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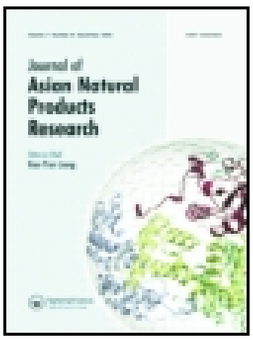

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Ameliorative effects of gallic acid on gentamicin-induced nephrotoxicity in rats

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ABSTRACT

The major side effect of gentamicin (GEN) is nephrotoxicity which in turn restricts the clinical use of this drug. In this study, the effect of gallic acid (GA) on gentamicin-induced nephrotoxicity was studied. A total number of 28 male Wistar rats were randomly divided into four experimental groups: control, GEN (100 mg/kg/day), GEN + GA (30 mg/kg/day), GA (30 mg/kg/day). All drug administrations were done intraperitoneally (i.p) for eight consecutive days. Twenty-four hours after the last administration, blood samples were collected to determine serum creatinine (Cr), blood urea nitrogen (BUN). The right kidney was used for histological examination. Malondialdehyde (MDA), glutathione (GSH), nitric oxide (NO) levels and catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activity were assayed in left renal tissue. Results showed a significant increase in the levels of MDA, NO, Cr, and BUN and decrease of GSH, CAT, GPx, and SOD by GEN administration. Co-administration with GA showed reduction in the levels of MDA, NO, Cr, and BUN and increase in GSH, CAT, GPx, and SOD. Also, the nephroprotective effect of GA was confirmed by the histological examination of the kidneys. The results of our study showed that GA exerts a significant nephroprotective effect against GEN-induced nephrotoxicity.

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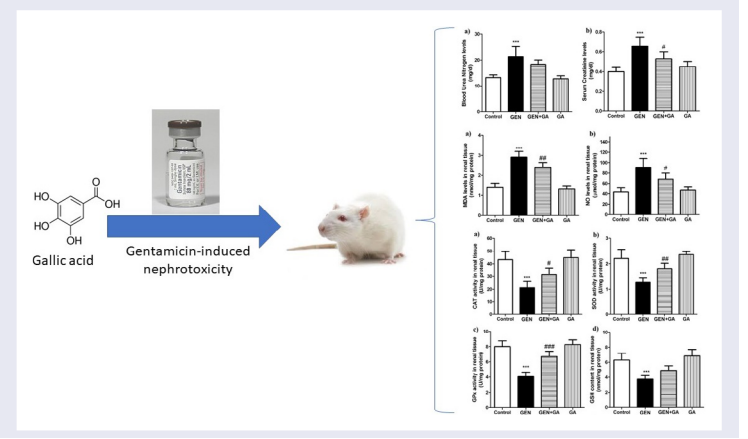

1. Introduction

Gentamicin (GEN) is an effective aminoglycoside antibiotic which has a major role in the treatment of Gram-negative microbial infections. The major side effect of aminoglycosides is nephrotoxicity and the prevalence of renal damage during treatment with this drug is about 30% despite careful drug monitoring [1]. Recent studies have demonstrated that the GEN-induced renal toxicity is related to its selective accumulation in proximal tubular cells, about 5–50 times higher than plasma levels [2]. Mechanisms underlying GEN-mediated nephropathy are not thoroughly understood. However, studies have shown that GEN administration increases the production of reactive oxygen species (ROS), renal lipoperoxidation, and renal nitric oxide (NO) synthesis [3]. On the other hand, GEN depletes protective antioxidants, such as glutathione (GSH) and inhibits the activities of enzymatic free radical scavengers, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) [4]. These complications are limiting factors for the clinical usage of GEN. Therefore, strategies for preventing or reversing the nephrotoxicity induced by GEN would have important clinical benefits [2]. GEN nephrotoxicity is a complex situation characterized by marked increase in levels of serum creatinine, blood urea nitrogen (BUN) concentration, extensive tubular epithelial edema, glomerular atrophy, and severe proximal renal tubular necrosis [5]. Oxidative stress induced by GEN seems to be an important molecular mechanism involved in the pathophysiology of GEN induced kidneys injury [3]. Antioxidant agents

