Original Article

Vitamin C attenuates vancomycin induced nephrotoxicity through the reduction of oxidative stress and inflammation in HK-2 cells

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Background: Oxidative stress is one of the possible mechanisms in vancomycin (VCM) induced nephrotoxicity. Some studies suggested that high dose Vitamin C (VC) has protective effect against the nephrotoxicity in mice, but the underlying molecular mechanism is not mentioned. We investigated the potential targets of high dose VC against oxidative stress and inflammation induced by VCM in renal tubular epithelial cells.

Methods: We conducted an *in vitro* study using an immortalized proximal tubule epithelial cell line from a normal adult human kidney (HK-2).

Results: VCM added to HK-2 cells caused an increase of cell death, oxidative stress and expression of inflammatory cytokines. Co-treatment with 0.5 and 1 mM VC attenuated 4–8 mM VCM induced cell death and increased the cell viability to 58–90%. VC significantly decreased lipid peroxidation and increased superoxide dismutase activity. The upregulations of NF-κB, TNF-α and IL-6 in HK-2 cells under 4 mM VCM were also reversed by 0.5 mM VC through the inhibition of oxidative stress.

Conclusions: This study showed for the first time that VC can attenuate the VCM induced nephrotoxicity by decreasing lipid peroxidation and expression of inflammatory cytokines, and increasing superoxide dismutase 2 (SOD2) activity, this effect may relate to the regulation of ROS/NF-κB pathway.

Keywords: Vancomycin (VCM); Vitamin C (VC); oxidative stress; inflammation; NF-κB

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Introduction

Vancomycin (VCM) is a very effective antibiotic for severe infections caused by Gram-positive bacteria. Clinical guidelines recommend that the trough concentration of VCM in adults with ordinary infections should be maintained at 10–15, and 15–20 mg/L would be needed in severe or complex infections to ensure its antibacterial effect (1,2). However, with rise in the trough concentration, risk of VCM related nephrotoxicity increased significantly. The incidence of nephrotoxicity was 29.6% and 8.9%, respectively, when trough concentrations of VCM were >15 and ≤15 mg/L (3). Patients receiving more than 4 g/day of VCM were 35% likely to occur renal injury according to the research by Lodise *et al.* (4). *In vitro* studies revealed that when concentration of VCM in the renal proximal tubule increases,

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reactive oxygen species (ROS) generates in the epithelial cells and activates the oxidative stress response, which leads to DNA damage, cell apoptosis, mitochondrial disorder and cell necrosis (5,6). Use of antioxidants significantly inhibited VCM induced oxidative stress and reduced the incidence of renal toxicity (7,8). However, there are currently few drugs clinically available to relieve nephrotoxicity in severe ill patients receiving high dose VCM.

Vitamin C (VC) is an important and classic antioxidant. Intravenous supplement of VC in large doses (100–200 mg/kg/day) has proven to be particularly effective in treatments of tumors and critical illnesses associated with oxidative stress (9,10) by rapidly improving the body's antioxidant capacity. Several studies (11,12) have found that in addition to eliminating ROS directly, high dose VC can ameliorate organ damage in hemorrhagic shock rats by inducing heme oxygenase HO-1 and deacetylase SIRT1, which indicated a possible mechanism of high dose VC. An analysis of 188 patients using VCM found that 2 g/day VC oral intake reduced the incidence of acute kidney injury (AKI) (13). A very recent study by Takigawa *et al.* (14) showed that 4 g/kg/day VC injection reduced VCM induced nephrotoxicity in mice by decreasing apoptosis of renal cell. However, overdose of VC intake may lead to oxalate nephropathy and aggravation of renal injury (15,16). Besides, the mechanisms of VC in attenuating oxidative stress, cell apoptosis or inflammatory response were not mentioned in these articles, the underlying molecular mechanism remains to be elucidated. Thus, the present study investigated the protective role of high dose VC against oxidative stress and inflammation induced by VCM in HK-2 renal tubular epithelial cells.

Methods

Materials

VCM (CAS: 1404-90-6) was purchased from Selleck (Shanghai, China). VC was purchased from Sangon Biotech (Shanghai, China). DME/F-12 was purchased from Hyclone (Logan, USA). Fetal bovine serum, penicillin and streptomycin were purchased from Gibco (NY, USA).

