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PHARMACOLOGY

Rutin Attenuates Vancomycin-Induced Nephrotoxicity by Ameliorating Oxidative Stress, Apoptosis, and Inflammation in Rats

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ABSTRACT Nephrotoxicity is the major limiting factor for the clinical use of vancomycin (VCM) for treatment of serious infections caused by multiresistant Gram-positive bacteria. This study investigated the renal protective activity of rutin in a rat model of VCM-induced kidney injury in male Wistar rats. VCM administered intraperitoneally at 200 mg/kg twice daily for 7 successive days resulted in significant elevation of blood urea nitrogen and creatinine, as well as urinary *N*-acetyl-β-D-glucosaminidase. Coadministration of VCM with oral rutin at 150 mg/kg significantly reduced these markers of kidney damage. Rutin also significantly attenuated VCM-induced oxidative stress, inflammatory cell infiltration, apoptosis, and decreased interleukin-1β and tumor necrosis factor alpha levels (all $P < 0.05$ or 0.01) in kidneys. Renal recovery from VCM injury was achieved by rutin through increases in Nrf2 and HO-1 and a decrease in NF-κB expression. Our results demonstrated a protective effect of rutin on VCM-induced kidney injury through suppression of oxidative stress, apoptosis, and downregulation of the inflammatory response. This study highlights a role for oral rutin as an effective intervention to ameliorate nephrotoxicity in patients undergoing VCM therapy.

KEYWORDS NF-κB, nephrotoxicity, oxidative stress, rutin, vancomycin

Vancomycin (VCM) is a glycopeptide antibiotic commonly used to treat methicillin-resistant Gram-positive bacteria, especially *Staphylococcus aureus* (1). However, between 5 and 35% of all VCM-treated patients suffer some form of nephrotoxicity (2–5). Current animal experiments suggest that VCM alters energy-dependent renal reabsorption in the proximal tubule that leads to mitochondrial dysfunction and that coadministration of antioxidants can prevent nephrotoxicity (6–12).

Reactive oxygen species (ROS) have been implicated in models of toxic renal failure due to cisplatin and aminoglycoside exposure. These compounds, as well as VCM, perturb proximal renal tubule epithelial cells that are the sites of reabsorption (6). The affects attributed to VCM involve renal tubular cell apoptosis through an increase in mitochondrial superoxide production, leading to a loss of mitochondrial membrane potential and elevated caspase activity (13). The filtration and energy transport mechanisms in the proximal tubule epithelia render the kidney highly susceptible to toxicant-induced renal injury (14). For instance, one of the most sensitive biomarkers of cell injury to the proximal tubules is the excretion of urinary *N*-acetyl-β-D-glucosaminidase (NAG) (15). Urinary NAG activity is also a sensitive indicator for glycopeptide antibiotic-induced nephrotoxicity (16).

Rutin is a citrus flavonoid glycoside consisting of the flavonol quercetin and the disaccharide rutinose (17). This polyphenolic bioflavonoid possesses a wide range of pharmacological activities and has substantial antioxidant properties. It has been

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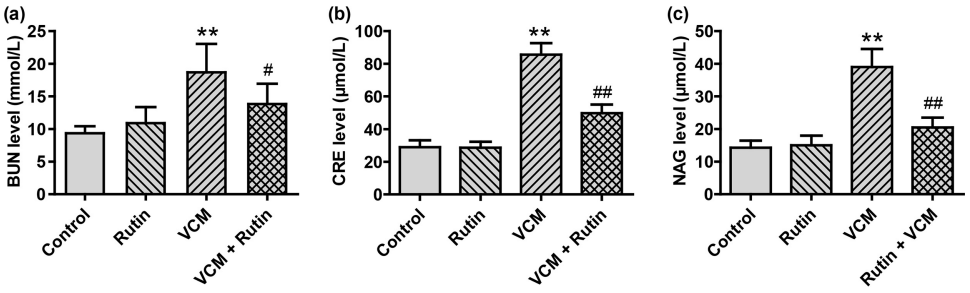