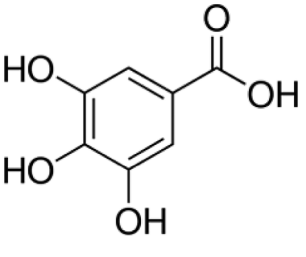

Figure 1. Chemical structure of gallic acid.

play an important role in preventing the oxidative damage by neutralizing the effect of reactive oxygen metabolites on cellular components. Little attention has been paid on the use of natural substances with potent antioxidant properties to protect against nephrotoxic damage induced by gentamicin. Some antioxidant compounds, such as melatonin, vitamin E, L-carnitine and naringenin have been used for the prevention of renal toxicity of GEN [3,6,7].

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) (Figure 1) is a well-known plant polyphenol found abundantly in tea, mango, grapes, different berries, areca nut, and other fruits. It is also found in wine and is widely used in foods, drugs, and cosmetics. GA is one of the most important polyphenolic compounds in plants with excellent antioxidant and anti-inflammatory activity. This compound also has a wide range of biological activities, such as anti-inflammatory, antimicrobial, anticancer, antidiabetic and antiangiogenic effects [8]. GA exerts antioxidant effect via scavenging free radicals, such as superoxide anions and hydroxyl radicals [9]. It was demonstrated that GA has protective effect in renal ischemia-reperfusion and ameliorates the oxidative kidney damage [10]. GA is a non-toxic substance which has wide therapeutic index and median lethality dose (LD_{50}) of 5 g/kg body weight in rats [11]. Also, toxicology studies in rats show that the no observed adverse effect level of GA is 120 mg/kg [12].

Thus, the objective of this study was to evaluate the potential effects of gallic acid on gentamicin-induced nephrotoxicity.

2. Results

2.1. Effects of gallic acid on BUN and Cr levels

Result showed that after GEN administration, rat developed severe nephrotoxicity that is reflected by a significant increase in the levels of BUN and Cr (all $p < 0.001$) (Figure 2). However, co-administration of GA and GEN for eight consecutive days decreased the level of BUN compared to GEN group, however, the difference was not statistically significant (Figure 2(a)). On the other hand, co-administration of GA and GEN for eight consecutive days decreased the level of serum Cr significantly compared to GEN group ($p < 0.05$) (Figure

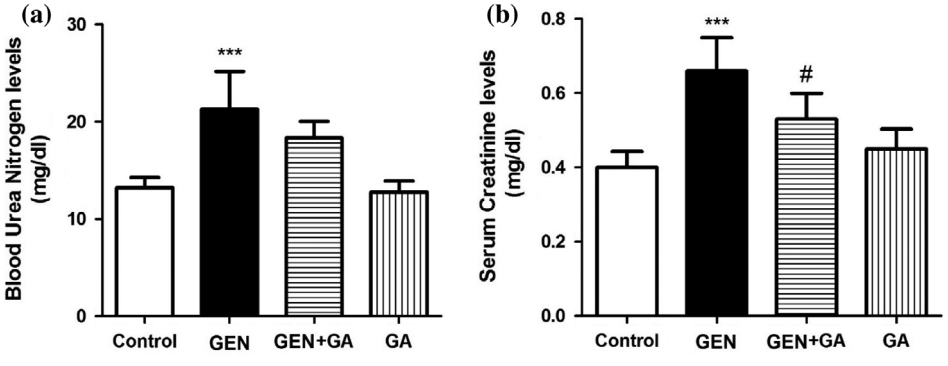

Figure 2. Effect of treatment with GA on BUN and serum creatinine levels in GEN-induced nephrotoxicity.
Notes: Values are means ±SD ($n = 7$). Data were analyzed by one-way ANOVA test followed by Tukey's *post hoc* test for multiple comparisons. *Significant difference in comparison with the control group (***$p < 0.001$). #Significant difference in comparison with the GEN group (#$p < 0.05$).