Cell culture

The immortalized proximal tubule epithelial cell line from normal adult human kidney (HK-2, RRID: CVCL_0302, ATCC: CRL-2190) was gifted from Nephrology Department of our hospital. Cells were grown in medium DME/F-12 supplemented with 10% fetal bovine serum, 100 μg/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 ℃. Cells were seeded in cell culture plates and used for experiments at 70–80% confluence.

Measurement of cell viability

Cell viability of HK-2 cells under VC were tested first, results showed that cell proliferation inhibition began to occur when VC ≥2 mM. Previously sub-cultured cells (10^4 cells/mL) were exposed to various concentrations of VCM in 0.1% DMSO and VC (4, 6, 8 mM of VCM and 0.5, 1 mM of VC), and 0.1% DMSO only as control group in a 96-well plate and incubated them for 24 h at 37 ℃ in 5% CO_2. Then the supernatants were removed, 10 μL CCK8 (APExBIO Technology, Houston, USA) was added to each well and the cells were incubated for another 4 h. We measured the optical density at 450 nm as the reference (Thermo, USA).

Measurement of MDA level and superoxide dismutase 2 (SOD2) activity

We exposed previously sub-cultured cells (10^5 cells/mL) to 4 mM VCM and 0.5 mM VC in a 24-well plate and incubated them for 24 h at 37 ℃ in 5% CO_2. Cells were lysed and the supernatants obtained from 3,000 rpm at 4 ℃ for 10 min centrifugation were used to measure SOD2 activity (Beyotime Biotechnology, Shanghai, China) and malondialdehyde (MDA) level (Nanjing Jiancheng Bioengineering, Nanjing, China) using commercial assay kits according to the manufacturer's instructions.

Real-time PCR

Cells were harvested for RNA extraction using TRIzol. The reverse transcription reaction was conducted using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, California, USA) to generate the single-strand cDNA. Quantitative real-time PCR was performed on the ABI 7300 Real-Time PCR System (Thermo Scientific, California, USA) using the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). The relative mRNA expression levels were normalized to GAPDH and calculated using the ΔΔCt method. The mRNA levels were expressed relative to the control group. The sequences of

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Table 1 Sequences of the upstream and downstream primers used in this study

Gene	Primer sequences (5'→3')
NF-κB	Upper GGATTTCGTTTCCGTTATGTATGTG
	Lower CATTCGTGCTTCCAGTGTTTCA
TNF-α	Upper GCTGCACTTTGGAGTGATCG
	Lower ATGAGGTACAGGCCCTCTGA
IL-6	Upper AAGCCAGAGCTGTGCAGATGA
	Lower CTGGCATTTGTGGTTGGGTCA
GAPDH	Upper GGAAGCTTGTCATCAATGGAAATC
	Lower TGATGACCCTTTTGGCTCCC

the upstream and downstream primers for nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and GAPDH are shown in *Table 1*.

Western-blot analysis

Cells were lysed and the supernatants obtained from 12,000 rpm at 4 ℃ for 10 min centrifugation were loaded onto a 12% resolving gel for electrophoresis. The proteins were transblotted onto a 0.45 μm PVDF membrane (Millipore, CA, USA). The membranes were blocked by incubation in phosphate-buffered saline containing 0.1%. Tween 20 and 5% non-fat milk for 1 h at room temperature. The blot was immuneprobed with IL-6 antibody (1:750 dilution, RRID: AB_10732306, Servicebio, Wuhan, China) and GAPDH antibody (1:45,000 dilution, RRID: AB_1616725, Proteintech Group, Rosemont, USA) overnight at 4 ℃. The blots were then incubated with an HRP-conjugated secondary antibody (Servicebio, Wuhan, China) for 1 hr at room temperature and then reacted with an enhanced chemiluminescence substrate (Servicebio, Wuhan, China). The resulting chemiluminescence was recorded with an imaging system (EPSON V300, Shanghai, China). To quantify the protein expression, the enhanced chemiluminescence signals were digitized using the Photoshop CS6 software (Adobe, USA).

Statistical analysis

Numerical variables are summarized as mean ± standard deviation (SD). Data were analyzed using SPSS statistical software (version 11.0, SPSS Inc., Chicago). Determination of the statistical differences in parameters between groups was performed by either Student's *t*-test or the Mann-Whitney U test. A P value <0.05 was considered to be statistically significant.

