High-Dose Vitamin C Preadministration Reduces Vancomycin-Associated Nephrotoxicity in Mice

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Summary Vancomycin is recommended for treating severe infections caused by Gram-positive cocci, including methicillin-resistant Staphylococcus aureus. However, renal damage often occurs as a side effect because vancomycin is mainly excreted via the kidneys. The mechanism of vancomycin-associated nephrotoxicity is thought to involve the elevation of oxidative stress in the kidneys. Vitamin C (VC) has strong antioxidant properties; therefore, we evaluated the effect of high-dose VC preadministration on vancomycin-associated nephrotoxicity. Vancomycin was intraperitoneally injected into mice once daily for 7 d. Additionally, high-dose VC was intraperitoneally injected into mice at 30 min before vancomycin administration for 7 d. The plasma creatinine and urea nitrogen levels were increased by vancomycin treatment; however, high-dose VC preadministration suppressed the increase in these levels. Histological examination also revealed that high-dose VC preadministration reduced the characteristics of vancomycin-associated nephrotoxicity, such as dilated renal tubules with casts, the dilation of renal proximal tubules, and tubular epithelial desquamation. Furthermore, high-dose VC preadministration reduced the appearance of apoptotic cells presumably derived from the epithelial cells in the dilated proximal tubules. Thus, intraperitoneally injected high-dose VC preadministration reduced vancomycin-associated nephrotoxicity in mice. These novel findings may indicate that vancomycin-associated nephrotoxicity in humans may be reduced by high-dose VC preadministration.

Key Words ascorbic acid, CD10, creatinine, kidney, nephrotoxicity, urine, vancomycin, vitamin C

Vancomycin is a glycopeptide antibiotic that has been used to treat infections caused by Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus (1). Although vancomycin is widely used, nephrotoxicity is the most serious adverse effect of vancomycin (2–4). Vancomycin-associated nephrotoxicity has been reported to occur at high rates in recipient patients (5, 6). Therefore, renal failure associated with vancomycin has become an important clinical problem. Although several risk factors, such as high vancomycin trough concentration, severity of illness, intensive care unit residence, chronic kidney disease, old age, and concurrent nephrotoxin exposure, have been reported for vancomycin-associated nephrotoxicity (7–9), the mechanism is not fully understood. We previously reported time-dependent alterations in vancomycin-associated nephrotoxicity in mice (10). Vancomycin administration showed renal damage, and incipient renal failure began soon after the first treatment and progressively worsened. We also reported age-dependent alterations in vancomycin-associated nephrotoxicity in mice (11). Elderly mice were more likely to develop renal disturbance due to vancomycin treatment. Moreover, the proximal tubular epithelial cells of elderly mice are likely to undergo apoptosis by vancomycin treatment.

Vitamin C (VC) is a water-soluble antioxidant that scavenges reactive oxygen species (ROS) and is an essential micronutrient co-factor for numerous biosynthetic enzymes (12–14). Recently, high doses of VC administration have been reported to be effective as a cancer therapy (15–17). Thus, VC can safely be administered in large amounts in vivo. Moreover, Kadkhodaee et al. (18) reported that oral VC and vitamin E intake reduced gentamicin-associated nephrotoxicity in mice. In addition, Ocak et al. (19) also reported that oral VC intake reduced vancomycin-associated nephrotoxicity in rat. However, when considering clinical application...
in humans patients receiving vancomycin have severe symptoms and it is very difficult to take high-dose VC from their mouth. Therefore, in this study, we aimed to clarify whether intraperitoneally injected high-dose VC preadministration can reduce the renal failure caused by vancomycin. If it can reduce kidney damage, it can be a very useful treatment for vancomycin-induced renal damage in humans.

MATERIALS AND METHODS

Animals. The present study was carried out in accordance with the animal care and use protocol approved by the Institutional Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology (TMIG) (Permit Number: 17053) and the TMIG Guidelines for the Care and Use of Laboratory Animals. C57BL/6NCrSlc male mice aged 6 mo were obtained from the animal facility at the TMIG, Tokyo, Japan. All mice were fed CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) and maintained under a controlled photoperiod (12-h light/12-h dark).