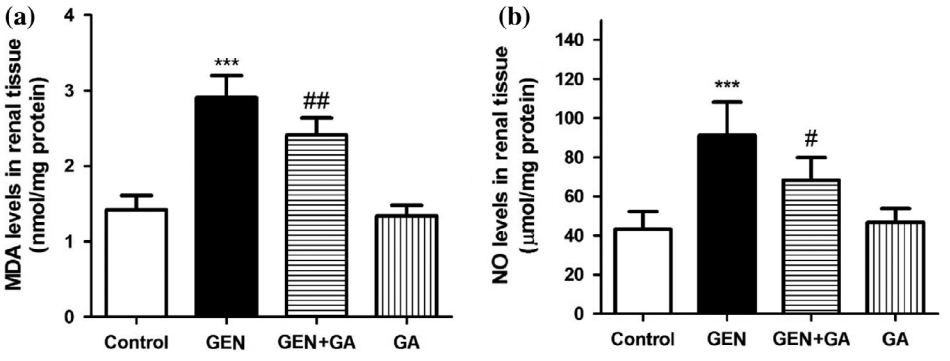

Figure 3. Effect of treatment with GA on MDA and NO levels in GEN-induced nephrotoxicity.
Notes: Values are means ±SD ($n = 7$). Data were analyzed by one-way ANOVA test followed by Tukey's *post hoc* test for multiple comparisons. *Significant difference in comparison with the control group (***$p < 0.001$). #Significant difference in comparison with the GEN group (#$p < 0.05$, ##$p < 0.01$).

2(b)). In addition, administration of GA to normal rats did not change the levels of BUN and Cr compared to rats in control group.

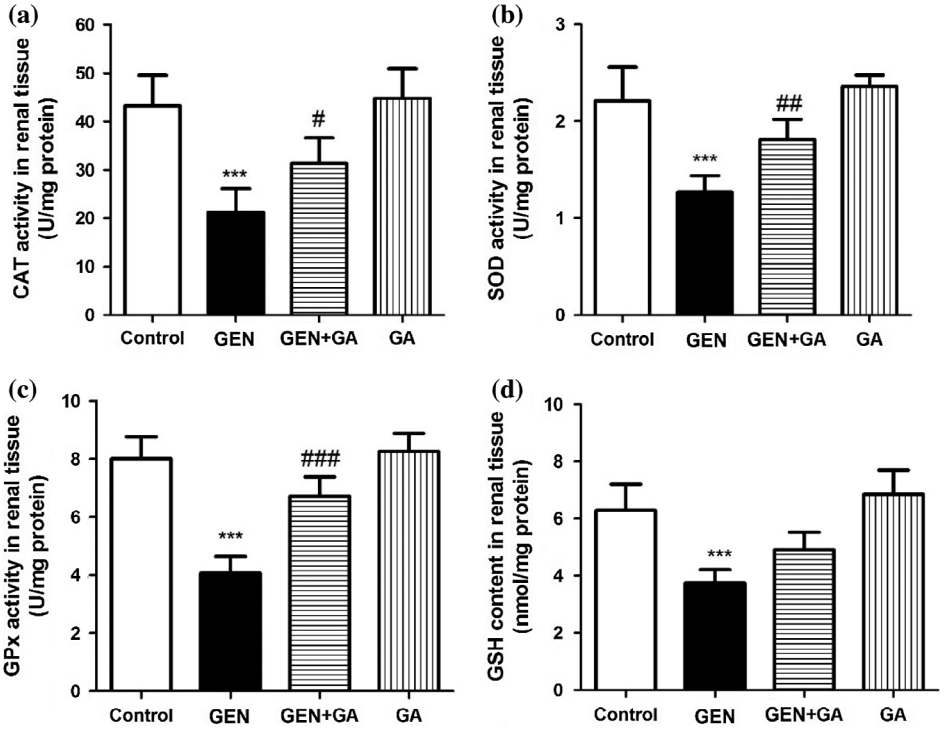

Figure 4. Effect of treatment with GA on CAT, SOD and GPx activity, and GSH content in GEN-induced nephrotoxicity.
Notes: Values are means ±SD ($n = 7$). Data were analyzed by one-way ANOVA test followed by Tukey's *post hoc* test for multiple comparisons. *Significant difference in comparison with the control group (***$p < 0.001$). #Significant difference in comparison with the GEN group (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$).

