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Protective Effects of Caffeic Acid Phenethyl Ester, Vitamin C, Vitamin E and *N*-Acetylcysteine on Vancomycin-Induced Nephrotoxicity in Rats

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Abstract: The objective of this study was to compare the beneficial effects of caffeic acid phenethyl ester (CAPE), vitamin C, vitamin E and *N*-acetylcysteine on vancomycin-induced nephrotoxicity. Thirty rats were randomly devided into six groups: (i) control; (ii) vancomycin, 200 mg/kg administrated via intraperitoneal route; (iii) vancomycin plus CAPE – vancomycin with 10 μmol/kg CAPE; (iv) vancomycin plus vitamin C – vancomycin (intraperitoneally) with 200 mg/dl vitamin C in drinking water; (v) vancomycin plus vitamin E – vancomycin with 1000 mg/kg body weight vitamin E (intramuscularly); and (vi) vancomycin plus *N*-acetylcysteine – vancomycin with 10 mg/kg body weight (intraperitoneally) of *N*-acetylcysteine. Vancomycin treatments were started 1 day after the first administrations of these agents and continued for 7 days. At the end of the experiments, catalase activity was significantly decreased by vancomycin in kidney homogenates ($P < 0.05$). Vitamin E, vitamin C, *N*-acetylcysteine and CAPE administrations decreased the blood urea nitrogen levels increased by vancomycin, although significant differences were detected only in the vitamins E and C groups ($P < 0.05$). Increased renal malondialdehyde and nitric oxide levels by vancomycin were significantly suppressed by agents used in the study ($P < 0.05$). Histopathological examination demonstrated prominent damages in the vancomycin-treated group. Vitamin E was the most beneficial agent on vancomycin-induced tubular damage, followed by vitamin C, *N*-acetylcysteine and CAPE treatments, respectively. The data suggest that vitamin E, as well as vitamin C, *N*-acetylcysteine and CAPE, could be useful for reducing the detrimental effects on vancomycin-induced toxicity in kidneys.

Vancomycin has been used more frequently in recent years because of the rising incidence of infections caused by methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* [1]. Vancomycin is mainly excreted via the kidneys. The dose and duration of administration are limited because vancomycin induces renal dysfunction [2,3]. The mechanism of vancomycin-induced renal injury is not fully understood. The reason might be indirectly generated reactive oxygen species (ROS) associated with inflammatory events *in vivo*. Oxidative stress may underlie in the pathogenesis of vancomycin-induced nephrotoxicity [4]. ROS have been reported to be the causative agents of cell death in various models of toxic renal failure, including vancomycin, aminoglycosides, lithium and cisplatin applications [5–9]. ROS may produce cellular injury via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage [10].

Caffeic acid phenethyl ester (CAPE), a main active ingredient of honeybee propolis, has been used in folk medicine in the treatment of various diseases. CAPE has several biological properties such as antioxidant, anti-inflammatory, antiviral and anticarcinogenic features. It has been shown that CAPE treatments protect tissues from ROS-mediated oxidative stress and reduce lipid peroxidation in ischaemia reperfusion and toxic injuries [5,7,11,12]. CAPE at a dose of 10 μmol/kg completely inhibits the production of ROS in human neutrophils, as well as blocks the xanthine oxidase system [13]. It has also been tested for prevention of kidney damage induced by numerous agents such as gentamicin [5,14], amikacin [15], cisplatin [9], lithium [8] and doxorubicin [16].

N-Acetylcysteine has antioxidant properties and, as a sulfhydryl donor, may contribute to the regeneration of endothelium-derived relaxing factor and glutathione [17,18]. *N*-Acetylcysteine reacts with the hydroxyl radical and hypochlorous acid and is poorly reactive with hydrogen peroxide and the superoxide radical [19]. *N*-Acetylcysteine inhibits the induction of pro-inflammatory cytokines and blocks the tumour necrosis factor-α (TNF-α)-induced apoptotic cell death [20]. Therefore, *N*-acetylcysteine is widely used in clinical practice, in a variety of disease states, including paracetamol and pulmonary oxygen toxicities, treatment of ischaemic damage toxicities, liver, and experimental ischaemic skin flaps [19]. *N*-Acetylcysteine has also been demonstrated to be effective in the prevention of hypoperfusion and toxin-induced renal failure [18,21].

