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Protective role of aminoguanidine on gentamicin-induced acute renal failure in rats

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Summary

The toxicity of aminoglycosides including gentamicin (GEN), the most widely used drug in this category, is believed to be related to the generation of reactive oxygen species (ROS) in the kidney. Aminoguanidine (AG) is known as an effective antioxidant and its free radical scavenger effects may protect GEN-induced acute renal failure (ARF). Therefore, this study was focused on investigating the possible protective effect of AG against GEN-induced nephrotoxicity in an in vivo rat model. We investigated the effects of AG on GEN-induced changes in renal tissue malondialdehyde (MDA) levels; nitric oxide (NO) generation; glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activities; glutathione (GSH) content; serum creatinine (Cr) and blood urea nitrogen (BUN) levels. Morphological changes in the kidney were also examined using light microscopy. GEN administration to control group rats increased renal MDA and NO levels but decreased GSH-Px, SOD, CAT activities and GSH content. AG administration with GEN injection resulted in significantly decreased MDA, NO generation and increased GSH-Px, SOD, CAT activities and GSH content when compared with GEN alone. Serum levels of Cr and BUN significantly increased as a result of nephrotoxicity. Also, AG significantly decreased Cr and BUN levels. Morphological changes in the kidney, including tubular necrosis, intracellular edema, glomerular and basement membrane alterations were evaluated qualitatively. Both biochemical findings and histopathological evidence showed that administration of AG reduced the GEN-induced kidney damage. We propose that AG acts in the kidney as a potent scavenger of free radicals to prevent the toxic effects of GEN both at the biochemical and histological level.

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Introduction

Aminoglycoside antibiotics are commonly used for the treatment of severe Gram-negative bacterial infections (Parlakpinar et al., 2003). Perhaps the most widely used drug in this category is gentamicin (GEN) (Reiter et al., 2002). A major complication of GEN treatment is nephrotoxicity, which accounts for 10–20% of all cases of acute renal failure (ARF) according to experimental results (Erdem et al., 2000). Also, 30% of the patients treated with GEN for more than 7 days show some signs of nephrotoxicity and this markedly limits its use (Pedraza-Chaverri et al., 2000). Although the change in GEN dosing from multiple-daily to once-daily doses has reduced the risk of nephrotoxicity, the incidence of GEN-induced ARF still remains high (Kopple et al., 2002).

Neprotoxicity induced by GEN is a complex phenomenon characterized by an increase in serum creatinine (Cr) and blood urea nitrogen (BUN) concentration and severe proximal renal tubular necrosis, followed by deterioration and renal failure (Cuzzocrea et al., 2002; Al-Majed et al., 2002). The toxicity of GEN seems to relate to the generation of destructive reactive oxygen species (ROS) in these cells (Reiter et al., 2002; Al-Majed et al., 2002). ROS have been proposed as a causative agent of cell death in many different pathological states (Mizrak et al., 2004) including glomerular disease (Cuzzocrea et al., 2002) and renal ischemia-reperfusion injury (Sahna et al., 2003).

Several studies have demonstrated that various agents, including melatonin (Ozbek et al., 2000), vitamin E, superoxide dismutase (SOD) (Pedraza-Chaverri et al., 2000), gum Arabic, lipoic acid (Al-Majed et al., 2002) and others, can prevent GEN-induced renal damage. To date there is no study into the protective effect of aminoguanidine (AG) – a known specific inhibitor of inducible nitric oxide synthase (iNOS), a potent antioxidant (Cigremis et al., 2005) and a free radical scavenger (Szabo et al., 1997) – on GEN nephrotoxicity.

Since iNOS production, oxyradicals and peroxynitrite ONOO generation are the main causes of GEN-induced renal injury, this study was planned to investigate the possible protective effect of AG on nephrotoxicity induced by GEN in a rat model, and to clarify the association between renal malondialdehyde (MDA) levels, nitric oxide (NO) generation, glutathione peroxidase (GSH-Px), SOD and catalase (CAT) activities, glutathione (GSH) content, Cr and BUN levels and GEN-induced histopathological changes. There are very different low and high AG dosage schemes reported in the literature, and we have reported previously that administration of AG at a dose of 200 mg/kg/day intraperitoneally (i.p.) prevents nephrotoxicity induced by a single dose of AK (Parlakpinar et al., 2004a). In the current study, we focused on the effects of repeated low-dose AG administered for more than 7 days.