Vancomycin and VC administration. All mice were randomly divided into three groups, as follows: (i) control (n = 10), (ii) vancomycin (n = 10), and (iii) vancomycin plus VC (n = 10); there were no significant differences in the mean body weights among the three groups. Each mouse in the vancomycin and vancomycin plus VC groups was intraperitoneally injected with vancomycin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) dissolved in saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) at a dose of 400 mg/kg body weight at 24 h intervals for 7 d (Fig. 1A). The dose and duration of vancomycin treatment were based on our previous study that confirmed the occurrence of renal damage in mice (10). Additionally, each mouse in the vancomycin plus VC group was intraperitoneally injected with VC (L(+)-ascorbic acid sodium salt. Nacalai Tesque, Inc., Kyoto, Japan) dissolved in saline, which was administered at 30 min before vancomycin administration at a high dose of 4 g/kg body weight at 24 h intervals for 7 d (Fig. 1A). To collect urine, the mice were housed individually in metabolic cages (Tecniplast Japan, Co., Ltd., Tokyo, Japan) for 24 h after the last injection at 7 d. The mice were then sacrificed under anesthesia with pentobarbital. As a control, the mice were sacrificed after being housed for 24 h in metabolic cages. The blood was collected from the inferior vena cava, anticogulated with ethylenediaminetetraacetic acid and centrifuged at 880 × g for 10 min at 4 ºC. The resulting supernatants were used as plasma for further analysis. The collected urine and plasma were stored at −80 ºC until use. The mice were then perfused systematically with ice-cold phosphate buffered saline through the left ventricle to wash the blood from the tissues. The kidneys were removed and then weighed.

Biochemical analysis. The plasma creatinine and blood urea nitrogen levels were measured for biochemical analysis (Oriental Yeast Co., Ltd.).

VC determination. The plasma, kidneys, and urine VC levels were measured by HPLC and an electrochemical detector as described previously (20).

Histological analysis. Each kidney was divided into longitudinal sections of the same size and fixed with 10% neutral-buffered formalin (FUJIFILM Wako Pure Chemical Corporation) for 48 h and embedded in paraffin. The sections were serially cut into 4 μm thick sections and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and azan staining. The virtual slide imaging system, NanoZoomer 2.0-LS (Hamamatsu Photonics, Hamamatsu, Japan), was used to evaluate kidney section. To estimate the degree of tubular dilation, twenty tubules in H&E-stained kidney sections were randomly selected, and the minor axis of each section was measured using NDP.view2 (Hamamatsu Photonics). Additionally, the number of casts in the renal tubules and tubular epithelial desquamation were counted in a single randomly selected 1 mm² field from the H&E- and PAS-stained sections, respectively. Furthermore, to estimate interstitial fibrosis, we measured the percentages of azan-positive areas using WinROOF Ver. 7.4.1 image analysis software (MITANI Corporation, Fukui, Japan) in a single randomly selected 0.3 mm² field from the azan-stained kidney sections.

Immunohistochemistry. To confirm the detailed site of kidney damage, we stained the kidney sections with a rabbit polyclonal anti-CD10 antibody (1 : 400 dilution, ab109275, Abcam plc, Cambridge, UK) to verify the proximal tubule damage (21); a rabbit polyclonal anti-single-stranded DNA (ssDNA) antibody (1 : 400 dilution, A4506, DAKO Japan Co., Ltd., Tokyo, Japan), which is a marker of apoptosis (22); and the VECTASTAIN Elite ABC kit (VECTOR Laboratories, INC., Burlingame, CA) with 3,3'-diaminobenzidine as the chromogenic substrate. The numbers of CD10-positive dilated proximal tubules were counted in one randomly selected 1 mm² field from the kidney sections stained with the anti-CD10 antibody. Additionally, the numbers of ssDNA-positive cells were counted in a single randomly selected 0.3 mm² field from the kidney sections stained with the ssDNA antibody.