It is well known that vitamin C (ascorbic acid) and vitamin E (α-tocopherol) have strong antioxidant properties and that they decrease oxidative stress. These vitamins have been injury [22]. Vitamin C and/or vitamin E have been tested for the prevention of kidney damage induced by drugs such as

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gentamicin [23], cisplatin [24], sodium chromate and thallium [25] and cyclosporine [26].

There are no reports concerning a protective role of CAPE, *N*-acetylcysteine, vitamin C and vitamin E on vancomycin-induced nephrotoxicity. In the present study, we aimed at investigating the effects of CAPE, *N*-acetylcysteine, vitamin C and vitamin E on vancomycin-induced nephrotoxicity in a rat model.

Materials and Methods

Animals and experimental conditions. Experimental procedures were approved by the Veterinary Faculty Ethics Committee, Mustafa Kemal University (Antakya, Turkey), for the use and care of laboratory animals. Thirty male Wistar albino rats, weighing 200–250 g, were purchased from the Laboratory Animal Production Unit of Firat University (Elazig, Turkey). All rats were kept in an environment with controlled temperature (21 ± 2°C) and humidity (55–60%), and they were acclimated for 2 weeks before the start of the experiment. They were housed in a facility with a 12-hr light:dark cycle. All animals were fed balanced rat chow (Elazig Yem Sanayi, Elazig, Turkey) and were provided tap water to drink *ad libitum*.

The rats were randomly divided into six groups consisting of five rats per group: (i) control, (ii) vancomycin, (iii) vancomycin plus CAPE, (iv) vancomycin plus vitamin C, (v) vancomycin plus vitamin E and (vi) vancomycin plus *N*-acetylcysteine. Vancomycin treatments were started 1 day after the first administrations of CAPE, vitamin C, vitamin E and *N*-acetylcysteine, and continued at 24-hr intervals for 7 days. CAPE, vitamin C, vitamin E and *N*-acetylcysteine treatments were repeated at 24-hr intervals for 8 days.

Vancomycin (Vancocin-CP flacon 0.5 g, Lilly, Istanbul, Turkey) was injected intraperitoneally at a dose of 200 mg/kg, which is the dosage scheme reported to cause marked nephrotoxicity in rats [1,7].

Caffeic acid phenethyl ester (Sigma Aldrich, Steinheim, Germany) was dissolved in ethanol and diluted with sterile saline solution to give a final concentration of 5% ethanol. CAPE was injected intraperitoneally at a dose of 10 μmol/kg, which is the dosage scheme reported to protect marked nephrotoxicity caused by other drugs such as gentamicin [5,14], amikacin [15] and doxorubicin [16].

Vitamin C (Redoxan effervescent tablet 1000 mg, Roche, Turkey) was added to the drinking water with 200 mg/dl. Kadkhodaee et al. reported that 100 mg vitamin C supplementation protects rats from marked nephrotoxicity caused by gentamicin [23].

Vitamin E (Evigen ampul 300 mg, Mefar Ilac San., Turkey) 1000 mg/kg body weight was injected intramuscularly. This dosage scheme was reported to protect from marked nephrotoxicity caused by gentamicin [23] and cisplatin [24].

N-Acetylcysteine (Asist ampul 300 mg, Husnu Arsan Ilac San., Turkey) was injected intraperitoneally at a dose of 10 mg/kg body weight, which was the dosage scheme reported to protect marked nephrotoxicity caused by gentamicin [18].

