Megalin Blockade with Cilastatin Suppresses Drug-Induced Nephrotoxicity

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ABSTRACT
Nephrotoxicity induced by antimicrobial or anticancer drugs is a serious clinical problem. Megalin, an endocytic receptor expressed at the apical membranes of proximal tubules, mediates the nephrotoxicity of aminoglycosides and colistin, key antimicrobials for multidrug-resistant organisms. The mechanisms underlying the nephrotoxicity induced by vancomycin, an antimicrobial for methicillin-resistant Staphylococcus aureus, and cisplatin, an important anticancer drug, are unknown, although the nephrotoxicity of these drugs and gentamicin, an aminoglycoside, is suppressed experimentally with cilastatin. In the clinical setting, cilastatin has been used safely to suppress dehydropeptidase-I–mediated renal metabolism of imipenem, a carbapenem antimicrobial, and thereby limit tubular injury. Here, we tested the hypothesis that cilastatin also blocks megalin-mediated uptake of vancomycin, cisplatin, colistin, and aminoglycosides, thereby limiting the nephrotoxicity of these drugs. Quartz crystal microbalance analysis showed that megalin also binds vancomycin and cisplatin and that cilastatin competes with megalin for binding to gentamicin, colistin, vancomycin, and cisplatin. In kidney-specific mosaic megalin knockout mice treated with colistin, vancomycin, or cisplatin, the megalin-replete proximal tubule epithelial cells exhibited signs of injury, whereas the megalin-deficient cells did not. Furthermore, concomitant cilastatin administration suppressed colistin-induced nephrotoxicity in C57BL/6J mice. Notably, cilastatin did not inhibit the antibacterial activity of gentamicin, colistin, or vancomycin in vitro, just as cilastatin did not affect the anticancer activity of cisplatin in previous studies. In conclusion, megalin blockade with cilastatin efficiently suppresses the nephrotoxicity induced by gentamicin, colistin, vancomycin, or cisplatin. Cilastatin may be a promising agent for inhibiting various forms of drug-induced nephrotoxicity mediated via megalin in the clinical setting.


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Drug-induced nephrotoxicity is an important factor for the development of AKI1 and CKD.2 Irrespective of the risks, however, the use of nephrotoxic antibiotics is unavoidable in patients with CKD and infection, and some nephrotoxic anticancer agents are usually not used or are used less often in patients with CKD, despite their excellent anticancer effects.

Multidrug-resistant organisms called carbapenem-resistant *Enterobacteriaceae*, such as *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, and *Klebsiella pneumoniae*, are on the rise and have become problematic worldwide.3 Infections caused by these bacteria lead to poor clinical outcomes and high mortality rates; especially in sepsis, mortality rates reach as high as 50%.4 Colistin, a cationic polypeptide antibiotic, has re-emerged as a last resort therapy for carbapenem-resistant *Enterobacteriaceae* infections and is frequently the only agent to which these organisms are susceptible.5 Despite its efficacy, the clinical use of colistin has been limited owing to its serious nephrotoxicity. Recent studies reported the nephrotoxicity rate in patients receiving colistin as 40%–45% using the RIFLE criteria.6

Vancomycin is also recognized as a nephrotoxic agent but remains a first-line antibiotic for infections caused by methicillin-resistant *Staphylococcus aureus*. In the last several years, there have been growing concerns about the emergence of methicillin-resistant *S. aureus* strains with reduced susceptibility to vancomycin,7 and high-dose vancomycin therapy has been conducted. These attempts, however, led to a higher incidence of renal injury.8 Therefore, nephrotoxicity has been one of the most pressing issues in infections caused by resistant organisms.

Cisplatin is one of the preferred initial chemotherapeutic agents for the treatment of various human malignancies. The most serious and common adverse event of cisplatin administration is kidney injury, which occurs in approximately one third of patients.9 Nephrotoxicity, the main dose-limiting factor of the drug, is associated with a history of diabetes mellitus and cardiovascular disease, and it worsens the prognosis.10

Megalin, a large (approximately 600-kD) glycoprotein member of the LDL receptor family,11 is expressed at the apical membranes of proximal tubule epithelial cells (PTECs).12 Megalin mediates intracellular signal transduction and plays a pivotal role in the reabsorption of glomerular-filtered substances.13 In a high-fat diet–induced mouse model of metabolic syndrome, megalin mediates the proximal tubular uptake of nephrotoxic substances, such as lipid-modified proteins, causing tubuloglomerular alterations.14 Megalin also mediates the uptake of nephrotoxic drugs, such as aminoglycosides,15 polymyxin B,15 and colistin.16 Vancomycin and cisplatin are not known to bind to megalin (Table 1).