Statistical analysis. Statistical analyses were carried out with GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA). All dates are presented as the means ± standard errors of the means (SE). The probability of significant differences among experimental groups was determined using a Tukey-Kramer test in post-hoc analysis. p < 0.05 was considered statistically significant.

RESULTS

Body and kidney weights

Vancomycin and high doses of VC were administered to the mice once a day for 7 d (Fig. 1A). Day 8 refers to 24 h after the last vancomycin administration. The mean body weights of the vancomycin group mice at 8 d were significantly lower than those of the control group mice (Fig. 1B), but the mean body weights of the vancomycin and vancomycin plus VC groups were comparable. The mean kidney weights of the vancomycin group mice were significantly higher than those of the vancomycin plus VC and control group mice (Fig. 1C).
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Biochemical analysis

The plasma creatinine and blood urea nitrogen levels of the vancomycin group mice were significantly higher than those of the vancomycin plus VC and control group mice (Fig. 1D, 1E).

VC levels in the plasma, kidneys, and urine

The VC levels in the plasma of the vancomycin group mice were significantly higher than those of the control group mice, but there was no difference between the VC levels in the plasma of the vancomycin and vancomycin plus VC groups (Fig. 2). Moreover, there were no differences in the VC levels in the kidneys among the three groups. On the other hand, the VC levels in the urine of the vancomycin group mice were significantly lower than those of the vancomycin plus VC group mice. Additionally, there was a significant difference between the vancomycin plus VC and control groups.

Histological analysis of the kidneys

H&E staining of the kidney sections from the vancomycin group mice revealed renal tubular degeneration, such as blush-border atrophy, epithelial desquamation and apoptosis/necrosis of the tubular epithelium (Fig. 3A, white arrowheads). However, the renal damage in the vancomycin plus VC group was mild. PAS staining of the kidney sections from the vancomycin group mice revealed tubular epithelial desquamation and hyaline casts (Fig. 3A, black arrowheads), and the renal damage in the vancomycin plus VC group was slight, although there was no damage in those from the control group mice. Moreover, the azan staining of the kidneys from the vancomycin group mice showed interstitial fibrosis in the kidneys (Fig. 3A). However, marked interstitial fibrosis was not observed in the kidneys of mice from the vancomycin plus VC and control groups.
The mean minor axes of the renal tubules in the vancomycin group mice were significantly larger than those in the vancomycin plus VC and control group mice (Fig. 3B). The numbers of casts in the renal tubules of the vancomycin group mice were significantly larger than those of the vancomycin plus VC group mice (Fig. 3C). There were no casts in the renal tubules from control mice. The number of tubules exhibiting epithelial desquamation in the kidneys from the vancomycin group mice was significantly higher than those from the vancomycin plus VC group mice (Fig. 3D). However, the control mice did not display detectable tubular epithelial desquamation. The percentage of areas with interstitial fibrosis in the kidneys from the vancomycin group mice were significantly higher than those from the vancomycin plus VC and control group mice (Fig. 3E).

**Immunohistochemical staining of CD10 in the kidneys**

Immunohistochemical staining of CD10 in the kidneys revealed the dilation of the renal proximal tubules in the kidneys from the vancomycin group mice (Fig. 4A, white arrows). The numbers of CD10-positive dilated proximal tubules from the vancomycin group mice were significantly higher than those from the vancomycin plus VC group mice (Fig. 4B). No detectable dilated proximal tubules were observed in the renal tubules from the control group mice.