Sacrifice of animals. Under ketamine hydrochloride anaesthesia blood samples were collected from the inferior vena cava, both kidneys were then extirpated and one of them stored in 10% formalin solution for histopathological evaluation. The other was washed with cold saline solution and stored at −20°C until processing for biochemical analyses. The whole blood samples were centrifuged at 1500 ×g at 4°C for 10 min., and the plasma was separated and stored at −20°C until processing for biochemical analyses.

Determination of blood urea nitrogen and creatinin levels. Blood urea nitrogen (BUN) and creatinine were determined in an autoanalyser (Olympus Instruments, Tokyo, Japan). Results were expressed as mg/dl.

Tissue homogenizations and protein determination. Approximately 1 g of kidney slice was homogenized (1:10, w/v) in ice-cold phosphate buffer (pH 7.4) containing 50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid and complete protease inhibitor cocktail using a glass Teflon homogenizer. The homogenate was centrifuged at 15,000 ×g for 20 min. to remove debris. The supernatant was used for analyses of selected biochemical parameters. The whole procedure was carried out at 4°C. Protein concentrations of supernatants were measured by the Bradford method [27] using bovine serum albumin as a standard.

Determination of malondialdehyde levels in kidney homogenates. Malondialdehyde levels, an indicator of lipid peroxidation, were estimated by the method described Yoshioka et al. [28]. The principle of the method is the spectrophotometric measurement of the colour generated by the reaction of thiobarbituric acid (TBA) with malondialdehyde. For this purpose, 2.5 ml of 100 g/l trichloroacetic acid solution and 1.0 ml of 6.7 g/l TBA solution were added to 0.5 ml supernatant in each centrifuge tube, and the tubes were placed in a boiling water bath for 30 min. The tubes were immediately cooled in ice-cold water, and 4 ml of n-butanol was added. After centrifugation at 1500 g for 10 min., the absorbance intensity of the upper n-butanol phase was measured by a spectrophotometer (Shimadzu UV-1208, Kyoto, Japan) at 535 nm. Values were compared to a series of standard solutions (1,1,3,3-tetramethoxypropane). Results were expressed as nmol/g protein.

Determination of nitric oxide levels in kidney homogenates. Tissue nitrite (NO_2^-) and nitrate (NO_3^-) levels were determined as an index of nitric oxide production because of the very short life of the nitric oxide molecule in biological specimens. The method for kidney nitrite and nitrate levels was based on the Griess reaction [29]. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite + nitrate) were measured after conversion of nitrate to nitrite by copperized cadmium granules. Absorbance values of samples were detected at 546 nm. Results were expressed as μmol/g protein.

Determination of catalase activity in kidney homogenates. Catalase (EC 1.11.1.6) activity was determined according to the method of Aebi [30]. The principle of the assay is based on the determination of the rate constant k (dimensions: $s - 1$, k) of hydrogen peroxide decomposition at 240 nm. By using the absorbance change per minute, the rate constant of the enzyme was determined. Activities were expressed as k (rate constant) per g protein.

Histopathological examination of kidneys. For light microscopic evaluation, portions of each kidney were sectioned and fixed in 10% neutral phosphate-buffered formaldehyde and embedded in paraffin. These specimens were cut into 6-mm thick section and stained with haematoxylin and eosin and examined blindly by a pathologist who was unaware of the treatment regimens used. Light microscopy (Olympus BH-2, Tokyo, Japan) was used to evaluate semi-quantitative analysis of the kidney sections. The kidneys were examined for tubular epithelial alterations (dilatation, desquamation, vacuolization, necrosis, atrophy), interstitial inflammation, oedema and glomerular alterations. All histopathological parameters were graded as follows: (–) showing no changes; mild (+) single cell necrosis, slight degenerative changes, few foci of dilatation, casts, inflammatory infiltration and oedema; moderate (++) for all changes at different foci throughout the kidney; and severe (+++) extensive and marked changes.

Statistical analyses. Biochemical data were analysed by one-way ANOVA test. Post-hoc comparisons were done using Tukey's tests. Results were expressed as mean ± S.D. Differences were considered significant at $P < 0.05$.