FIG 1 Rutin attenuates VCM-induced nephrotoxicity in rats. (a to c) Serum BUN, CRE, and urinary NAG levels, respectively, in rats treated with VCM in the presence or absence of rutin. The results are presented as group means ± the SD ($n = 10$ in each group). **, $P < 0.01$ compared to group 1; # and ##, $P < 0.05$ and $P < 0.01$, respectively, compared to the VCM treatment group.

utilized as an antioxidant as well as an anticancer, anti-inflammatory, antidiabetic and antihyperpietic agent (18). Its toxicity is significantly less than other bioflavonoids, and it is a nonoxidizable molecule. These qualities suggest that rutin could be a highly potent and relatively safe antioxidant.

Rutin supplementation in experimental diets has demonstrated its *in vivo* antioxidant, anti-inflammatory, antiapoptotic, and antiautophagic properties and has been used to mitigate gentamicin-induced nephrotoxicity in a rat model (19). This protective effect has been linked to NF-κB, tumor necrosis factor alpha (TNF-α), and Bcl-2 against benzo(*a*)pyrene-induced lung toxicity and genotoxicity in mice (20). Rutin administration also lowered plasma glucose and decreased oxidative stress via the Nrf2 signaling pathway in a rat model of diabetic neuropathy (21). Coincidently, rutin significantly increased NF-E2-related factor 2 (Nrf2) and heme oxygenase (HO-1) expression in biliary obstruction-induced liver injury in a rat model (22). A protective effect of rutin was also demonstrated in a rheumatoid arthritis model that involved the downregulation of the NF-κB p65 protein (19).

In the present study, activities of rutin against VCM-induced nephrotoxicity, along with potential molecular mechanisms for its mode of action were evaluated in a rat model.

RESULTS

Assessment of kidney function. Serum blood urea nitrogen (BUN) and creatinine (CRE) levels have been reported as an initial indication of VCM-induced nephrotoxicity (7). We found no differences between groups 1 and 2. However, both BUN and CRE levels in the VCM-treated rats (group 3) were significantly increased compared to groups 1 and 2 ($P < 0.01$). Interestingly, BUN and CRE levels in group 4 (VCM plus rutin) were significantly decreased in comparison with group 3 ($P < 0.05$ and $P < 0.01$, respectively) but still higher than controls (Fig. 1a and b). In addition, urinary NAG excretion in group 3 was higher than in controls ($P < 0.01$), and rutin coadministration significantly reduced urinary NAG activity ($P < 0.01$) (Fig. 1c).

Rutin ameliorates VCM-induced oxidative stress in kidney tissue. In rats treated with VCM alone (group 3), we found a significant induction of oxidative stress in kidneys with a marked increase in malondialdehyde (MDA), inducible nitric oxide synthase (iNOS), and nitric oxide (NO) levels (all $P < 0.01$). This was accompanied by significant decreases in superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) activities (all $P < 0.01$). In the rutin-plus-VCM group 4, the oxidative stress markers were significantly attenuated ($P < 0.05$ and $P < 0.01$, respectively). These oxidative stress indicators did not noticeably change between the rutin and control groups (Fig. 2).

Rutin attenuates VCM-induced activation of caspase-9 and -3 and inflammatory mediators in kidneys. Because VCM can activate renal caspase-9 and -3, we examined whether rutin can alter this process or not (23). Rats that received VCM alone (group 3) had significant increases in caspase-9 (3.14-fold) and caspase-3 (2.36-fold)