2.2. Effects of gallic acid on MDA and NO levels

The obtained results clearly revealed that GEN administration markedly increased the amount of MDA in kidney tissues ($p < 0.001$) (Figure 3(a)). GA-GEN co-administration for eight consecutive days decreased the level of MDA significantly ($p < 0.01$). In addition, renal NO levels were significantly increased in the GEN group in comparison with the control group ($p < 0.001$) (Figure 3(b)). Co-administration of GA and GEN for eight consecutive days caused a significant reduction in renal NO level ($p < 0.05$) in comparison with the GEN group. In addition, administration of GA to normal rats for eight consecutive days did not affect the levels of MDA and NO compared to rats in control group.

2.3. Effects of gallic acid on antioxidant enzymes

GEN administration significantly decreased the CAT, SOD, and GPx activity as compared to the control group (all $p < 0.001$) (Figure 4(a)–(c), respectively). Co-administration of GA and GEN significantly increased the GEN-induced reduction of CAT, SOD, and GPx activity ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). However, the GSH level was significantly decreased in the kidneys of rats exposed to GEN ($p < 0.001$), but treatment with GA could not significantly inhibit the GEN-induced reduction of GSH content (Figure 4(d)). In addition, administration of GA to normal rats for eight consecutive days did not change the CAT, SOD, and GPx activity and GSH content compared to the rats in control group.

2.4. Effects of gallic acid on SOD2 and PGx1 mRNA expression

The expression of SOD2 and PGx1 mRNA was assessed in rat kidney tissues using quantitative real-time RT-PCR (Figure 5). GEN administration significantly decreased the expressions of SOD2 and PGx1 mRNA compared to the control group (all $p < 0.001$). Co-administration of GA and GEN significantly elevated the levels of SOD2 and PGx1 mRNA compared to the GEN group ($p < 0.05$ and $p < 0.01$, respectively). In addition,

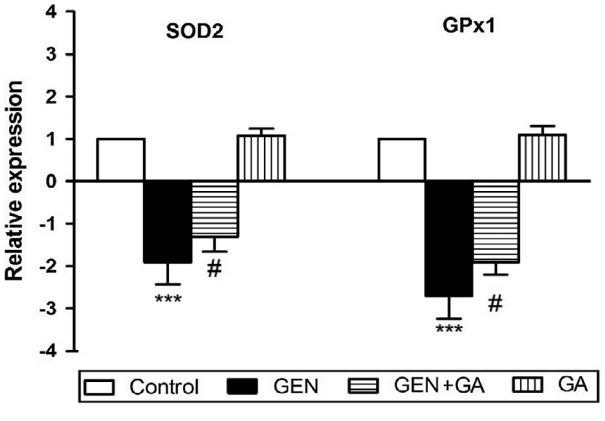

Figure 5. Expression of SOD2 and GPx1 genes in kidney tissues of the studied groups.
Notes: Values are means ±SD ($n = 7$). Data were analyzed by one-way ANOVA test followed by Tukey's *post hoc* test for multiple comparisons. *Significant difference in comparison with the control group (***$p < 0.001$). #Significant difference in comparison with the GEN group (#$p < 0.05$).

