular cells, oxidative damage caused by high oxalate levels promotes the epithelial-mesenchymal transition (EMT). Given the tight association between oxalate loading and oxidative stress and the correlation between ROS levels and the EMT, the oxalate-induced EMT in vascular epithelial cells might be considered the potential mechanism of vascular calcification in patients with ESRD. Interestingly, our study did not observe a significant effect of high oxalate concentrations on HASMCs, which was probably related to the lack of the oxalate transporter SLC26 in HASMCs [36].

The *O. formigenes* intervention significantly decreased serum and urine oxalate levels, which was proven to be effective in reducing the risk of calcium oxalate kidney stones [37]. *O. formigenes* decreased both exogenous and

endogenous oxalate levels. Based on our results, the expression of *O. formigenes* in feces was determined by qPCR (online suppl. Fig. 3), and *O. formigenes* effectively reduced the oxalate overload in uremic mice. After the *O. formigenes* intervention, the serum oxalate levels and oxalate levels in aortic calcification plaques were significantly decreased. Moreover, the administration of *O. formigenes* significantly reduced serum MDA, AOPP, and TNF- α levels and led to an increase in serum SOD activity, indicating that *O. formigenes* ameliorated the oxidative stress caused by hyperoxalemia.

The present study has some limitations. First, our study failed to verify a significant therapeutic effect of the *O. formigenes* intervention on the overall area of calcified plaques and glomerulosclerosis (online suppl. Fig. 4). The uremic mouse model was established for only 3 months. Due to the relatively short course of uremia, serum oxalate levels in this uremic mouse model were not as high as the levels we usually observe in uremic patients. This discrepancy might contribute to the result that vascular oxalate levels were not very high and the ratio of oxalate to phosphate levels was relatively low. Thus, we observed a trend of a decrease in the plaque area in the UA + OxF group, which unfortunately did not reach statistical significance. Nevertheless, *O. formigenes* significantly reduced the vascular oxalate level and the serum inflammatory factor levels and improved oxidative stress. Second, we did not measure the atherosclerotic plaque by Oil Red O staining in the present study. Since high-fat diet could also induce atherosclerotic plaques in the apoE^{-/-} mice, it would be also interesting to see if atherosclerotic plaque burden can be modified by *O. formigenes* treatment. Third, we observed associations between oxalate overload and oxidative stress and inflammation but did not further verify the putative cause-effect relationship by directly intervening in the inflammatory process related to oxalate loading. We did not further explore the mechanism of oxalate-induced vascular endothelial injury to determine whether it is mediated by oxalate transporters

or the deposition of calcium oxalate crystals in the vascular wall. However, after considering these limitations, we propose that oxalate overload induces inflammation, oxidative stress, and vascular calcification in subjects with ESRD, and the present study provides insights into another important harmful effect of oxalate overload on patients with ESRD.

Our study is the first to suggest an important role for hyperoxalemia in vascular calcification in ESRD by inducing oxidative stress and inflammatory gene expression, leading to oxalate deposition in calcified areas. The administration of *O. formigenes* may reduce the serum oxalate level and improve the inflammatory state of patients with ESRD, which is of great clinical significance.

Statement of Ethics

The animal study was reviewed and approved by the Shanghai Changzheng Hospital's Biomedical Research Ethics Committee.

Conflict of Interest Statement

There are no conflicts of interest to declare. The results presented in this article have not been published previously.

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Author Contributions

Bin Wen and Changlin Mei designed the research; Ke Sun, Xiaojing Tang, and Changlin Mei analyzed the data; Ke Sun, Shuwei Song, Yuan Gao, Hongjing Yu, and Ningyun Sun performed the research; and Ke Sun, Xiaojing Tang, Bin Wen, and C. Mei wrote the manuscript.

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