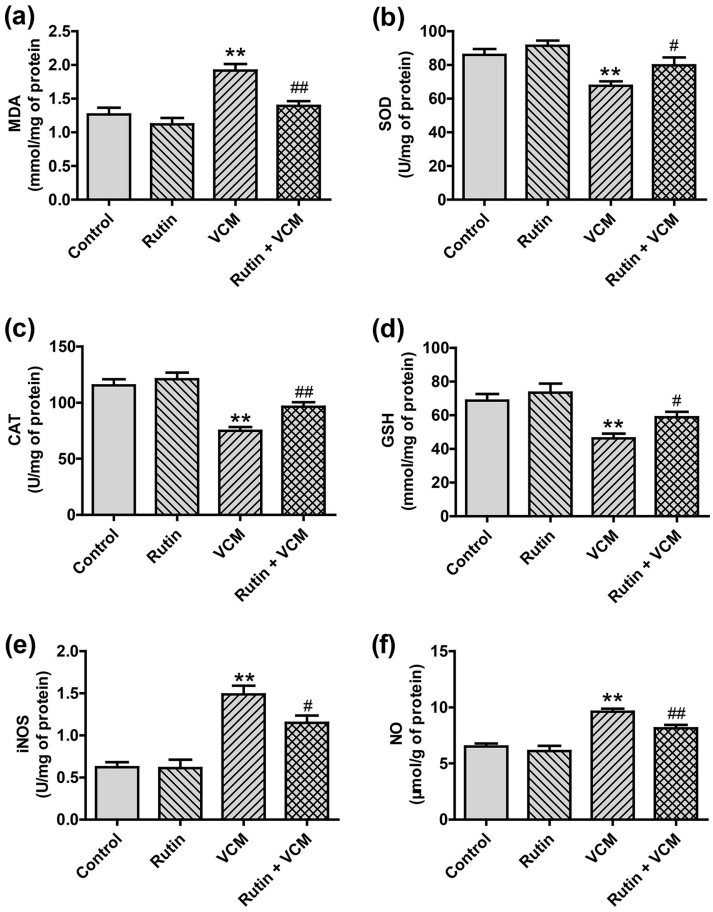

FIG 2 Impact of rutin on the levels of oxidative and nitrosative stress markers in the kidney tissues of rats treated with VCM. The results are presented as group means ± the SD ($n = 10$ in each group). **, $P < 0.01$ compared to group 1; # and ##, $P < 0.05$ and $P < 0.01$, respectively, compared to the VCM treatment group.

activities (both $P < 0.01$) compared to control group 1. Rutin prophylaxis resulted in a marked attenuation of VCM-induced caspase activation caspase-9 levels decreased by 1.97-fold and caspase-3 decreased by 1.66-fold compared to VCM group 3. The administration of rutin alone (group 2) did not alter caspase levels from control values (group 1) (Fig. 3a and b).

The levels of the inflammatory mediators intereleukin-1β (IL-1β) and TNF-α were also significantly increased by VCM treatment. However, rutin coadministration with VCM decreased IL-1β 38% and TNF-α 27% (Fig. 3c and d).

Histopathological evaluation. Kidney histopathological examination showed that rutin prophylaxis markedly attenuated VCM-induced tissue damage (Fig. 4). The kidney tissues of rats treated with VCM alone displayed extensive damage that included tubular degeneration, tubular dilation, necrosis, cast formation, and infiltration of inflammatory cells (Fig. 4c). Rutin prophylaxis attenuated the VCM-induced kidney damage, as evidenced by a marked attenuation of the infiltration of inflammatory cells and decreased tubular necrosis in the renal cortex (Fig. 4d). A semiquantitative scoring reinforced these findings and revealed a significant attenuation of damage with rutin coadministration with VCM ($P < 0.01$) (Fig. 4e). There were no marked histopathological changes in the rutin alone group, and the histology was similar to the saline vehicle control group 1 (Fig. 4a and b).