Material and methods

Experimental conditions

Female Wistar rats aged 8–12 weeks, each weighing 200–250 g, were placed in temperature ($21 \pm 2\ ^{\circ}C$) and humidity ($60 \pm 5\%$) controlled room in which a 12:12 h light: dark cycle was maintained. Twenty-one rats were randomly assigned to three groups of seven animals each: (I) control group; injected i.p. with saline for 12 days, (II) GEN-treated group (GEN); injected for 2 days with saline, then injected i.p. with 100 mg/kg GEN (Genta 80 mg, I.E. ULAGAY, Istanbul, Turkey) for 8 days, then again with saline for 2 days, (III) AG-treated group (GEN+AG); injected i.p. with 100/mg/kg AG in 0.9% w/v saline (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) for 2 days, then with GEN and AG for 8 days, then AG alone for the final 2 days. Twenty-four hours after the last injection, a time point chosen on the basis of our previous GEN-related study (Parlakpinar et al., 2004a, b), rats in all groups were killed and the kidneys were quickly removed, decapsulated and divided longitudinally into two equally sized pieces. One piece was placed in formaldehyde solution for processing to paraffin wax by routine methods for histopathologic examination by light microscopy. The other piece was placed in liquid nitrogen and stored at $-85\ ^{\circ}C$ until assayed for MDA, NO, GSH-Px, SOD, CAT and GSH contents. Trunk blood was extracted to determine the serum levels of Cr and BUN. All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research at Inonu University, Malatya, Turkey.

Biochemical determination

The methods used for biochemical determinations, described briefly below, have been used and reported in our previous studies (Parlakpinar et al., 2002, 2005a, b; Ozer et al., 2005).

MDA levels in the kidney tissue were determined by the method described by Mihara and Uchiyama (1978). Kidney tissue (200 mg) was homogenized with ice-cold 1.15% KCl to form a 10% homogenate.

Then, 0.5 ml of this homogenate was pipetted into a 10 ml centrifuge tube and 3.0 ml of 1% w/v H_3PO_4 and 1.0 ml of 0.6% v/v aqueous thiobarbituric acid solution were added. The tubes were heated for 45 min in a boiling water bath and the reaction mixture was then cooled in an ice-bath. This was followed by the addition of 4.0 ml of *n*-butanol. The contents were mixed for 40 s using a vortex mixer, centrifuged at 1200*g* for 10 min and the absorbance of the organic layer was measured at wavelengths of 520 and 535 nm.

Since tissue nitrite (NO_2^-) and nitrate (NO_3^-) levels can be used to estimate NO production (Wennmalm et al., 1992), we measured the concentration of these stable NO oxidative metabolites. Quantification of NO_2^- and NO_3^- was based on the Griess reaction, in which a chromophore with a strong absorbance at 545 nm is formed by reaction of NO_2^- with a mixture of naphthlethylenediamine and sulfanilamide. Results are expressed as μmol/g.

GSH-Px activity was measured by the method of Paglia and Valentina (1967). Briefly, in the presence of glutathione reductase and NADPH, oxidized glutathione (GSSG) is immediately converted to its reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured. GSH-Px activity was expressed as U/mg protein.

CAT activity was determined according to the method of Aebi (1984). Briefly, 10 μl of kidney tissue supernatant was added to 2.99 ml of phosphate-buffered saline (PBS) and the absorbance was read at 240 nm using a UV spectrophotometer. The principle of the assay is based on the determination of the rate constant (s^{-1} k) or the H_2O_2 decomposition rate at 240 nm. Results were expressed as k (rate constant)/g protein.

SOD enzyme activity determination was based on the production of H_2O_2, from xanthine by xanthine oxidase and reduction of nitroblue tetrazolium, as previously described Aladag et al. (2003). The product was evaluated spectrophotometrically at 560 nm. Results are expressed as U/g protein.