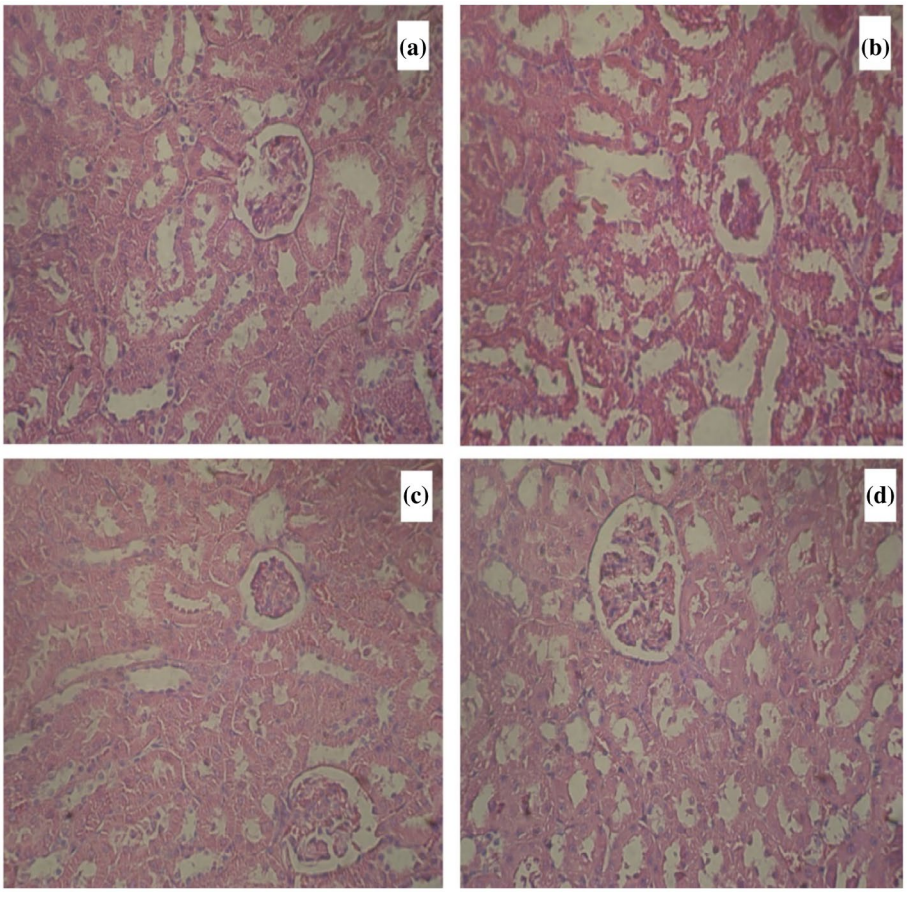

Figure 6. Effect of GA on the morphology of the rat kidneys with GEN-induced nephrotoxicity (X 300). Control group: healthy kidney structure was seen. The glomerulus and tubules are normal (a). GEN group: kidney is severely damaged. Glomerular atrophy, acute tubular necrosis, and extensive tubular degeneration were seen (b). GEN + GA group: Showed dramatic improvement in the morphologic appearance. Minimal tubular necrosis and degeneration was observed. Slight glomerular atrophy is still seen (c). GA group: The glomerulus and tubules are normal (d).

administration of GA to normal rats did not change the levels of SOD2 and PGx1 mRNA expression.

2.5. Histopathological changes of kidney

The histopathological study of kidney in the control group showed a normal architecture. In this group, the structure of glomerular was clear, capsular space was small, the structure of epithelial cells in proximal convoluted tubules and distal convoluted tubules were normal and the boundaries of both visceral, as well as parietal layers of renal capsule were clear (Figure 6(a)). In the GEN group, the glomerular was atrophied and disintegrated, capsular spaces got widened obviously, both visceral and parietal layers of renal capsule were destroyed and some of them disappeared. The epithelial cells in proximal convoluted tubules

and distal convoluted tubule were swollen and some epithelial cells had vacuolization. The structure of epithelial cells was unclear and there were some cell fragments in the tubules (Figure 6(b)). Co-administration of GA and GEN showed considerable improvement in proximal and distal convoluted tubules. The extent of glomerular atrophy was attenuated and the size of capsular spaces was reduced compared to the GEN group. The boundaries of visceral and parietal layers of renal capsule were clear, the epithelial cells in proximal and distal convoluted tubules were slightly swollen (Figure 6(c)). In addition, administration of GA did not cause any detectable alteration in the renal structure of the normal rats (Figure 6(d)).