Expression of HO-1, Nrf2, and NF-κB mRNA in kidney tissues. To examine the effects of rutin more closely, we measured steady-state mRNA levels of genes that are induced in kidneys by VCM. Both Nrf2 and HO-1 mRNAs were markedly increased in the

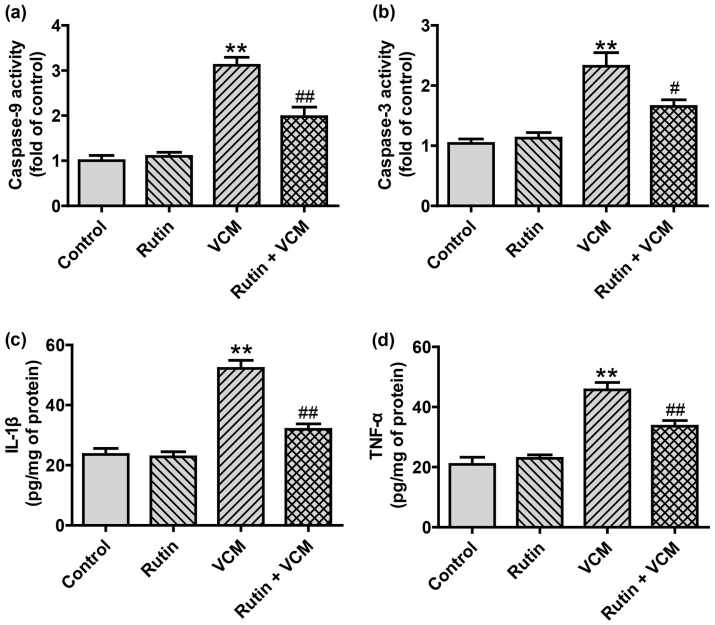

FIG 3 Rutin attenuates VCM-induced activation of caspase-9 (a) and caspase-3 (b) and the inflammatory mediators IL-1β (c) and TNF-α (d) in the kidneys of rats treated with VCM. ELISA results are presented as group means $\pm$ the SD ($n = 10$ for each group). **, $P < 0.01$ compared to group 1; # and ##, $P < 0.05$ and $P < 0.01$, respectively, compared to the VCM treatment group.

VCM group. Interestingly, these levels were even greater in all rutin-treated groups compared to that in the saline-treated control rats (Fig. 5) ($P < 0.05$ and $P < 0.01$, respectively). In contrast, the expression of NF-κB mRNA was significantly decreased in the VCM-plus-rutin group compared to the VCM group. Unlike the results of upregulation of Nrf2 and HO-1, NF-κB displayed no marked changes following rutin treatment.

DISCUSSION

VCM is used for treatment of serious infections, and its therapeutic effects are significantly improved by dose escalation. However, high-dose therapy with VCM is limited by its cumulative risk of nephrotoxicity (24). Although there are studies indicating the molecular mechanisms of VCM nephrotoxicity, no definitive conclusions have been drawn. Accordingly, the development of strategies to avoid this unwanted side effect is of the utmost importance.

Rutin is an active citrus flavonoid compound that possesses a variety of pharmacological activities (25, 26). In the present study, we provided evidence that oral rutin prophylaxis significantly reduces VCM-induced nephrotoxicity in a rat model. Rats that were coadministered oral rutin (150 mg/kg) had decreased serum BUN and CRE levels. This indicated that rutin decreased the overall damage caused by high-dose VCM. This was corroborated by a decrease in kidney damage (Fig. 1 and 4). In addition, the significant increase in urinary NAG excretion detected after VCM administration was neutralized with rutin coadministration (Fig. 2). There are newer glycopeptide biomarkers for acute kidney injury that are more sensitive than NAG, and these can be tested in future trials of VCM-induced tubular injury (27–29).

Previous studies have indicated that oxidative stress plays a critical role in VCM-induced kidney damage in cultured proximal tubular cells and *in vivo* in rats (30). In support of this, we found decreased SOD and CAT activities and lower levels of GSH in the kidneys of VCM-treated rats. Moreover, the peroxidized lipid decomposition marker MDA and nitrosative stress-related NO and iNOS activities were significantly elevated in the kidney tissues of the VCM-treated rats. Pretreatment with rutin strongly diminished damage from both these factors, indicating that oral rutin has a nephroprotective effect (Fig. 2).

