Effects of co-supplementation of vitamins E and C on gentamicin-induced nephrotoxicity in rat

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Gentamicin (GM) is an effective antibiotic against severe gram-negative infections. However, it can produce nephrotoxicity in human. Reactive oxygen species (ROS) have been proposed as the causative factors of the renal side effects the drug. This study was performed to investigate the protective role of antioxidant vitamins against GM-mediated nephropathy in an in situ model of isolated rat kidney. Male Sprague-Dawley rats were randomly assigned to one of the following groups of seven rats: group 1 (Control) was perfused with Tyrode solution; group 2 (GM), 200 µg ml⁻¹ GM was added to the perfusate; group 3 (GM + Vit C), as group 2 with vitamin C added to the drinking water for 3 days (200 mg l⁻¹) and to the perfusate (100 mg l⁻¹); group 4 (GM + Vit E), as group 2 with vitamin E (100 mg (100 g body weight)⁻¹, I.M.) injected 12 h before the start of the experiment; group 5 (GM + Vit C + Vit E) a group 2 with vitamin E and C co-administered (concentrations and conditions as in groups 3 and 4). To compare the groups, urinary lactate dehydrogenase (LDH), N-acetylcetyl-β-D-glucosaminidase (NAG) and alkaline phosphatase (ALP) activities, inulin clearance (glomerular filtration rate, GFR) and renal tissue glutathione (GSH) content were measured. GM caused a significant nephrotoxicity demonstrated by an increase in urinary enzyme activities. Reduction in GSH content and a marked decrease in GFR were observed compared to controls. Vitamin C inhibited the GM-induced increase in urinary enzyme activities but did not show a significant effect on the GSH content or GFR. Vitamin E prevented the GM-induced reduction in GSH level without a significant improvement in GFR. Co-administration of vitamins C and E significantly prevented the GM-induced nephrotoxicity demonstrating by preservation of GFR and GSH levels and prevention of increase in urinary enzyme activities. We conclude that co-administration of moderate doses of vitamins C and E has beneficial effects on renal preservation in GM-induced nephrotoxicity.

(Received 20 December 2004; accepted after revision 10 March 2005; first published online 15 March 2005)

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Gentamicin (GM) is an aminoglycoside antibiotic which is used in clinical practice to treat severe gram-negative infections. However, its nephrotoxic action has limited the extent of its use (Mingeot & Tulkens, 1999). There have been many studies in recent years suggesting a significant role for reactive oxygen species (ROS) in GM-induced nephrotoxicity (Cuzzocrea et al. 2002). Sha & Schacht (1999a) have suggested that aminoglycoside antibiotics can stimulate formation of free radicals. In addition, ROS scavengers and antioxidants are used to ameliorate the GM-induced nephrotoxicity (Mazzon et al. 2001; Maldonado et al. 2003). Superoxide dismutase treatment has shown some promise in protection against GM-induced nephrotoxicity in rats (Ali & Bashir, 1996).

Recently it has been shown that both vitamins E and C decreased lipid peroxidation and augmented the activity of antioxidant enzymes in the kidneys of diabetic rats (Kedziora-Kornatowska et al. 2003). Prior vitamin E dietary supplementation suppresses oxidative stress and glomerulosclerosis in rat remnant kidney (Hahn et al. 1999). Single-dose administration of vitamin E had protective effects on cisplatin-induced nephrotoxicity in developing rats (Appenroth et al. 1997).
There have been several studies in recent years suggesting more effectiveness of combination therapy by co-supplementation of two antioxidants (Ademuyiwa et al. 1990; Kavutcu et al. 1996; Abdel-Naim et al. 1999). In a state of oxidative damage in rat erythrocytes induced by chlorpyrifos-ethyl, combination of vitamins C and E reduced lipoperoxidative effects (Gultekin et al. 2001). The aim of the present study was to evaluate the effects of co-supplementation of vitamins E and C on antioxidative state in rat GM-induced nephrotoxicity.

Methods

Subjects

Male Sprague-Dawley rats weighing 200–300 g were housed under controlled environmental conditions (24 ± 2°C and 12 h light–dark cycle) and allowed free access to standard rat chow and tap water. Animal care was in compliance with the guidelines of the Animal and Human Ethical Committee of Tehran Medical Sciences University.

Animal preparation: in situ isolated perfused kidneys

Animals were anaesthetized with i.p. injection of ketamine hydrochloride (70 mg kg−1). To perfuse the kidneys, the abdominal aorta above and below the renal artery was ligated. After the cannulation of the aorta, kidneys were perfused using a peristaltic pump (IBS P803, Integra Bioscience, Switzerland). The ureters were transected, cannulated and placed in 2-ml tubes for urine collection. After infusion of heparin, perfusion was started with oxygenated Tyrode solution containing inulin (60 mg dl−1) at a rate of 8 ml min−1 at 37°C.