3. Discussion

In the present study, we investigated the effect of GA on GEN-induced nephrotoxicity in rats. Results of our study confirmed that rats received GEN at a dose of 100 mg/kg (i.p.) for eight consecutive days exhibit nephrotoxicity, as evidenced by increase in serum creatinine and BUN levels, and this impairment of renal function was accompanied by an increase in renal NO and MDA levels, as well as a reduction in renal GSH level, SOD, GPx, and CAT activity which corroborated with previous reports [13,14]. We also found that these alterations in biochemical parameters are well correlated with the renal histological features. Our data also showed that the treatment of rats with GA (30 mg/kg/day, i.p.) for 8 days attenuates GEN-induced nephrotoxicity by preventing GEN induced increase in NO, MDA, BUN and creatinine levels and increasing the activity and level of antioxidative defense enzymes, such as SOD, GPx and CAT. Moreover, we demonstrated that administration of GA (30 mg/kg/day, i.p.) in normal rats for 8 days, affected neither the morphology nor the function of kidney. The results of the present study, for the first time, indicates that GA has a significant nephroprotective effect on GEN-induced nephrotoxicity in rats.

Aminoglycosides are effective agents commonly used against severe Gram-negative bacterial infections. Among the aminoglycoside antibiotics, GEN has been used widely and nephrotoxicity is a major complication of this drug which restricts its clinical application [1].

The results of this study showed that administration of GEN for 8 days increases the serum creatinine and BUN levels. There are some reports demonstrating the strong relationship between GEN-induced nephrotoxicity and oxidative stress, especially ROS [3]. GEN elicits mitochondrial release of iron in renal cortex and iron-GEN complex enhances the ROS formation [15]. Overproduction of ROS impairs the kidney functions which is accompanied by increased serum creatinine and BUN levels [16]. We also observed that co-administration of GA and GEN reduces the level of serum creatinine and BUN compared to the GEN group. Various studies have demonstrated that antioxidant substances inhibit these elevated levels of serum creatinine and BUN in GEN-treated rats [3,6]. It is well established that GA exerts antioxidant effects directly by scavenging free radicals or indirectly by increasing the activity and expression of antioxidant enzymes [9]. Jadon et al. showed that GA reduced the oxidative damage in liver and kidney via this antioxidant effect in carbon tetrachloride-treated rats [17]. In another study, Kim et al., indicated that the melanogenesis inhibitory effect of GA is associated with its antioxidant property. They found that GA effectively downregulated the ROS generation in B16 melanoma cells via up-regulation of GSH [18].

In the present study, we measured the MDA and nitric oxide (NO) as the main indices of oxidative stress. MDA is one of the most commonly used markers of lipid peroxidation which is a well-known mechanism of cellular injury. On the other hand, MDA levels were increased after GEN treatment and this phenomenon may partly be due to the free radicals generated by GEN [19]. NO is a molecular mediator of many physiological and pathological processes including vasodilatation and inflammation. Over production of NO plays a major role in oxidant stress and tissue damage via interacting with superoxide to generate the potent cytotoxic agent peroxynitrite. Moreover, GEN induces iNOS leading to increased production of NO in renal tissue [20]. In addition, increased peroxidation of membrane lipids and enhanced levels of inflammatory mediators, such as NO can cause morphological and functional changes in the kidney [21]. Consistent with these studies, we observed that GEN increases renal MDA and NO levels. Our results also showed that GA treatment significantly decreases MDA and NO level in rats treated with GEN. Previous studies showed the inhibitory effects of GA on lipid peroxidation and NO level. Oyagbemi et al., showed that GA could reduce MDA and NO level in cyclophosphamide-induced hepatotoxicity in Wistar rats [22]. Moreover, El-Hussainy et al., showed that GA significantly reduces MDA in myocardial tissue of rats that exposed to aluminum oxide [23].