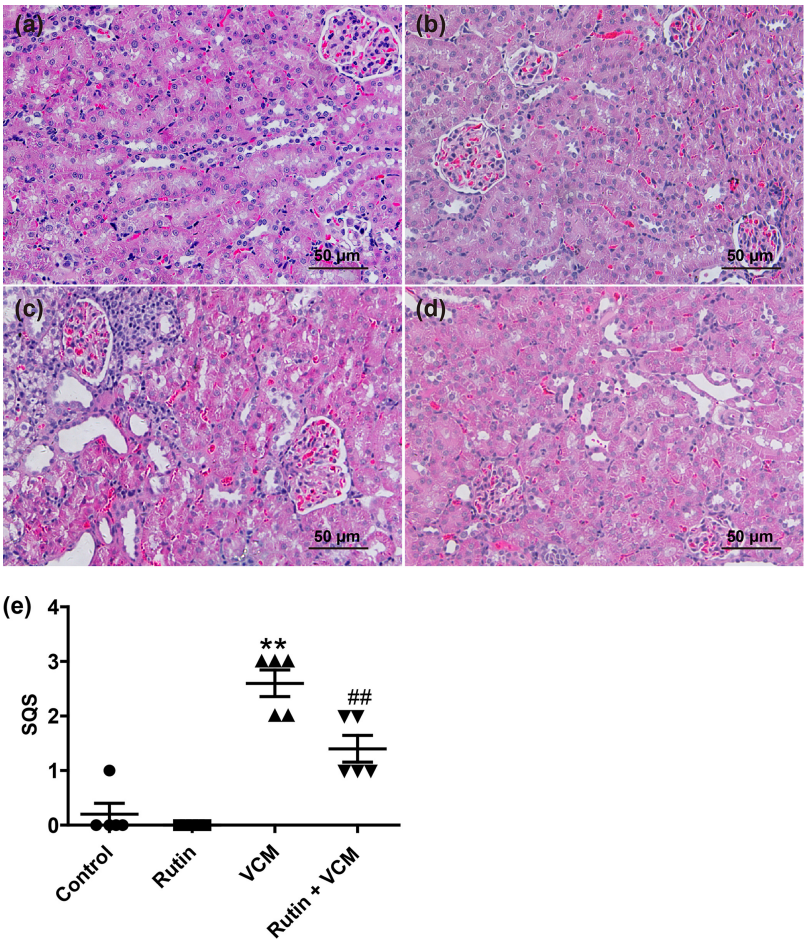

FIG 4 Representative histopathological changes in kidneys of rats treated with VCM and rutin. (a to d) H&E-stained rat kidney sections from control group 1 (a), rutin group 2 (b), VCM group 3 (c), and the rutin-plus-VCM group (d). (e) Semiquantitative scores of kidney damage (group means ± the SD, $n = 5$). **, $P < 0.01$ compared to group 1; ##, $P < 0.01$ compared to the VCM treatment group.

Nrf2 is a transcription factor that centrally regulates oxidative stress response genes (31). The upregulation of Nrf2 effectively prolongs the cellular oxidative damage response by activating genes that provide phase II detoxifying enzymes and antioxidant enzymes, including CAT, SOD, HO-1, and glutathione peroxidase (32). In the present study, we found that rutin treatment increased Nrf2 mRNA levels, as well as one