Table 1.

Blood urea nitrogen (BUN) and creatinine (Cr) levels in plasma and malondialdehyde (MDA) and nitric oxide levels and catalase (CAT) activity in kidney homogenates of rats.

Parameters	Control group	VCM	VCM + CAPE	VCM + Vitamin C	VCM + Vitamin E	VCM + NAC
BUN (mg/dl)	28.6 ± 4.2	53.2 ± 4.1*	45.8 ± 3.6	44.0 ± 2.0†	43.2 ± 1.3†	46.0 ± 2.4
Creatinine (mg/dl)	0.53 ± 0.02	0.78 ± 0.03*	0.63 ± 0.02*	0.61 ± 0.03†	0.58 ± 0.01†	0.65 ± 0.02†
MDA (nmol/g protein)	2.63 ± 0.5	3.65 ± 0.5*	3.34 ± 0.4†	3.21 ± 0.3†	3.16 ± 0.4†	3.30 ± 0.5†
NO (μmol/g tissue)	113 ± 5.4	158 ± 4.0*	135 ± 3.3†	133 ± 3.7†	131 ± 1.8†	134 ± 3.1†
CAT (k/g protein)	1.31 ± 0.04	1.14 ± 0.06*	1.18 ± 0.04	1.20 ± 0.05	1.21 ± 0.05	1.16 ± 0.05

CAPE, caffeic acid phenethyl ester; NAC, *N*-acetylcysteine; NO, nitric oxide; VCM, vancomycin. *$P < 0.05$ versus control group; †$P < 0.05$ versus vancomycin group.

Results

The plasma BUN level in the vancomycin group were significantly increased compared to the control group ($P < 0.05$). The vancomycin-increased plasma BUN level was significantly decreased by vitamins C and E treatments only ($P < 0.05$). Plasma creatinine levels were elevated compared to the control group in the vancomycin group ($P < 0.05$). Significant differences were detected in plasma creatinine levels of vancomycin plus CAPE, vitamin C, vitamin E and *N*-acetylcysteine groups compared to the vancomycin group ($P > 0.05$) (table 1).

Vancomycin significantly increased the malondialdehyde and nitric oxide levels in kidney tissue homogenates in comparison to control ($P < 0.05$). Co-administration of CAPE, vitamin C, vitamin E or *N*-acetylcysteine with vancomycin decreased the malondialdehyde levels compared to the vancomycin group ($P < 0.05$). The lowest levels of malondialdehyde and nitric oxide were observed in the vancomycin plus vitamin E group, followed by vitamin C, *N*-acetylcysteine and CAPE groups, respectively (table 1).

Vancomycin administration decreased catalase activities in kidney tissues compared to control group ($P<0.05$). Co-administration of CAPE, vitamin C, vitamin E or *N*-acetylcysteine in vancomycin-treated animals increased the catalase activities, although this differences were not statistically significant ($P > 0.05$) (table 1).

During the histopathological examinations tubular necrosis, degeneration, vacuolization, interstitial oedema, tubular atrophy and inflammatory cell infiltration were detected and scored (table 2). Vancomycin administration caused histopathologically prominent damage in the kidneys (fig. 1). These histopathological damages were reversed by CAPE, vitamin C, vitamin E and *N*-acetylcysteine treatments (table 2). The weak histopathological damages were observed in the vancomycin plus vitamin E group, followed by the vitamin C, *N*-acetylcysteine and CAPE groups, respectively. The histopathological figures of kidneys in vancomycin plus CAPE, vitamin C, vitamin E and *N*-acetylcysteine groups are presented in fig. 2.

Discussion

The nephrotoxicity related to vancomycin therapy has been reported to be 5–25% and even 35% when combined with an aminoglycoside antibiotic [2,3]. The mechanism of vancomycin-induced renal dysfunction is not completely known [2,31].