Cilastatin, an inhibitor of renal dehydropeptidase-I (DHP-I), was originally introduced to clinical practice in 1987 combined with imipenem, a carbapenem antimicrobial. It suppresses the renal metabolism of imipenem and thereby, tubular injury.17 Cilastatin was also found to be an inhibitor of organic anion transporter 3.18 Cilastatin competes with the renal uptake of vancomycin and cisplatin and their consequent nephrotoxicity (Table 1).19,20 However, there is no evidence indicating that the nephrotoxicity of vancomycin and cisplatin is caused by DHP-I– or organic anion transporter 3–mediated mechanisms. Indeed, these drugs were reported to be taken up by PTECs via basolateral organic cation transporters (OCTs), whereas the effects of OCT inhibitors to suppress their renal uptake and nephrotoxicity are partial.9,21 The nephrotoxicity of gentamicin, an aminoglycoside, also reportedly competed with cilastatin,22 but the mechanism is also unknown (Table 1).

Thus, we hypothesized that cilastatin may be a megalin blocker that competes with megalin binding to vancomycin and cisplatin as well as aminoglycosides and colistin, thereby inhibiting the nephrotoxicity of these drugs. To test this hypothesis, we have evaluated the binding of megalin to such agents, particularly vancomycin, cisplatin, and cilastatin. We have also analyzed the megalin-dependent nephrotoxicity of these drugs using kidney-specific megalin knockout (KO) mice and examined the competitive inhibitory effect of cilastatin in vitro and in vivo.

### RESULTS

**Gentamicin, Cilastatin, Vancomycin, and Cisplatin Are Bound by Megalin, Which Is Competitively Inhibited by Cilastatin**

We firstly performed quartz crystal microbalance (QCM) analysis to assess the direct binding of megalin with these drugs. To assess their interactions accurately, we used megalin protein purified from rat renal microvillar membranes by affinity chromatography using a megalin-specific mAb as described previously.23 Affinity-purified megalin, immobilized on a sensor chip, was bound directly by gentamicin and colistin as reported previously24 as well as by vancomycin, cisplatin, and cilastatin (Figure 1, A–E, respectively). However, megalin was not bound by a comparative amount of tenofovir (Figure 1F), an anti-HIV drug that is taken up by PTECs via basolateral OCT1 to induce cellular toxicity.24 Recombinant glutathione S-transferase (GST) protein immobilized on a sensor chip as a negative control was bound by an anti-GST antibody but was not bound by cilastatin (Figure 1G) and the other megalin-bound drugs (data not shown). In addition, a sensor chip, on which no proteins were immobilized, was also not bound by cilastatin (Figure 1H) and the other megalin-bound drugs (data not shown).

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Binding to Megalin</th>
<th>Renoprotection by Cilastatin</th>
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<tr>
<td>Gentamicin</td>
<td>Ref. 15</td>
<td>Ref. 22</td>
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<tr>
<td>Colistin</td>
<td>Ref. 16</td>
<td>Unknown</td>
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<tr>
<td>Vancomycin</td>
<td>Unknown</td>
<td>Ref. 19</td>
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<tr>
<td>Cisplatin</td>
<td>Unknown</td>
<td>Ref. 20</td>
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<tr>
<td>Cilastatin</td>
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Affinity analysis showed that the $K_d$ values for the binding of megalin with colistin and vancomycin were 0.0764±0.0118 and 1.159±0.168 mmol/L, respectively (Supplemental Figure 1). For gentamicin, cisplatin, and cilastatin, the $K_d$ values for binding to megalin were not determined due to their limited dissolution in the reaction solution used for saturation analysis. The binding of gentamicin, colistin, vancomycin, and cilastatin to megalin was all competed by cilastatin (Figure 1, I–L, respectively). These findings indicate that all of these drugs bind specifically to neighboring sites of megalin.