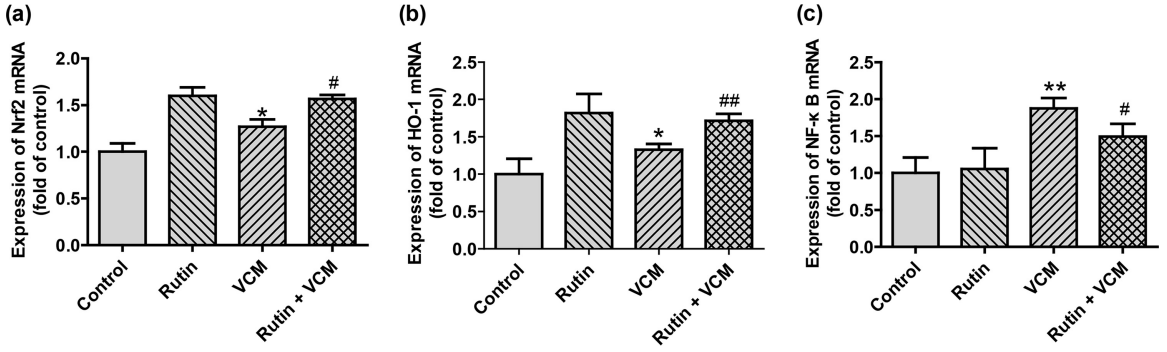

FIG 5 (a to c) Steady-state mRNA levels of Nrf2 (a), HO-1 (b), and NF-κB (c) in rat kidney samples. Values are presented as group means ± the SD ($n = 10$ in each group). * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to group 1; # and ##, $P < 0.05$ and $P < 0.01$, respectively, compared to the VCM treatment group.

of its downstream target genes HO-1. Rutin also increased SOD and CAT activities (Fig. 2). These data indicated that the Nrf2/HO-1 pathway contributes to the nephroprotective effect of rutin against VCM.

Previous studies have shown that rutin coadministration alleviated VCM-induced reproductive damage via suppression of oxidative stress and apoptosis in adult male rats. Rutin also ameliorated intestinal toxicity induced by methotrexate linked with antioxidative and anti-inflammatory effects (33, 34). Furthermore, testicular degeneration and damage and decreased sperm viability due to cisplatin treatment in rats was attenuated by rutin (30). In contrast, the NF-κB p65 subunit can inhibit the Nrf2-antioxidant response element pathway at the transcriptional level by depriving the CREB binding protein from the Nrf2 promoter and recruiting histone deacetylase 3 to MafK (35). Our results indicated that the activation of the Nrf2/HO-1 pathway contributes to the ability of rutin to inhibit the NF-κB-mediated inflammatory response (Fig. 5).

Rutin is also an effective antioxidant flavonoid that prevents the adverse effects of ethanol on cadmium-induced oxidative stress by increasing glutathione and glutathione peroxidase activities in the testes of adult rats (36). Rutin was a significant inhibitor of iron-dependent lipid peroxidation systems by acting as an iron chelator that formed an inert iron complex unable to initiate lipid peroxidation. Rutin was able to suppress free radical processes at three stages: the formation of superoxide ion, the generation of hydroxyl (or crypto-hydroxyl) radicals in the Fenton reaction, and the formation of lipid peroxy radical (37). Furthermore, rutin effectively protected the human hepatoma cell line from H_2O_2-induced oxidative stress and apoptosis in a dose-dependent manner. This ability involved mechanisms related to the regulation of ROS production, the inhibition of lipid peroxidation, the protection of the intracellular antioxidant system, and its modulation of the Bcl-2/Bax and NF-κB/p65 signaling pathways (38). Overall, the direct radical scavenging activity of rutin and its ability to increase the resistance of the kidneys by activating their intrinsic antioxidant defense mechanisms are all activities attributed to rutin and are responsible for dampening the VCM-induced nephrotoxicity.