Reactive oxygen species (ROS) have been proposed as the causative factors of the renal side-effects of the drug [5–7,9,23]. Lipid peroxidation, mediated by oxygen-free radicals, is believed to be an important cause of destruction and damage to cell membrane. Malondialdehyde is formed during oxidative degeneration and is accepted as an indicator

Table 2.

Score levels for histopathological changes of vancomycin administration to rats with or without caffeic acid phenethyl ester (CAPE), vitamin C, vitamin E and *N*-acetylcysteine.

Histopathological parameters	Control	VCM	VCM + CAPE	VCM + Vitamin C	VCM + Vitamin E	VCM + NAC
Interstitial oedema	–	+++	+	+	+	++
Interstitial inflammation	–	++	+	+	+	+
Glomerular damage	–	+	–	–	–	–
Disarrangement of tubule	–	++	++	+	+	+
Tubular atrophy	–	++	+	+	–	+
Epithelial vacuolization	–	+++	++	++	+	++
Epithelial desquamation	–	+++	++	+	+	++
Tubular necrosis	–	++	–	–	–	–
Tubular dilatation	–	++	+	–	–	–

NAC, *N*-acetylcysteine; VCM, vancomycin. Histopathological evaluation of the experimental parameters was scored as follows: score (–): no meaningful histopathological change; score (+): mild degree; score (++): moderate degree; score (+++): severe degree

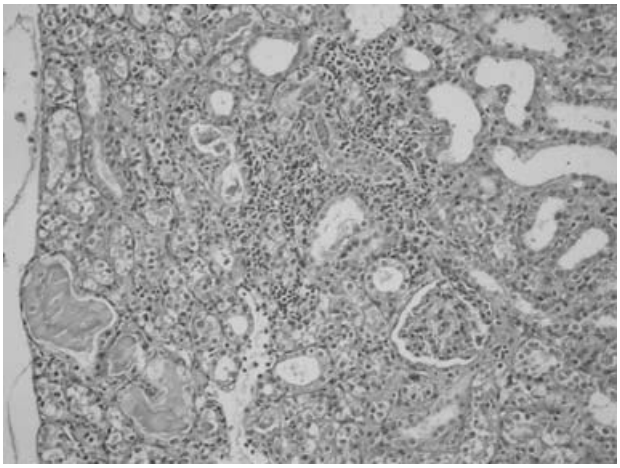

Fig. 1. Epithelial desquamation, tubular distortion, epithelial vacuolization, tubular necrosis, tubular dilatation, interstitial oedema and inflammatory cell infiltration were detected in kidney slices of the vancomycin-treated group (haematoxylin and eosin staining, magnification ×200).

of lipid peroxidation [32,33]. The low catalase activity could aggravate the oxidative damage in the renal tissue. Over-production of nitric oxide results in peroxynitrite, a potent and aggressive cellular oxidant. High nitric oxide production might be related to the inflammatory response of the tissue [16], and oxidative stress to the pathogenesis of vancomycin-induced nephrotoxicity [1,4,7,32]. Some studies have reported that vancomycin administration significantly increased plasma BUN and creatinine levels [1,32,34]. Oktem et al. have shown that vancomycin administration decreases catalase and increases BUN, creatinine and malondialdehyde levels [7]. In the present study, we also found that vancomycin-induced nephrotoxicity was associated with low activity of catalase ($P < 0.05$) and increased levels of malondialdehyde and nitric oxide in the renal tissues ($P < 0.05$). Moreover, we found that BUN and creatinine levels were increased compared to the control group ($P < 0.05$). These results suggest that oxidative stress may underlie the pathogenesis of vancomycin-induced nephrotoxicity.