It is well established that excessive production of ROS in GEN-induced nephrotoxicity is associated with depletion of renal antioxidant enzymes, such as CAT, SOD, GPx and GSH [4]. GSH is one of the main intracellular defense systems against oxidative damage via scavenging of hydroxyl radicals and singlet oxygen [19]. SOD is an intracellular antioxidant enzyme, which rapidly and specifically reduces superoxide to hydrogen peroxide. Then, GPx and CAT, another endogenous antioxidants, detoxify hydrogen peroxide to water [21]. For evaluating the antioxidant activity in renal tissue, we measured the renal GSH level, SOD, GPx, and CAT activity. Our results are in line with the previous studies that a decline in renal antioxidant enzymes, such as CAT, SOD, GPx and GSH are apparent in animals treated by GEN [4,6]. Results also indicated that GA administration increases the activity of antioxidative defense enzymes, such as SOD, GPx and CAT but not the GSH level. We also demonstrated that GEN decreases the SOD and GPx protein content and mRNA levels. Intriguingly, this effect was significantly prevented by GA. These results were in agreement with recent studies reporting that GA effectively increases the activity of antioxidant enzymes such as CAT, SOD, GPx, and GSH. Rather and Saravanan showed that GA increases the antioxidant levels such as SOD, CAT, and GPx in brain and heart tissue of rats experienced immobilization-induced stress [9]. In another study, administration of GA to cyclophosphamide-treated rats was found to increase the activities of CAT, SOD, GPx, GSH, and GST in liver [22]. Moreover, rats exposed to aluminum oxide with GA displayed significantly higher levels of SOD and CAT activities, as well as more GSH concentration in myocardial tissue [23].

In line with the results of biochemical assessment, histopathological findings demonstrated structural changes in renal tissue of GEN-treated rats. We found that GEN administration causes histopathological lesions in kidney, like glomerular atrophy, cell fragments in the tubules and swollen epithelial cells in proximal and distal convoluted tubules. Histopathological results are in rapport with the previous studies [5]. In addition, the protective effects of GA were confirmed by histopathological studies of kidney which indicates considerable improvement in proximal and distal convoluted tubules and glomerular atrophy in pre-treated groups. We also found no significant histopathological modifications

in kidney of GA-treated rats. This shows that, the histopathological lesions in the present study were due to the GEN injection.

In conclusion, we clearly demonstrated that i.p administration of GA to GEN-treated rats significantly decreased BUN and serum creatinine and efficiently reduced renal NO and MDA levels. It also increased SOD, GPx, and CAT activity in kidney tissue. GA treatment mitigated renal damage associated with GEN treatment and this is attributed to its potent antioxidant activity and its ability to protect the cell membrane integrity and to prevent inflammation. Future studies are needed to unveil the cellular mechanisms responsible for the therapeutic effect of GA.

4. Experimental

4.1. Chemicals

Gentamicin (GM) was purchased from Darupakhsh, Co. (Tehran, Iran). Gallic acid (GA) (the purity: 97.5%), reduced glutathione (GSH), trichloroacetic acid, thiobarbituric acid (TBA), bovine serum albumin (BSA), and Bradford reagent were all purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A).

4.2. Animals

Twenty-eight male Wistar rats (180–200 g) were obtained from animal house of Ahvaz Jundishapur University of Medical Science, Iran. Rats were kept in polypropylene cages and given standard rat chow and drinking water *ad libitum*. Animals were maintained under a controlled temperature (20 ± 2 °C) with a 12 h light: 12 h dark cycle. Rats were acclimated to the environment for a minimum of one-week prior to the beginning of experiments. All procedures were performed in accordance with the ethical standards of the Committee for Animal Care and Use of Ahvaz Jundishapur University of Medical Sciences (Ethic code: IR.AJUMS.REC.1395.39).

4.3. Experimental design

In this, rats were randomly divided into four experimental groups ($n = 7$ in each group). The control group received normal saline. The GEN group received 100 mg/kg/day gentamicin. The GEN + GA group received 100 mg/kg/day gentamicin plus 30 mg/kg/day gallic acid (dose equivalent to 2.1 g in a 70 kg man). The GA group received 30 ml/kg/day gallic acid. All drugs were intraperitoneally (i.p) administered for eight consecutive days.