Previous studies identified that the mitochondrial, oxidative stress, and PAR and PARP-1 protein expression pathways are involved in VCM-induced nephrotoxicity in rats and in human kidney proximal tubular cells (30, 39). Both the intrinsic (mitochondrial) and the extrinsic (death receptor) pathways could activate the key apoptotic mediator caspase-3 (40). Caspase-9 plays a key role in the mitochondrial apoptosis pathway (40). We have demonstrated that rutin supplementation significantly attenuated VCM-induced increased activity of caspase-3 and -9 in kidney tissues. Indeed, several other studies have indicated that the majority of pharmacological activities of rutin are closely related to its role in modulating mitochondrial function and dynamics (41, 42).

Inflammation is an additional factor that facilitated VCM-induced nephrotoxicity (43). NF-κB is a crucial transcriptional mediator of the proinflammatory cytokine response that is regulated by ROS and plays a pivotal part in damage to tubular epithelial cells (44). In the present study, we found that VCM-evoked inflammatory responses in kidneys resulted in infiltration of inflammatory cells and elevated NF-κB expression, as well as the production of the proinflammatory cytokines IL-1β and TNF-α (Fig. 3c and d). Rutin supplementation markedly diminished NF-κB expression and attenuated IL-1β and TNF-α generation to control levels (Fig. 3c and d). Coincidently, rutin suppressed the production of TNF-α and IL-6 and the activation of NF-κB and extracellular signal-regulated kinases 1 and 2 (ERK1/2) by the high-mobility group box 1 (HMGB) factor. This indicates that rutin could be a candidate therapeutic agent for treatment of various severe vascular inflammatory diseases (45). In order to improve anti-inflammatory activity of the rutin, it was microencapsulated in a chitosan matrix using the spray-drying technique, and the resulting drug delivery system was investigated (46). We used a high dose of the nephrotoxic agent VCM, rending a poor clinical relevance to this particular study. Our data demonstrate that a high dose of rutin provides valuable protection against VCM nephrotoxicity, while developing new drug delivery systems to improve bioavailability could be a great approach. Notably, various

TABLE 1 Primer sequences used for real-time qRT-PCR

Gene	Primer sequence (5′–3′)
Nrf2	CAC ATT CCC AAA CAA GAT GC TCT TTT TCC AGC GAG GAG AT
HO-1	CGT GCT CGA ATG AAC ACT CT GGA AGC TGA GAG TGA GGA CC
NF-κB	CAC TGT CTG CCT CTC TCG TCT AAG GAT GTC TCC ACA CCA CTG
GAPDH	ACA GTC CAT GCC ATC ACT GCC GCC TGC TTC ACC ACC TTC TTG

formulations such as a rutin-loaded nanostructured lipid carrier have improved the stability and bioavailability of oral rutin (47).

In conclusion, we found that rutin had significant prophylactic effects on VCM-evoked nephrotoxicity. Rutin inhibited oxidative/nitrosative stress, inflammation, and apoptosis in the kidneys of rats. Rutin did not have any undesirable effects on normal rats, indicating that this compound should be considered as a candidate of novel agents to mitigate the nephrotoxic effects of VCM therapy.

MATERIALS AND METHODS

Chemicals. Abbott Laboratories (North Chicago, IL) provided vancomycin. Rutin (purity 95%), Tris-HCl, EDTA-Na_2, sucrose, and saline were purchased from Sigma (St. Louis, MO).

Animal experiments. This animal study followed the Guidelines of Experimental Animal Care issued by the Animal Welfare and Research Ethics Committee at Qingdao Agriculture University. We used male Wistar rats weighing 250 to 300 g (Vital River Animal Technology, Beijing, China). The rats were kept in a controlled environment at 23 ± 2°C with humidity at 60% ± 5% and a controlled light cycle (12-h light/12-h dark). The rats were acclimated to this environment for 5 days prior to the start of the experiments and had free access to food and water during the experiments.