Caffeic acid phenethyl ester has recently been shown to possess free radical scavenging ability, anti-inflammatory, and lipid peroxidation reduction effects [5,14,16]. It has been reported that 10 μmol/kg of CAPE act as a potent scavenger of free radicals in the prevention of kidney damage induced by drugs such as gentamicin [5,14], amikacin [15] and doxorubicin [16]. It has been shown that CAPE administration reduces gentamicin-induced renal injury, malondialdehyde and nitric oxide production [5,14]. Similarly, we have demonstrated that CAPE decreases vancomycin-elevated plasma creatinine ($P < 0.05$) and homogenate malondialdehyde and nitric oxide levels ($P < 0.05$) and slightly increases catalase activity ($P > 0.05$). The data suggest that free radicals may have a critical role in vancomycin-induced injury and that supplementation with CAPE may be helpful in reducing vancomycin nephrotoxicity.

One of the vital roles of vitamin C is to act as an antioxidant to protect cellular components from free radical damage as also does vitamin E [35,36]. Administration of vitamin E results in inhibition of lipid peroxidation level on gentamicin, and cisplatin-induced nephrotoxicity in rats [37,38]. Co-administration of vitamins C and E has protective effects on gentamicin-induced nephrotoxicity [23]. In the current study, vitamins C and E suppressed vancomycin-induced malondialdehyde, BUN and creatinine levels, and prevented high nitric oxide production ($P < 0.05$). The results suggest that supplementation of vitamins C and E may be useful in reducing vancomycin-induced nephrotoxicity.

N-Acetylcysteine has been shown to be effective in the prevention of nephrotoxicity in human beings and in animal models because of its antioxidant properties [17,18], but its effect on vancomycin nephrotoxicity has not yet been evaluated. Administration of *N*-acetylcysteine has beneficial effects on renal preservation in adriamycin-induced nephrotoxicity [39]. *N*-Acetylcysteine also has protective effects in gentamicin and lithium-induced nephrotoxicity in rats [18,40]. In this study, *N*-acetylcysteine decreased homogenate malondialdehyde and nitric oxide levels ($P < 0.05$) and plasma creatinine ($P < 0.05$). Therefore, it might be that *N*-acetylcysteine prevents vancomycin-induced nephrotoxicity.

Several studies on the mechanism of nephrotoxicity have generally focused on the role of the renal tubules in the excretion of vancomycin [31,41]. The cause of tubular cell degeneration might be explained by an increased uptake of vancomycin by kidneys [42]. In the present study, the glomeruli appeared normal in all groups. The vancomycin group exhibited interstitial oedema, epithelial vacuolization and epithelial desquamation. This shows that vancomycin causes degeneration of the tubular cells. CAPE, vitamin C, vitamin E and *N*-acetylcysteine decreased the histological damage caused by vancomycin-induced nephrotoxicity, but they did not completely prevent the renal disorders. The vancomycin plus vitamins C and E groups showed mild epithelial desquamation, interstitial oedema and disarrangement of tubules. Vancomycin plus CAPE administration reduced less the extent of tubular damage than did *N*-acetylcysteine. The largest reduction in tubular damage caused by vancomycin was found in the vancomycin plus vitamin E group, followed by the vitamin C, *N*-acetylcysteine and CAPE groups, respectively. These findings suggest that vitamins E and C may have beneficial effects on the tubulointerstitial damage in vancomycin-induced nephrotoxicity.

We conclude that oxidative tubular damage plays an important role in vancomycin-induced nephrotoxicity. Our results also show that vitamin E, vitamin C, *N*-acetylcysteine and CAPE could be used for reduction of the nephrotoxic effects of vancomycin. However, vitamin E was the most effective agent as evaluated on the basis of the biochemical and pathological findings.