Results

VC reduced VCM induced cell injury in HK-2

CCK8 assay was performed to determine the protection effect of VC on cell death induced by VCM of HK-2 cells. VCM inhibited the cell viability of HK-2 cells to 34–66% in a dose-dependent manner, and VC reduced the cell toxicity of VCM, as shown in *Figure 1A*. Cell viability in all VC co-exposure groups were significantly increased from the relative VCM groups (P<0.05), 0.5 and 1 mM VC increased cell viability of 6 mM VCM by 30% and 37%, respectively. The viability of cells between groups of DMSO and 4 mM VCM combined 0.5 mM VC showed no difference (P>0.05), so we chose 4 mM VCM and 0.5 mM VC for the following tests.

VC reversed VCM induced oxidative stress in HK-2 cells

MDA is one of the major secondary products of lipid peroxidation, and also a reflection of ROS-induced cell damage. The antioxidant activity of SOD2 was reduced and the MDA level was increased in the 4 mM VCM group (P<0.05 compared to DMSO group), which revealed significant induction of VCM on oxidative stress. Compared with the 4 mM VCM only group, SOD2 activity was significantly increased by 25% and MDA level was significantly reduced by 23% (P<0.05) in the 0.5 mM VC combined group. MDA level and SOD2 activity showed no difference between the DMSO, VC and VCM+VC group (*Figure 1B,C*).

VC attenuated protein expression of NF-κB and pro-inflammatory cytokines in HK-2 cells activated by VCM

VCM treatment on HK-2 cells resulted in significant increases in NF-κB mRNA level, a threefold increase in pro-inflammatory cytokines TNF-α, and a 13.9-fold in IL-6 (P<0.05 compared to DMSO group). With 0.5 mM VC combined, the mRNA levels of NF-κB and TNF-α greatly reduced to no difference from control, and IL-6 level significant decreased compared to the VCM group (P<0.05). There were no obvious changes in NF-κB, TNF-α and IL-6

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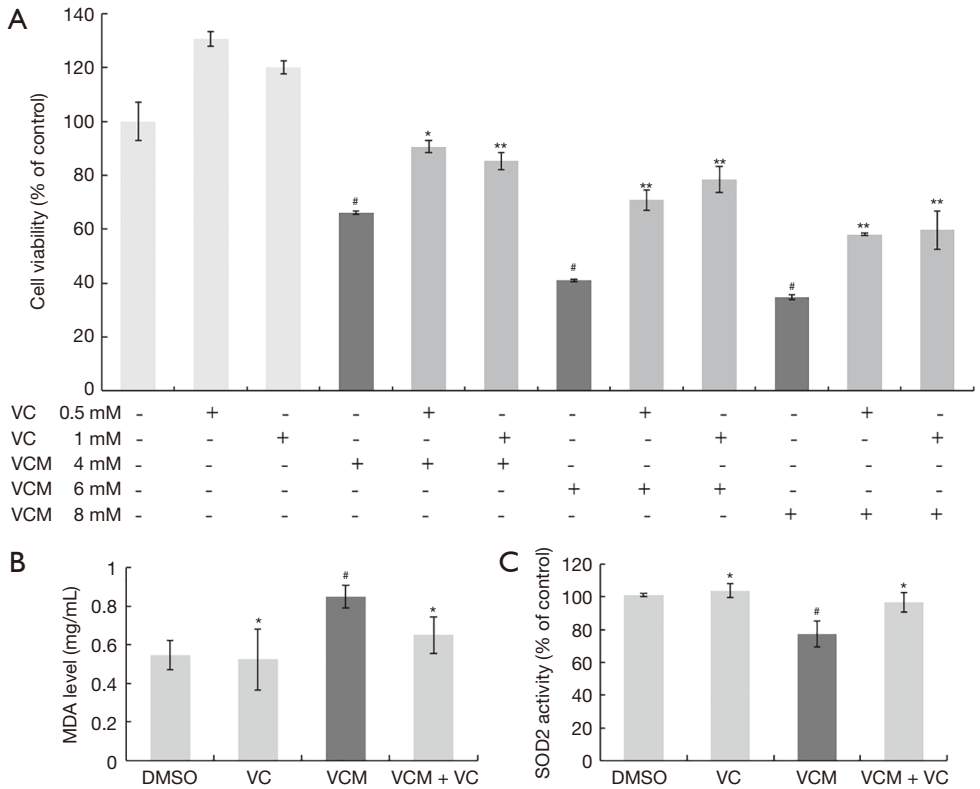