GSH was determined by a spectrophotometric method, based on the use of Ellman's reagent (Gupta et al., 1999). Tissue homogenates were mixed with 50% trichloroacetic acid in distilled water in glass tubes and centrifuged at 3000 rpm for 15 min. The supernatants were mixed with 0.4 M Tris buffer, pH 8.9, and 0.01 M 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) was added. After shaking the reaction mixture, its absorbance was measured at 412 nm. The absorbance values were expressed as μmol/g tissue.

Serum levels of Cr and BUN were determined using the Olympus Autoanalyser (Olympus Instruments, Tokyo, Japan) according to manufacturers' instructions.

Histological evaluation

6 μm-thick sections of formalin fixed, paraffin wax embedded tissues were stained with hematoxylin and eosin (H–E) and periodic acid Schiff (PAS) reagent, using standard protocols, and examined by light microscopy using an Olympus BH-2 microscope. The PAS reaction stains carbohydrates and carbohydrate-rich macromolecules. It is used to demonstrate the basement membrane that underlies epithelia. Five coded slides from each group were examined in a blinded manner. Tubular necrosis, intracellular edema, glomerular and basement membrane alterations were evaluated qualitatively as follows: +: slight, ++: moderate, +++: severe changes, according to the extent of damage observed.

Statistical analysis

The results are expressed as mean ± standard deviation (SD). A Shapiro–Wilk test was performed to demonstrate normality of the data. Kidney MDA, NO, GSH-Px, SOD, CAT, GSH, serum Cr and BUN levels were analyzed using a one-way ANOVA. Post-hoc comparisons were performed using Tukey's test. Differences were considered significant when $p < 0.05$.

Results

The results are summarized in Tables 1 and 2. GEN administration significantly increased MDA and NO levels, and decreased GSH-Px, SOD, CAT activities and GSH content. AG administration resulted in a significant decrease in MDA and NO generation, and an increase in GSH-Px, SOD and CAT activities, and GSH content when compared with GEN administration alone. Serum levels of Cr and BUN were significantly higher in the GEN-treated animals, when compared to the control group. Pretreatment of the animals with AG significantly reduced the high serum Cr and BUN levels resulting from GEN administration.

Histological examination of the kidneys from animals in the control group revealed, as expected, entirely normal histological features, illustrated in Fig. 1A. However, there was marked necrosis in kidneys from animals in the GEN-treated group. The lumena of the tubules were filled with degenerate and desquamated epithelial cells,

Table 1. The effects of aminoguanidine (AG) administration to rats with or without gentamicin (GEN)

Parameters	Control ($n = 7$)	GEN ($n = 7$)	GEN+AG ($n = 7$)
MDA (nmol/g tissue)	61.99 ± 9.65	89.19 ± 10.68[a]	63.42 ± 10.18[b]
NO (μmol/g tissue)	104.92 ± 20.45	159.81 ± 36.56[a]	106.41 ± 13.22[b]
GSH-Px (U/mg protein)	2.27 ± 0.16	1.76 ± 0.29[a]	2.17 ± 0.24[b]
CAT (k/g protein)	242.91 ± 50.74	77.16 ± 22.51[a]	228.99 ± 23.83[b]
SOD (U/g protein)	1.69 ± 0.13	1.04 ± 0.03[a]	1.23 ± 0.08[b]
GSH (μmol/g tissue)	11.88 ± 1.53	8.66 ± 1.74[a]	14.88 ± 2.38[b]
BUN (mg/dL)	15.57 ± 1.27	27.71 ± 2.86[a]	15.14 ± 1.19[b]
Cr (mg/dL)	0.34 ± 0.05	0.77 ± 0.11[a]	0.37 ± 0.03[b]

Values are expressed as mean ± standard deviation.
[a] $p < 0.05$ vs. control group.
[b] $p < 0.05$ vs. GEN.