Groups

For the study, 35 rats were arranged randomly in five groups of seven rats: group 1 (Control), kidneys were perfused with Tyrode solution; group 2 (GM), gentamicin (200 µg ml−1) was added to the perfusate 15 min after initiation of perfusion; group 3 (GM + Vit C), as group 2 with vitamin C was added to the drinking water for 3 days (200 mg l−1) and to the perfusate (100 mg l−1); group 4 (GM + Vit E), as group 2 with vitamin E (100 mg (100 g body weight)−1, i.m.) injected 12 h before the start of the experiments; group 5 (GM + Vit C + Vit E), as group 2 but vitamins C and E were both administered (concentrations and conditions as in group 3 and 4).

Experimental protocol

In all groups, perfusion was performed for 90 min in which the first 15 min was considered as the stabilizing period. Urine samples were collected in 15-min periods in separate tubes (at 30, 45, 60 and 75 min after initiation of perfusion). Adequate volumes of urine and buffer samples were kept at −30°C for inulin assays. Urinary lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG) and alkaline phosphatase (ALP) activities were measured in fresh urine samples. Inulin clearance (glomerular filtration rate, GFR) and renal tissue glutathione (GSH) levels were also measured.

At the end of the perfusion, kidneys were removed from the body and weighed. Each kidney was halved; one part was homogenized for GSH measurements (see below) and the other was fixed in 10% formalin buffer then embedded in paraffin. Sections of the kidneys were stained with haematoxylin and eosin. Histology for all kidneys was scored per section in at least 10 randomly selected non-overlapping fields at × 400 magnification. The results were scored as the percentage of the damaged tubules: no damage; mild, areas of tubular damage less than 25%; moderate, 25–50% tubular damage; severe, more than 50% tubular damage. The presence of luminal debris, cellular vacuolation and reduction in tubular patency were used as evidence of tubular damage.

Biochemical assays

GSH content of kidney tissues was measured according to the method of Kuo & Hook (1982) which was based on...
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Figure 2. Comparison of NAG release from kidneys perfused with Tyrode solution with and without any treatment

The NAG release from kidneys perfused with Tyrode solution (Control); and Tyrode solution containing: 200 µg ml⁻¹ gentamicin (GM); 100 mg l⁻¹ vitamin C after pretreatment with 200 mg l⁻¹ vitamin C in the drinking water for 3 days (Vit C); Vitamin E pretreatment (Vit E); and co-administration of vitamin C and E (Vit C + E). The data are presented as means ± s.e.m. *P < 0.01 compared to control; †P < 0.01 compared to GM (n = 7).

Activities of ALP and LDH were measured using an autoanalyser (Hitachi 704, Japan) with commercial kits. ALP activity was assayed using a method based on conversion of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoate (TNB). Formation of TNB was measured by spectrophotometry at 412 nm (Kuo & Hook, 1982).

ALP activity was assayed using a method based on conversion of P-nitrophenol (PNP)-phosphate to PNP at 405 nm (Weisshaar et al., 1975). ALP activity was assayed using a method based on conversion of P-nitrophenol (PNP)-phosphate to PNP at 405 nm (Weisshaar et al., 1975). The assay for urinary NAG activity was based on the enzymatic hydrolysis of p-nitrophenyl-n-acetyl-glucosaminide at pH 4.4 and the subsequent detection of liberated p-nitrophenol at 405 nm by spectrophotometry (Horak et al., 1981). Inulin was measured in urine and perfusate by colorimetric analysis using anthrone complexation (Poola et al., 2002). GFR was then calculated using a standard formula.

Statistics

Data are expressed as mean ± s.e.m. for the groups, analysed by two-way analysis of variance (repeated measurements) for urinary enzyme activities and one-way analysis of variance for inulin clearance and GSH content. To show the difference among the groups, Tukey's post
The *hoc* test was used. $P < 0.05$ was considered statistically significant.

**Results**

GM caused a significant nephrotoxicity demonstrated by increase in urinary LDH (Fig. 1), NAG (Fig. 2) and ALP (Fig. 3) activities. Decline in GSH activity and a marked decrease in GFR (Fig. 4A and B) were observed compared to controls. Vitamin C inhibited the GM-induced increase in urinary enzyme activities but did not show a significant effect on GSH content and GFR. Vitamin E prevented the GM-induced decline in GSH content without a significant improvement in GFR. Co-administration of vitamins C and E significantly prevented the GM-induced nephrotoxicity which was demonstrated by the preservation of GFR and GSH levels. Co-administration also prevented the increases in urinary enzyme activities.