### Vancomycin, Cisplatin, and Colistin Induce PTEC Injury Dependent on Megalin

We next investigated whether PTEC injury, the major pathologic feature induced by vancomycin, cisplatin, and colistin, is mediated via megalin using kidney-specific mosaic megalin KO mice (apoE cre, megalin lox/lox). Megalin gene deletion occurs in approximately 60% of PTECs in these mice, thereby allowing a direct comparison between megalin KO and intact PTECs in the same mice. As shown in Figure 2, megalin-expressing PTECs were extensively vacuolized or destroyed in mice administered colistin, vancomycin, or cisplatin, whereas megalin KO PTECs were protected from injury in the same mice. In addition, kidney injury molecule-1 (KIM-1), an established kidney injury marker, was expressed concomitantly with megalin in the same PTECs of mice administered colistin, vancomycin, or cisplatin (Figure 3). Comparatively, KIM-1 was not expressed even in megalin-expressing PTECs in mice injected with saline (vehicle), and the expression of megalin and KIM-1 was not always colocalized in a unilateral ureteral obligation model of the mice (Figure 3). These results indicate that the colocalized expression of megalin and KIM-1 in colistin-, vancomycin-, or cisplatin-administered mosaic megalin KO mice is not an artifact but verifies the megalin-dependent nephrotoxicity induced by these drugs.

### Concomitant Administration of Cilastatin Suppresses Colistin-Induced Nephrotoxicity in C57BL/6J Mice

We also found that cilastatin suppresses colistin-induced nephrotoxicity in C57BL/6J mice (Figure 4), as shown previously in renal injury models induced by gentamicin, vancomycin, and cisplatin. Concomitant administration of cilastatin with colistin to the mice suppressed colistin-induced tubulointerstitial injury, including tubular vacuolization, tubular dilation or atrophy, brush border loss, tubular cell lysis, and cast formation (Figure 4A); urinary excretion of N-acetyl-$\beta$-(D)-glucosaminidase (NAG), a PTEC injury marker (Figure 4B); and KIM-1 expression in PTECs (Figure 4C).
DISCUSSION

The original target of cilastatin, DHP-I, is localized at the brush border membranes of PTECs, where megalin is also located and likely to be encountered by the drug in vivo. Because the serum protein binding rates for gentamicin, colistin, and vancomycin are 3.4%, 66%, and 34%, respectively, according to their drug information forms, significant fractions of the administered drugs should consist of free forms that are filtered by glomeruli into the proximal tubular lumens and encounter megalin. A low urinary recovery rate of colistin has been reported; however, significant colistin clearance by glomerular filtration has been approximated, and thus, the extensive renal tubular reabsorption of colistin has been estimated. In contrast, a high plasma protein binding rate, approximately 65%–98%, was reported for cisplatin. Although minor amounts of glomerular filtration were speculated, the remarkable excretion of cisplatin in urine was identified due to its uptake via OCTs in the basolateral membranes of PTECs followed by tubular secretion. Hence, each drug accumulates in significant amounts by glomerular filtration or tubular secretion in the tubular lumens, where they should be encountered by megalin for reabsorption.

In this study, we have shown the direct binding of megalin with colistin, gentamicin, vancomycin, cisplatin, and cilastatin using QCM analysis. This real-time analysis also revealed that prior administration of cilastatin competitively inhibited the binding of megalin with the other megalin-bound drugs. We have already shown that the receptor-ligand binding kinetics analyzed by QCM were comparable with those of other methods, such as surface plasmon resonance. A previous study by Suzuki et al. indicated that colistin bound to megalin with a $K_d$ of 130.3 ± 79.0 nmol/L; however, the study had some limitations: they used megalin-containing brush border membrane vesicles for competitive binding assays, and thus, the direct binding of megalin with colistin was not clear. In this study, we used high-grade quality affinity-purified megalin, which provided more accurate binding specificity and quantification of its binding kinetics. The assay temperature (23°C) in our study was closer to the body environment compared with that in the previous study (4°C), which was also likely responsible for the determination of the greater affinity of colistin for megalin.