Table 2. Histological findings

Histological findings	Control	GEN	GEN+AG
Proximal tubule			
Necrosis	–	+++	+
Intracellular edema	–	+++	++
Desquamation	–	+++	+
Glomeruli			
Narrowing of the Bowman's space	–	+++	+++
Basement membrane			
Interruption	–	+++	–

Damage was evaluated qualitatively as +: slight, ++: moderate, +++: severe.

illustrated in Fig. 1B. In addition to the necrosis of proximal tubules, basal membrane interruption was also observed in the affected tubules, illustrated in Fig. 1C. In the GEN+AG-treated group, sparse tubular changes were observed. In this group, the affected tubules showed intracellular edema, slight necrosis and slight desquamation, seen in Fig. 1D. Narrowing of the Bowman's space was detected in kidneys from all treated animals in comparison to those of the control group, seen in Fig. 1D and E. Distal tubules were intact in all groups, and no significant inflammatory infiltration was identified. Some histological changes such as apoptosis, mitotic figures and nuclear polymorphism were observed only in the GEN-treated group, as illustrated in Fig. 1F and G.

Discussion

The toxicity of aminoglycosides including GEN seems to be a result of generation of destructive ROS (Halliwell et al., 1992). It has been found that GEN induces superoxide (O_2^-) anion-peroxide (H_2O_2) and hydroxyl radical (HO) production from renal mitochondria, and lipid peroxidation and nitrotyrosine and protein carbonyl content are increased. Additionally, O_2^- can react with NO to form $ONOO^-$, a cytotoxic oxidant radical species. As a result of this, production of O_2^- could lead to a decrease in glomerular filtration rate (GFR) by another mechanism (Sugimoto and Yagihashi, 1997; Rao et al., 1999). As treatment with some antioxidants protects against GEN-induced renal injury, we studied here the effect of AG, a potent antioxidant and free radical scavenger, on the renal damage and oxidative injury induced by GEN.

AG has several biological activities that together account for its beneficial effects. AG inhibits diamine oxidase (Tilton et al., 1993), which catalyzes degradation of biologically active diamines such as histamine and putrescine. AG inhibits iNOS in a selective manner, leading to decreased generation of NO (Parlakpinar et al., 2004a). It is of note that previous studies pointed to the beneficial antioxidant effects of AG and scavenger effects of $ONOO^-$ which is a reactive oxidant produced from NO and O_2^- in various forms of tissue injury (Mansour et al., 2002). Yildiz et al. (1998) also found that AG had direct scavenging activities against HO. Recently, Giardino et al. (1998) reported that AG acts as an antioxidant in vivo, preventing ROS formation and lipid peroxidation in cells and tissues, thus preventing oxidant-induced apoptosis. In addition, AG may also inhibit lipid peroxidation by inducing GSH-Px and SOD, or scavenging and inactivating H_2O_2 and HO (Babu et al., 1995). More recently, we have shown that AG improved GSH content and inhibited lipid peroxidation after single-dose AK-induced renal injury, (Parlakpinar et al., 2004a) and this is consistent with the above-mentioned reports.

In the current study, we evaluated the following endpoints of renal damage: (1) renal hemodynamics, (2) determination of detailed antioxidant enzyme activities and lipid peroxidation and (3)