Figure 1 Protective effects of Vitamin C (VC) on vancomycin (VCM) induced cytotoxicity and oxidative stress in HK-2 cells. (A) Cell viability of dose-dependent VCM and VC. (B) 0.5 mM VC significantly reduced malondialdehyde (MDA) levels induced by 4 mM VCM. (C) 0.5 mM VC significantly reversed SOD2 activity inhibited by 4 mM VCM. Results are presented as group mean ± SD (n=6 in each group). *, indicates P>0.05 compared to DMSO group, **, indicates P<0.05 compared to DMSO group and relative VCM group, # indicates P<0.05 compared to DMSO group.

mRNA levels in the VC only group compared with control (*Figure 2A,B,C*). According to the western-blot test of IL-6, 0.5 mM VC significantly attenuated the protein expression activated by 4 mM VCM (*Figure 2D*). These results verified the inhibitory effect of VC on pro-inflammatory cytokines activation induced by VCM.

Discussion

VCM is considered as the first-line treatment against infections with Gram-positive bacteria (17), but its use is mostly limited by the nephrotoxicity. Animal experiments showed that VCM leads to pathological changes in kidney tissue in connection with oxidative stress (18,19). The antioxidant effects of high doses (100–200 mg/kg/day) of VC were effectively confirmed in tumor, severe diseases treatment and organ injuries *in vivo* (10,11). Overdose VC (4 g/kg) pretreatment had protective effects on VCM induced pathological changes in kidney tissue of mice by reducing renal cell apoptosis (14). However, the underlying mechanism is not clear. In the present study, we demonstrated that VC treatment led to a marked improvement in oxidative stress and inflammatory response in HK-2 cells, and the protective effect of VC might relate to its inhibition of ROS and NF-κB.

HK-2 cells incubated at 4, 6 and 8 mM VCM for 24 hrs appeared a dose-dependent decrease in cell viability from 66% to 34%. Co-treatment with 0.5 and 1 mM VC attenuated VCM induced cell death and increased the cell viability to 58–90%. 4 mM VCM exposure also resulted in a significant increase in oxidative stress indicator MDA and reduction in SOD2 according to our results, and was significantly reversed by 0.5 mM VC. These results indicated that VC attenuated VCM induced oxidative stress and prevented cell death.

The non-homeostatic states "oxidative stress" is caused

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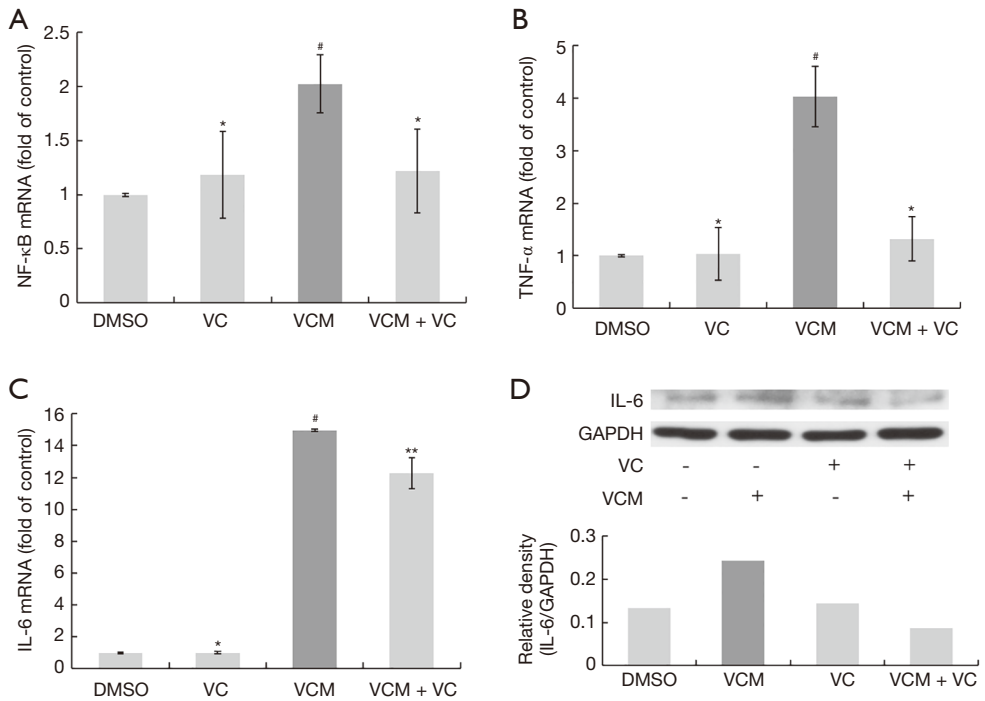