**The detection of apoptotic cells in the kidneys**

Numerous ssDNA-positive apoptotic cells or cell bodies were present in the renal tubules of the kidneys from the vancomycin group mice (Fig. 4A, white arrowheads). Additionally, few ssDNA-positive apoptotic cells or cell bodies were present in the dilated renal tubules from the vancomycin plus VC group mice. However, no detectable ssDNA-positive apoptotic cells were observed in the kidneys from the control mice (Fig. 4A). The numbers of ssDNA-positive apoptotic cells from the vanco-
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In this study, high-dose VC preadministration reduced vancomycin-associated nephrotoxicity, such as dilated renal tubules with casts, tubular epithelial desquamation, and the dilation of renal proximal tubules. Furthermore, high-dose VC preadministration reduced the appearance of apoptotic cells that were presumably derived from epithelial cells in dilated proximal tubules.

In the vancomycin group mice, the body weights were decreased and, conversely, the kidney weights were increased at 8 d. The cause of body weight loss was considered to be renal failure, and the kidney weight increase resulted from interstitial fibrosis and renal damage. In fact, plasma creatinine and blood urea nitrogen levels were increased by vancomycin treatment in the vancomycin group mice at 8 d. Previously, we studied the time-dependent alteration in kidney damage due to vancomycin administration and confirmed that body weight decreases and kidney lesions worsened as kidney damage progressed (10). Thus, high-dose VC preadministration is believed to reduce the renal damage that results in body weight loss and kidney lesions from vancomycin treatment.

Histologically, the renal damage, such as dilated renal tubules with casts, tubular epithelial desquamation, and interstitial fibrosis, was severe in the kidneys of the vancomycin group mice, and slight damage was observed in the vancomycin plus VC group mice, but the degree was very mild. Thus, high-dose VC preadministration was believed to reduce renal damage by vancomycin administration.

Arimura et al. (23) reported that vancomycin caused the increased production of intracellular ROS by the inactivation of mitochondrial complex I in cultured LLC-PK1 cells. Additionally, Sakamoto et al. (24) reported that cardiolipin peroxidation mediates vancomycin-associated intracellular ROS production and the initiation of apoptosis in proximal tubular epithelial cells. In this study, the numbers of ssDNA-positive apoptotic cells from the vancomycin plus VC group mice were significantly lower than those from the vancomycin group mice. These results suggested that high-dose VC preadministration reduced oxidative stress and reduced vancomycin-associated nephrotoxicity. Further, Oacak et al. (19) reported that oral VC administration reduced vancomycin-associated nephrotoxicity in rat. In this report, oral VC administration decreased the plasma creatinine and blood urea nitrogen levels increased by vancomycin, however, it did not completely reduce vancomycin-associated nephrotoxicity to the same level as controls that did not receive vancomycin. In the present study, the plasma creatinine and blood urea nitrogen levels of vancomycin plus VC and control group mice were almost same levels. These results suggested that intra-peritoneal high-dose VC preadministration is more effective for reduction of vancomycin-associated nephrotoxicity than oral VC administration.

In the present study, the VC levels in the plasma and kidneys from the vancomycin group mice were higher than those from the vancomycin plus VC and control group mice, despite the barely detectable VC levels in the urine. VC is usually be excreted from the kidneys into the urine (25). Therefore, the higher VC levels in the plasma and kidneys of the vancomycin group mice is thought to be due to the inhibition of VC excretion from the kidneys into the urine due to renal failure.

The present study has one limitation: we did not assess the blood vancomycin concentration of the mice. Therefore, we were not able to exclude the possibility that the blood vancomycin concentration differed between the vancomycin and vancomycin plus VC group mice. Additional studies are required to clarify this point.

In conclusion, high-dose VC preadministration reduced vancomycin-associated nephrotoxicity in mice. This novel method suggests that vancomycin-associated nephrotoxicity in humans can be reduced by high doses of VC preadministration.

Disclosure of state of COI

No conflicts of interest to be declared.

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Author contributions

MT, TY, YT, SM, TK, JL, TA, HT, TI, YM, and AI designed the research. MT, TY, YT, and AI conducted the experiments, and MT, TY, YT, TM, TK, JL, TA, HT, TI, YM, and AI analyzed the data. MT, TY, YT, SM, TK, JL, TA, HT, TI, YM, and AI wrote the manuscript and had primary responsibility for the final content of the manuscript.

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