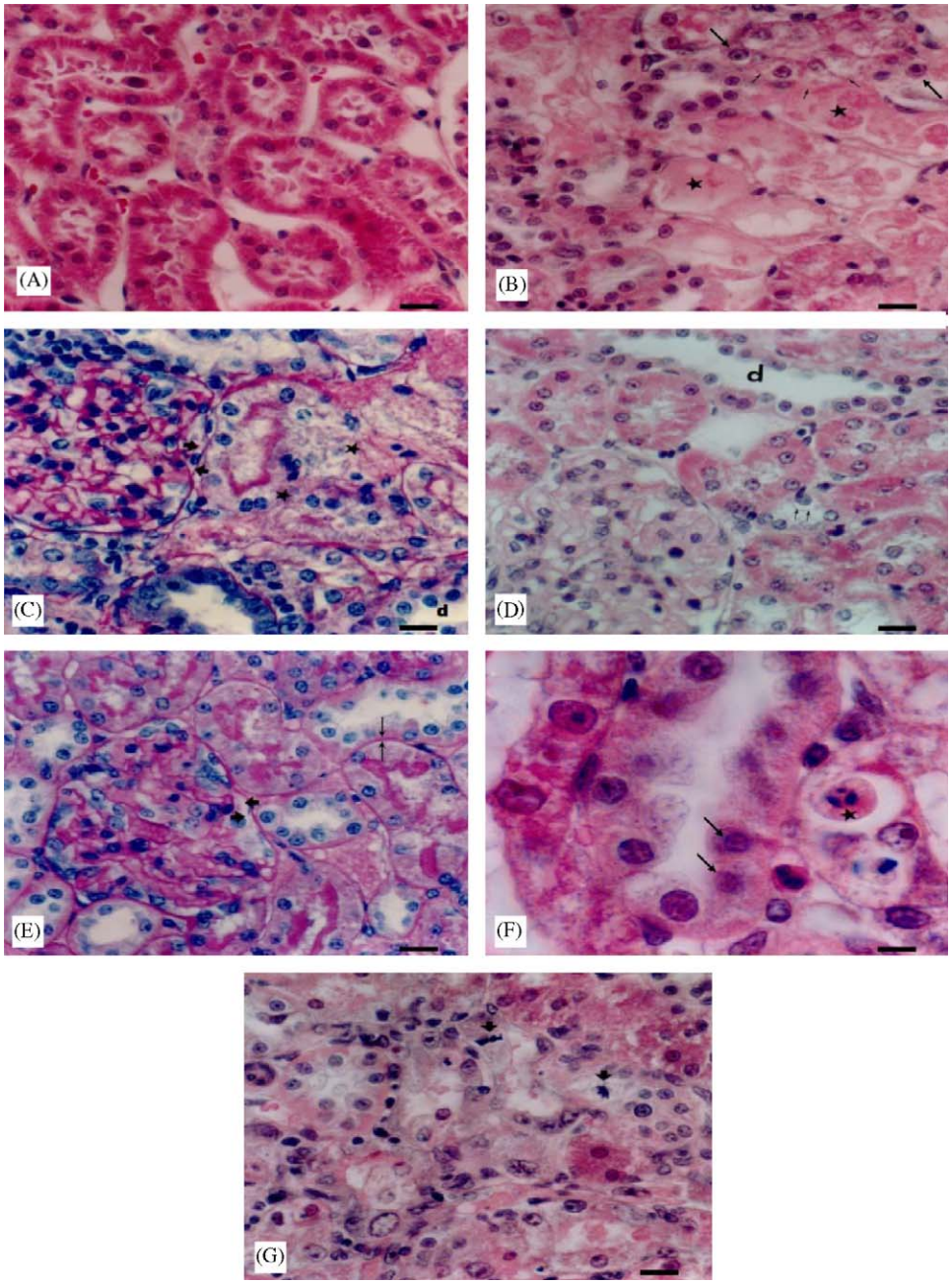

Figure 1. Histological appearance of kidney samples. A: Glomeruli and tubules have a normal appearance in samples from the control group. Hematoxylin and eosin stain. Bar, 100 μm. B: Marked tubular necrosis is observed in samples from the GEN-treated group. Desquamated and degenerated epithelial cells are visible in the lumena of necrotic tubules (*). Some of the tubular epithelial cells have large nuclei (large arrows). Notice the swelling cytoplasm, which is a result of edema (small arrows). Hematoxylin and eosin stain. Bar, 100 μm. C: Sample from GEN-treated group featuring interruption in the basement membrane around the necrotic tubules (*) and narrowing of the Bowman's space (arrows). Distal tubules (d) appear almost normal. Periodic acid Schiff stain. Bar, 100 μm. D: Sample from GEN+AG-treated group showing a marked reduction in tubular damage. Distal tubules (d) appear normal while some of the proximal tubules show moderate intracellular edema (arrows). Hematoxylin and eosin stain. Bar, 100 μm. E: Sample from GEN+AG-treated group. Basement membrane appears orderly and continuous (thin arrows) and narrowing of the Bowman's space is also evident (thick arrows). Periodic acid Schiff stain. Bar, 100 μm. F: Sample from GEN-treated group. Apoptotic body (*) and picnotic nuclei (arrows) are visible in the proximal tubules. Hematoxylin and eosin stain. Bar, 250 μm. G: Sample from GEN-treated group. Notice the two mitotic figures (arrows). Hematoxylin and eosin stain. Bar, 100 μm.

kidney histopathology. Several dosage schemes have been reported for GEN administration. We administrated GEN at the dose of 100 mg/kg/day for 8 days i.p., which is the dosage scheme reported to cause marked nephrotoxicity (Erdem et al., 2000).