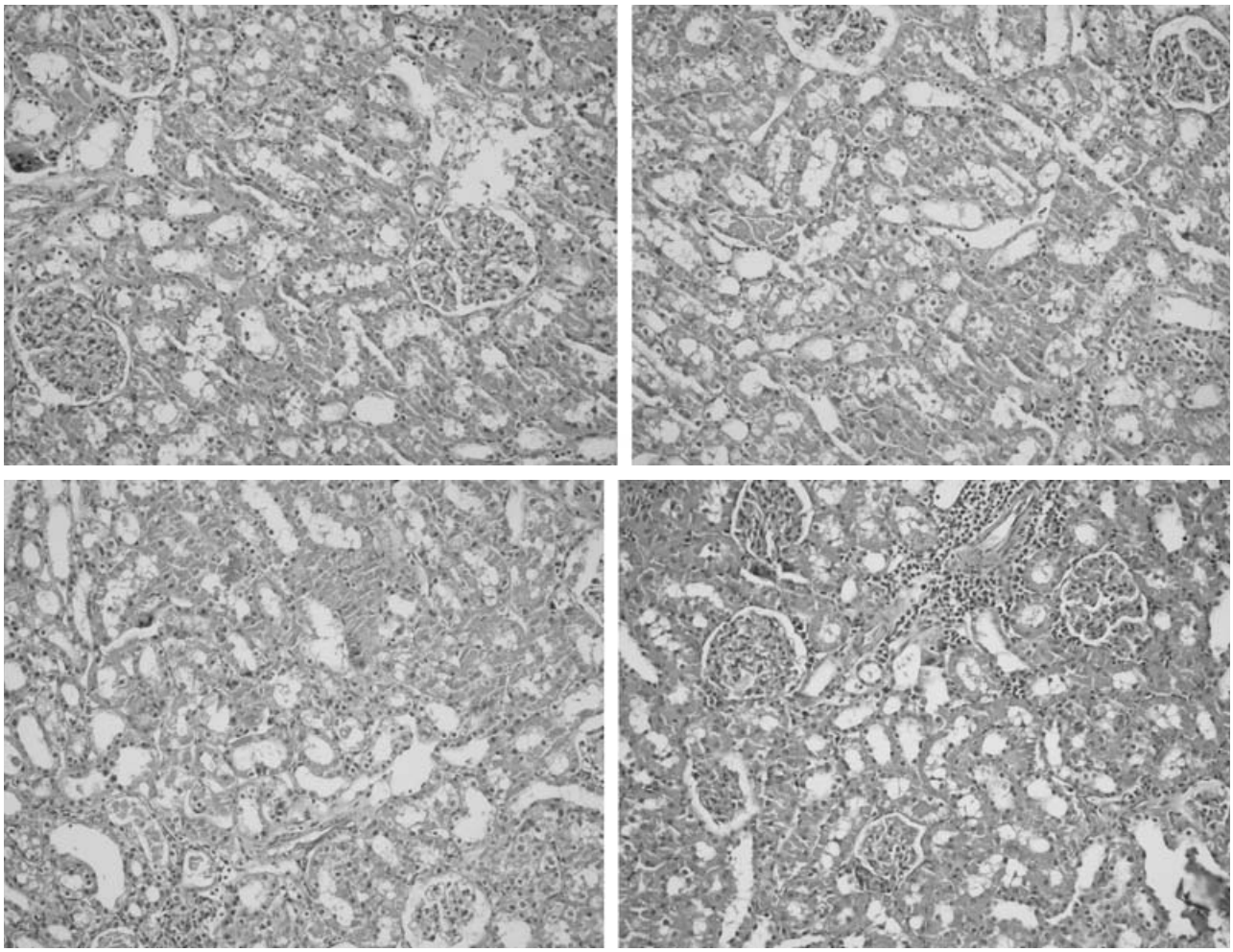

Fig. 2. Top left panel represents lowest histopathological damages such as minimal inflammation, minimal tubular distortion, epithelial vacuolization and desquamation in the vancomycin plus vitamin E group. Top right panel represents interstitial oedema, epithelial vacuolization and tubular dilatation in the vancomycin plus vitamin C group. Bottom left panel represents interstitial oedema, minimal inflammation, epithelial vacuolization and desquamation in the vancomycin plus *N*-acetylcysteine group. Bottom right panel represents interstitial oedema, tubular dilatation, epithelial vacuolization and desquamation in the vancomycin plus CAPE group (haematoxylin and eosin staining, magnification ×200).

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