In addition, our results suggest that cilastatin suppresses colistin-induced nephrotoxicity through competition for binding to megalin. Cilastatin did not show a complete reno-protective effect in the mouse model. However, to induce apparent nephrotoxicity including renal morphologic changes and KIM-1 expression in C57BL/6J mice, which are occasionally resistant to nephrotoxic agents, a high dose of colistin (30 mg/kg per day), which is close to its subcutaneous injection-mediated median lethal dose ($LD_{50}$) in mice (48.6 mg/kg; www.caymanchem.com/msdss/17584m.pdf), was needed for the experiment. In addition, the dose of cilastatin (intravenous injection–mediated $LD_{50}$ in mice: 8700 mg/kg; www.merck.ca/assets/en/pdf/products/PRIMAXIN-PM_E.pdf) used to compete with the nephrotoxic action of colistin was relatively low: the molar ratio between cilastatin and colistin in this experiment was approximately 1:25.

In the clinical setting, colistimethate, the non-nephrotoxic prodrug of colistin, is used at a concentration of 400–800 mg per 60 kg/d, and 30% of the administered colistimethate is converted to colistin, indicating that much less colistin is used clinically than in this study. Furthermore, the applicable molar quantity of cilastatin in clinical practice is estimated to be approximately 61- to 123-fold greater than the amount of colistin converted from colistimethate, leading to the expectation that cilastatin should be able to suppress colistin-induced
nephrotoxicity in the clinical setting. In addition, the maximum dosage of imipenem/cilastatin in current clinical use is limited by imipenem-mediated toxicity (intravenous injection–mediated LD50 in mice: 1500 mg/kg; www.merck.ca/assets/en/pdf/products/PRIMAXIN-PM_E.pdf); therefore, cilastatin would be used at a higher dose if its monotherapy was available. Cilastatin also reportedly inhibits the nephrotoxicity of vancomycin, cisplatin, and gentamicin. These findings thus indicate that cilastatin is a promising agent for the management of a variety of drug-induced nephrotoxicities and expected to be applied widely in clinical practice.

The structure of the domain of megalin involved in its binding with gentamicin has been studied. The authors solved the nuclear magnetic resonance structure of the tenth complement type repeat in the second ligand binding cluster of human megalin to investigate its interaction with gentamicin. However, the overall mechanisms by which megalin is bound by multiple drugs and how cilastatin competes with the binding of other drugs to megalin remain unknown. Additional structural studies of the ligand binding domains of megalin are needed to elucidate the mechanisms.

Aminoglycosides, colistin, vancomycin, and cisplatin are also ototoxic. Because megalin is also expressed in the inner ear and because single-nucleotide polymorphisms at the megalin gene might be associated with individual susceptibility to cisplatin-induced ototoxicity, megalin blockade treatment with cilastatin may also be useful for preventing the ototoxicity induced by these drugs.

In conclusion, megalin blockade with cilastatin suppresses efficiently the nephrotoxicity induced by gentamicin, colistin, vancomycin, and cisplatin. The clinical safety of cilastatin has been established by its long-term combined use with imipenem. Future pharmacologic development of cilastatin alone as a megalin blocker will be expected for its broad clinical application to inhibit the nephrotoxicity mediated by these drugs and probably some other compounds, such as cyclosporin.

**CONCISE METHODS**

**Materials**

Colistin, gentamycin, and vancomycin were purchased from Sigma-Aldrich. Cilastatin was purchased from Wako Pure Chemical Industries, Ltd. and Sigma-Aldrich. Cisplatin was obtained from Wako Pure Chemical Industries, Ltd. and NIPPON KAYAKU Co. Ltd., and tenofovir was from Wako Pure Chemical Industries, Ltd.