Figure 2 0.5 mM VC reversed 4 mM VCM induced protein expression of NF-κB, TNF-α and IL-6 in HK-2 cells. (A) Nuclear factor-κB (NF-κB) mRNA, (B) tumor necrosis factor-α (TNF-α) mRNA and (C) interleukin-6 (IL-6) mRNA in HK-2 cells are expressed relative to the level measured in DMSO group. (D) IL-6 protein expression in HK-2 cells was detected using Western-blot. Densitometric analysis was used to calculate the normalized protein ratio (IL-6 to GAPDH). * indicates P>0.05 compared to DMSO group, ** indicates P<0.05 compared to DMSO group and VCM group, # indicates P<0.05 compared to DMSO group.

by the over production of ROS (20). Superoxide dismutase SOD2 can effectively convert superoxide (one type of ROS) to less active hydrogen peroxide, the loss of SOD2 activity will lead to some metabolic diseases (21). ROS generated in cell can damage cellular macromolecules, including nucleic acids, phospholipids and proteins. ROS stimulation of mitochondrial lipid peroxidation may lead to inhibition of mitochondrial metabolism and mitochondrial dysfunction, such as abnormal in mitochondrial membrane potential and Ca^{2+} buffering capacity which plays a key role in certain modes of cell death (20,22). Overexpressed of lipid peroxidation product MDA and downregulation of SOD2 were found in many studies on VCM induced nephrotoxicity (18,23), and also in our results. Meanwhile, we found for the first time that the VC combination reduced cell death and MDA levels, and increased SOD2 activity in HK-2 cells under VCM treatment. This effect may be related to the antioxidant activity of VC.

Interstitial inflammations were found in histopathological examinations of kidney tissues from animals treated with VCM (19,24). Studies suggested that increased ROS, membrane lipid peroxidation and production of inflammatory chemokines may be the pathogenesis of VCM nephrotoxicity (25). According to the researches we found, NF-κB activated by ROS, the transcription factor that has pivotal roles in the immune response, inflammation, cell proliferation and apoptosis (26), may be involved in the inflammatory chemokines overexpression and also cell death. Phosphorylation and deactivation on inhibitors of NF-κB (IκB) caused by ROS stimulation of tyrosine protein kinase (27), and stimulation of NF-κB modulators IKKα and IKKβ through TNF-α induction (28) were considered as possible mechanisms for ROS acted as a messenger in NF-κB activation. The increased expression of NF-κB activates IL-6 promoter (29) and other pro-inflammatory cytokines including IL-1β and TNF-α, to promote inflammatory cascade and relative cell death (30). NF-κB signaling pathway has now become one of the potential targets for tumors associated with chronic inflammation (29,31). Inflammation response and upregulation of inflammatory

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cytokines were widely discovered in the researches on VCM induced nephrotoxicity (6,19,32). In our results, 4 mM VCM markedly induced the mRNA expression of NF-κB, IL-6, TNF-α and protein expression of IL-6 in HK-2 cells. The addition of VC reversed this effect with the inhibition of ROS. These findings suggested that VC can inhibit the inflammatory cascade associated with NF-κB through the reduced of oxidative stress.

In conclusion, we confirmed that VCM caused cell death by inducing oxidative stress and inflammatory response in HK-2 cells. VC attenuated the nephrotoxicity through decreasing lipid peroxidation and expression of inflammatory cytokines, and increasing SOD2 activity. This study showed for the first time that VC may alleviate VCM related renal cell injury by regulating ROS/NF-κB *in vitro*. Further studies are needed to discover the signals involved in the pathway. VC treatment may be a promising and effective method for the prevention of VCM induced nephrotoxicity.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/apm-20-694). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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