A marked tubular dilatation was noticed that was assumed to be the result of 90 min isolated perfusion.

In addition, sections from control group showed normal histology (Fig. 5A). In sections from the GM groups, glomeruli displayed no significant changes detectable by light microscopy, but there were signs of tubular damage including loss of brush borders, tubular debris and vast cellular vacuolations (Fig. 5B). All three treated groups exhibited markedly less histological damage than in rats of GM group, with Vit E + C showing least damage; however, this was not significant (Fig. 5C–E).

**Discussion**

Aminoglycoside antibiotics including GM can produce nephrotoxicity in human. Proximal tubular cells are a major site of damage in patients treated with GM or the antibiotic amikacin (Wiland & Szechcinski, 2003). GM binds to the cell wall phospholipids, blocking the chain reactions of phosphatidylinositol which impairs cell integrity (Walker & Duggin, 1988). It has been shown that aminoglycoside antibiotics exert their adverse renal effects by generation of ROS. Formation of ROS following bioactivation of GM has been reported (Sha...
& Schacht, 1999b). Some studies demonstrated that antioxidant administration have ameliorated GM-induced nephropathy (Pedraza-Chaverri et al. 2003; Atessahin et al. 2003). A role for superoxide in GM-mediated nephropathy was suggested when different superoxide dismutase treatments were shown to be effective in ameliorating renal injury in GM-induced nephrotoxicity (Ali & Bashir, 1996; Cuzzocrea et al. 2002). In another study, lipid peroxide levels were reduced and levels of antioxidant enzymes and thiol compounds were increased following administration of α-tocopherol and ascorbic acid in lead-induced oxidative stress (Patra et al. 2001).

In the present study, the role of ROS in GM-induced nephrotoxicity was assessed by administration of antioxidant vitamins and further evaluation of alterations in GFR and histological changes as well as measurements of urinary NAG, LDH and ALP activities. These cellular enzymes exist in many tissues. NAG is a hydrolytic lysosomal enzyme, LDH is a key enzyme in energy metabolism located in the cell cytoplasm and ALP is a phosphohydrolase enzyme attached to the cell wall by glycosyl phosphatidyl inositol anchors. Activities of these enzymes in urine are physiologically very low. Therefore, any increase in their activities suggests proximal tubular cell damage (Horak et al. 1981; Schreiber et al. 1997).

In this study, GM caused significant nephrotoxicity demonstrated by increases in urinary LDH, NAG and ALP activities, a decline in GSH content and a marked decrease in GFR compared to controls. Vitamin C inhibited the GM-induced increase in urinary enzymes but did not show a significant effect on the GSH and GFR. Prior administration of vitamin E, in addition to inhibition of GM-induced increase in urinary enzyme activities, prevented the GM-mediated decline in GSH content without a significant improvement in GFR. Co-administration of vitamins C and E significantly prevented the GM-induced nephrotoxicity demonstrated by preservation of GFR and GSH levels and prevention of the increase in urinary enzyme activities. Antioxidant vitamins have been shown to inhibit pathological conditions at sufficient concentrations. Vitamin E is the main endogenous antioxidant which reacts with oxygen radicals preventing free radical chain reactions to protect the membranes. However, storage of endogenous antioxidants such as vitamin E decreases gradually while reacting with free radicals. Due to the recycling property of vitamin E by vitamin C, administration of vitamin C helps to replenish the storage of vitamin E. High doses of vitamin C are reported to act as an oxidant agent (Paolini et al. 1999). Thus in this study to have the maximum antioxidative effects, moderate doses of vitamins E and C were co-administered.

In the other studies, protective effects of co-administration of antioxidants against GM-induced nephrotoxicity are reported. Similar to our results, a combination of vitamin E and probucol was effective in amelioration of renal functional parameters and antioxidant enzyme levels in GM-induced toxicity (Abdel-Naim et al. 1999). In another study, synergism was suggested between vitamin E and selenium in attenuating renal damage (Ademuyiwa et al. 1990). In a heart model of GM-induced toxicity, vitamin E could protect the guinea-pig heart tissues against the free radical-induced injury (Ozturk et al. 1997). GM significantly disturbed the enzymatic antioxidant defense system and suppressed Mn-superoxide dismutase, GSH-peroxidase and catalase in guinea-pig kidney tissues. While administration of vitamin E or vitamin C showed some beneficial effects, a combination of vitamin E and C completely abrogated the enzymatic suppression (Kavutcu et al. 1996).

This study revealed that the GM-induced renal toxicity, as measured by multiple functional, structural and enzymatic factors is significantly reduced by co-supplementation of vitamins C and E. The results of this study suggest the potential of antioxidant vitamins to protect against GM-induced nephrotoxicity.

References
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