The animals were randomly divided into four groups ($n = 10$ in each group). Group 1 served as control that received intraperitoneal (i.p.) injections of physiological saline at 1 ml/kg. Group 2 was the rutin control group that was administered rutin (150 mg/kg, p.o.). Group 3 was the VCM group that received VCM (200 mg/kg, i.p. twice daily), and group 4 was treated with rutin (150 mg/kg, p.o.) 2 h prior to VCM (200 mg/kg, i.p., twice daily) administration. All groups were treated for 7 consecutive days (6, 19). At 12 h following the last dose, the rats were transferred to metabolic cages for spontaneous micturition to collect urine samples. Rats were then euthanized by i.p. sodium pentobarbital injection (80 mg/kg; Sigma-Aldrich, St. Louis, MO). Blood samples were obtained from renal veins into polypropylene centrifuge tubes and allowed to clot spontaneously at room temperature. Serum was then separated by centrifugation at 2,000 × *g* at 4°C for 10 min and stored at −80°C until analysis. CRE, BUN, and urinary NAG levels were assayed using commercial assay kits according to the manufacturer's instructions (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China). Kidney tissue samples were collected, either formalin fixed or frozen for biochemical, histopathological, enzyme-linked immunosorbent assay (ELISA), and quantitative RT-PCR (qRT-PCR) analysis as described below.

Measurement of markers of oxidative stress in kidney tissues. Sections of kidney tissue were homogenized in 9 volumes of cold Tris buffer (0.01 M Tris-HCl, 0.1 mM EDTA-Na_2, 0.01 M sucrose, 0.9% saline [pH 7.4]) and a 10% (wt/vol) tissue homogenate was prepared. The supernatant obtained from 3,000 × *g* at 4°C for 15 min of centrifugation was used to measure MDA, CAT, NO, iNOS, GSH, and SOD using commercial assay kits (Nanjing Jiancheng). Protein concentrations were measured using a bicinchoninic acid protein assay kit (Beyotime, Haimen, China).

Histopathological examination. For light microscopy examination of histological sections, the right kidneys from 5 rats in each group were processed and fixed in 10% neutral buffered formalin. The formalin fixed tissue was embedded in paraffin, sliced into 4-μm tissue sections and stained with hematoxylin-eosin (H&E) (48, 49). Five coded sections from each group were evaluated blindly by a pathologist who was not revealed of the treatment scheme implemented as described previously (50). A semiquantitative evaluation of kidney injury was conducted and a semiquantitative score was given to grade the pathological changes severity for each kidney sample. Tissues were examined for tubular epithelial alterations (dilatation, desquamation, vacuolization, and casts) and interstitial inflammatory cell infiltration. All histopathological parameters were graded as follows: 0, no meaningful histopathological damage; +1, mild degree of damage; +2, mild to moderate degree of damage; +3, moderate degree of damage; +4, moderate to severe degree of damage; and +5, severe degree of damage.

Measurement of caspase-9 and -3 activities and TNF-α and IL-1β levels. The supernatants (stored at −80°C) were used to determine the activities of caspase-3 and caspase-9 (Nanjing Jian Cheng Biotechnology Co., Ltd., Nanjing, China) and TNF-α and IL-1β (R&D Systems, Minneapolis, MN) using commercial ELISA kits according to manufacturers' instructions (51).

qRT-PCR analysis. For the determination of mRNA levels of genes of interest, total RNA from kidney tissue samples was isolated using TRIzol reagent (Life Technologies, Grand Island, NY) following manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using a Prime Script RT-PCR kit (TaKaRa, Dalian, China). The quality of RNA was verified by measuring the OD at 260/280 nm. Primer sets used for amplification of target genes are detailed in Table 1. Real-time qRT-PCR was performed using an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA). Target genes expression was analyzed relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels.

Statistical analyses. Values are presented as means ± the standard deviation (SD) of at least five experiments. Data were analyzed using SPSS v15.0 (SPSS, Inc., Chicago, IL), and the differences between groups were compared via one-way analysis of variance, followed by Dunnett's multiple-comparison tests. A P value of <0.05 was considered statistically significant.

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The authors declare no conflicting financial interest.

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