**QCM**

Binding of the drugs to megalin was examined using a highly sensitive 27-MHz QCM instrument (AFFINIX Q, QCM2000; ULVAC) as described previously. Briefly, QCM sensor chips were activated with a 1:1 mixture of 100 mg/ml N-hydroxysuccinimide and 100 mg/ml 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride in

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**Figure 3.** Megalin mediates colistin-, vancomycin-, and cisplatin-induced PTEC injury shown by KIM-1 expression. Double immunofluorescence of megalin and KIM-1 in the kidney sections showed that KIM-1 expression is only seen in megalin-expressing PTECs (arrowheads) and is not seen in megalin KO PTECs (arrows) in the kidney-specific mosaic megalin KO mice administered colistin, vancomycin, or cisplatin. Vehicle administration did not induce KIM-1 expression, even in megalin-expressing PTECs (arrowheads). In the unilateral ureteral obstruction (UUO) model of the mice, KIM-1 was expressed in not only megalin-expressing PTECs but also, megalin KO PTECs (arrows). Scale bar, 40 μm.
water. Immunoaffinity-purified rat megalin was then immobilized on the sensor chips at a concentration of 65 μg/ml in buffer B (10 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM CaCl₂). The sensor chips were soaked in the incubation chamber at 23°C in 8 ml buffer B. Each drug dissolved in buffer B was added to the chamber to test binding to megalin. As a negative control, recombinant GST protein was prepared by using a prokaryotic expression system with the pGEX-6p-1 vector (GE Healthcare) according to the manufacturer’s instructions and immobilized on the sensor chip. An anti-GST antibody, used as a ligand of GST, was purchased from GE Healthcare. Cilastatin was used for competition with the other drugs for binding to megalin: cilastatin was first added to the chamber and reached the steady state of its binding to megalin, and then, each of the drugs was injected into the chamber to assess competitive binding inhibition by cilastatin. The resonance frequency of QCM at equilibrium was defined as the zero position. The stability and drift of the 27-MHz QCM frequency in solution were both ±3 Hz. Receptor-ligand binding kinetics were analyzed by the saturation method using AFFINIX Q8 (QCM2012; ULVAC) as described previously.

Treatment of Kidney-Specific Megalin KO Mice with Nephrotoxic Drugs

Male kidney-specific megalin KO mice (apoE cre, megalinlox/lox) established by Thomas Willnow (Max Delbrück Center for Molecular Medicine) were used in this study. The mice were housed in a temperature-controlled room and allowed free access to a standard diet and tap water. To induce the kidney injury model, 11-week-old mice received a subcutaneous injection of colistin (30 mg/kg body wt) once daily for 4 days or an intraperitoneal injection of vancomycin (400 mg/kg body wt) once daily for 4 days or cisplatin (20 mg/kg body wt) once for 1 day. At 24 hours after the final treatment with colistin or vancomycin and 72 hours after cisplatin treatment, the animals were euthanized under intraperitoneal pentobarbital sodium anesthesia (50 mg/kg body wt) or by cervical dislocation. Then, the right kidneys were removed and processed for periodic acid–Schiff (PAS) and immunofluorescence staining. Vehicle saline-injected male kidney-specific megalin KO mice were used as negative controls. All animal experiments in this study were on the basis of the

Figure 4. Cilastatin suppresses colistin-induced nephrotoxicity in C57BL/6J mice. (A) Representatively, tubular vacuolization, tubular dilation or atrophy, brush border loss, tubular cell lysis, and cast formation are observed in PAS-stained kidney sections of colistin-administered C57BL/6J mice, but these findings were ameliorated drastically by the concomitant administration of cilastatin. Semiquantitative morphometric analysis of tubular damage revealed that cilastatin administration significantly suppressed colistin-induced nephrotoxicity; n=8, n=10, and n=10 for the vehicle, colistin, and colistin and cilastatin groups, respectively. Scale bar, 40 μm. *P<0.05; ***P<0.001. (B) Colistin-induced increase of urinary N-acetyl-b-(D)-glucosaminidase excretion in C57BL/6J mice was significantly suppressed by the concomitant administration of cilastatin. A significant interaction was found by two-way ANOVA (P<0.01), and subsequent Bonferroni post hoc analysis identified significant differences between the indicated points (n=5 each). **P<0.01; ***P<0.001. (C) Representative photomicrographs showing that the concomitant administration of cilastatin suppresses colistin-induced KIM-1 expression in PTECs of C57BL/6J mice. G, glomerulus. Scale bar, 40 μm.
Kidney tissue sections were deparaffinized and rehydrated through a graded ethanol series. Antigen retrieval was accomplished by heating the slides in a microwave in 10 mM citrate buffer, pH 6.0. After incubation with 5% rabbit serum in PBS to block nonspecific binding, the sections were incubated overnight at 4°C with a goat anti-mouse KIM-1 antibody (1:500; AF1817; R&D Systems) in PBS containing 0.1% Triton X-100 and, in immunopositive reactions, was visualized by using fluorescein anti-goat IgG (Vector Laboratories). Sequentially, the sections were incubated with 5% goat serum in PBS and an antimegalin antiserum (1:2000) in PBS containing 0.1% Triton X-100 at 4°C overnight. Immunopositive reactions were visualized by using Texas red anti-rabbit IgG (Vector Laboratories) in the dark. Nonimmune rabbit or goat serum was used as negative control without using a primary antibody. After staining, the samples were mounted with the SlowFade Gold Antifade Reagent (Life Technologies), and images were captured by using a fluorescence microscope (BZ-9000; Keyence).