There are some experimental data suggesting that nephrotoxic drugs may also change levels of MDA, GSH-Px, CAT, SOD, GSH, Cr and BUN (Ozbek et al., 2000; Parlakpinar et al., 2003), which are commonly used to monitor the development and extent of renal tubular damage due to oxidative stress. Results of this study confirmed that GEN at a dose of 100 mg/kg/day produces nephrotoxicity, as evidenced by the reduction in GFR which is indicated by increase in serum Cr. Recently, it has been reported that for humans, serum Cr in association with certain other clinical characteristics, may be a more accurate measure of the GFR than Cr clearance (Kopple et al., 2002). This impairment in glomerular function was accompanied by an increase in BUN. Serum Cr concentration is more significant than BUN level in the earlier phases of kidney disease. On the other hand, BUN begins to rise only after marked renal paranchymal injury occurs (Erdem et al., 2000). In the present study, GEN-induced increase in serum Cr and BUN levels was significantly blocked by AG administration. The protective effect of AG on Cr and BUN concentrations can be attributed to its antioxidant properties as it has been found that ROS may be involved in the impairment of GFR (Pedraza-Chaverri et al., 2000).

In the current study, GEN caused significantly decreased MDA and NO, while GSH-Px, CAT, SOD, GSH levels were reduced in the kidney tissue. Similar results were also observed by Ozbek et al. (2000). GEN nephropathy was associated with low activities of antioxidant enzymes in renal cortex. This decreased renal antioxidant enzymatic defense could aggravate the oxidative damage.

Administration of AG resulted in a significant decrease in MDA levels in the kidney when compared with GEN alone. Also, AG treatment prevented the decrease in GSH-Px, which is synthesized almost exclusively in kidney proximal tubular cells, and may be used as a marker of tubular damage. AG exert a beneficial effect by increasing the activities of SOD, CAT, GSH-Px and GSH content.

The findings of this study strongly indicate that AG is important in protecting the kidney from GEN-induced injury. Recent studies have tested the beneficial effects of AG in the prevention of diabetic complications in several tissues, including kidney. Sugimoto et al. (1999) reported that treatment of rats with AG alone (100 mg/kg per day p.o.) for 10 days did not induce any change in the measured biochemical parameters. On the other hand, Mansour et al. (2002) reported that administration of AG in drinking water (100 mg/kg p.o.) renders rats less susceptible to kidney damage induced by treatment with a single dose of cisplatin.

According to the histological results reported here, the GEN-treated rats suffered extreme cortical damage, tubular epithelial alterations, basal membrane interruption, desquamated epithelial cells, apoptotic cells, picnotic nuclei and damaged glomerular structure in their kidneys. Similar changes were also reported by Kumar et al. (2000) and Al-Majed et al. (2002) who demonstrated structural changes in renal tissue in GEN-treated animals and protection by various agents.

The results of this study confirm the earlier reports that GEN-treated rats show accelerated lipid peroxidation in the renal tissue, as reflected by an increase in MDA and reduced antioxidant enzyme activities. Pretreatment with AG afforded significant protection against nephrotoxicity induced by GEN treatment. The beneficial effect of AG in GEN toxicity implies the involvement of free radicals in the renal damage, although other destructive processes may also be involved. According to our biochemical findings, which were supported by histopathological evidence, administration of AG abolished some nephrotoxic effects of GEN. These findings indicate that AG supplementation may reduce GEN-induced renal injury. We propose that AG acts in the kidney as a potent scavenger of free radicals to prevent the toxic effects of GEN both at the biochemical and histological level.

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