Preparation of Kidney Sections for PAS and Immunofluorescence Staining
After removal of the kidneys, 3-mm-thick horizontal cross-sections containing the hilum were obtained immediately and fixed in a 4% paraformaldehyde phosphate buffer for 72 hours at room temperature. After fixation, the tissues were dehydrated in a graded ethanol series from 70% to 100%, cleared in xylene, and embedded in paraffin. The discs containing cilastatin represent no inhibitory zone, and the combined discs revealed no antagonistic effects. Scale bar, 10 mm.

Semiquantitative Morphometric Analysis of Tubular Injury
Semiquantitative renal morphometric analysis was performed using 2-μm-thick paraffin sections stained with PAS. Tubular damage was scored by evaluating the percentage of injured tubules showing dilatation or atrophy, brush border loss, cell lysis, and cast formation in the corticomedullary junction and outer medulla. Lesions were graded on a scale from zero to four: zero, none; one, <25%; two, 25% and <50%; three, ≥25% and <75%; four, ≥50% of 15 randomly selected viewing fields (original magnification, ×400) each section per kidney. Morphometry was conducted in a blinded fashion by an experienced pathologist.

Renal Immunohistochemistry for KIM-1
For the detection of KIM-1 in kidney tissues, 4-μm-thick paraffin sections were stained with the avidin–biotin–peroxidase complex method. A goat anti-mouse KIM-1 antibody (1:800; AF1817; R&D Systems) was used as the primary antibody. After deparaffinization, antigen retrieval was performed by using citrate buffer, and endogenous peroxidase activity was eliminated with 3% H2O2. Nonspecific binding was blocked with 5% rabbit serum, and endogenous biotin binding was blocked with an Avidin/Biotin Blocking Kit (Vector Laboratories). Finally, immunoreactivity was developed with the DAB Substrate-Chromogen System (Dako), and the sections were counterstained with Mayer hematoxylin. Negative control staining was always performed in parallel with PBS instead of the primary antibody.
Antibacterial Activity Assay
The agar disc diffusion method was performed according to Clinical and Laboratory Standards Institute guidelines. Briefly, a test organism (E. coli ATCC 25922 or S. aureus ATCC 25923) was cultured in Mueller–Hinton broth and adjusted to a McFarland Standard of 0.5. Bacterial suspensions were streaked onto Mueller–Hinton agar plates. Paper discs containing colistin (10 mg), gentamicin (10 mg), vancomycin (30 mg), and cilastatin (10 mg) were deposited on the inoculated plates, and the plates were incubated at 37°C overnight. The plates were subsequently inspected for bacterial growth, and the zone of growth inhibition surrounding the paper disc was measured.

Statistical Analyses
In the animal experiments, semiquantitative morphometric data were analyzed by Kruskal–Wallis one-way ANOVA by ranks and comparisons between each group were made by Dunn Multiple Comparison Test. Urinary NAG excretion data were analyzed using two-way ANOVA and comparisons between each group were made by Bonferroni post hoc analysis. All statistical analyses were carried out using Predictive Analytics SoftWare Statistics 18 (SPSS, Inc.) and GraphPad Prism5 (MDF). The level of significance was P<0.05.

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