4.4. Sample collection

24 h after the last drug injection, animals were anesthetized with diethyl ether and blood samples were collected from the jugular vein. Serum was separated by centrifugation of blood sample for 10 min at 1500 rpm at +4 °C and stored at −20 °C until analysis. Then animals were sacrificed by decapitation and kidneys were isolated. For histological studies, the right kidneys were washed with saline and fixed in 10% phosphate buffered formalin. The left kidneys were washed with ice-cold saline quickly and divided into two parts. One

part was homogenized (1/10 w/v) in ice-cold Tris-HCl buffer (0.1 M, pH 7.4) and kept in –20 °C for biochemical estimations. The other section was shock-frozen in liquid nitrogen and kept in –80 °C for real-time PCR studies. Protein content of all homogenates samples was determined by the method of Bradford [24] using BSA as standard.

4.5. Histopathological assessment

For the histological examination, the right kidneys were fixed in 10% formalin and then kidneys tissues were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 μm sections, mounted on glass slides, and stained with hematoxylin-eosin dye (H&E). These sections were then examined under a light microscope for the presence of necrosis, edema, hemorrhage, tubular degeneration, and narrowing of Bowman's capsule space [5].

4.6. Quantitative real-time RT-PCR

Total RNA was purified from rat kidney tissue using Trizol method as described by the suppliers (Cinnagen, Tehran, Iran). One microgram total RNA and random primers were used for cDNA synthesis using the Script™ cDNA synthesis kit (Bio-Rad, Hercules, CA, U.S.A). For real-time PCR analysis, the cDNA samples were run in triplicate. Real-time PCR reactions were performed using Power SYBR® Green (Life Technologies, Camarillo, CA, U.S.A) and conducted on the Applied Biosystems 7500 Instrument. The typical thermal profile used was at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. After PCR amplification, the ΔCt was calculated by subtraction of the β-actin Ct from each sample Ct. The primers (Table 1) were taken from the literature [25]. β-actin, as a housekeeping gene, was used to normalize the amplified signals of the target genes.

4.7. Biochemical assessment

4.7.1. Serum assessments

BUN and creatinine levels were determined according to Technicon kits (Bayer Company, Leverkusen, Germany) using an autoanalyzer (RA-XT, Technicon, Tarrytown, NY, U.S.A).

4.7.2. Antioxidant assessments

MDA and NO levels and the activities of antioxidant enzymes, such as SOD, GPx, GSH and CAT were measured by methods as described in detail earlier [4,6,13].

CAT activity was determined according to the method of Aebi by measuring the decomposition of H_2O_2 (0.066 M) at 240 nm for 60 s. The CAT activity was expressed as μmol of

Table 1. The primers used in real-time RT-PCR.

Primer	Sequence
SOD2	Forward: 5′- AGCTGCACCACAGCAAGCAC-3′ (Sense)
	Reverse: 5′- TCCACCACCCTTAGGGCTCA-3′ (AntiSense)
GPx1	Forward: 5′- CGGTTTCCCGTGCAATCAGT-3′ (Sense)
	Reverse: 5′- ACACCGGGGACCAAATGATG-3′ (AntiSense)
β-actin	Forward: 5′- GGCATCCTGACCCTGAAGTA-3′ (Sense)
	Reverse: 5′- GGGGTGTTGAAGGTCTCAAA-3′ (AntiSense)

H_2O_2 consumed per min per mg of protein. SOD activity was determined by measuring the inhibition of autoxidation of hematoxylin according to the method described by Martin and the results were expressed as units/mg protein. GSH peroxidase activity was determined by the method described by Ellman which is based on the formation of a yellow colored complex with Ellman's Reagent. The absorbance of this yellow component was measured at 412 nm and compared with the standard curve to determine GSH concentration. GSH content was expressed as nmol/mg protein. Lipid peroxidation was evaluated by the measurement of Malondialdehyde (MDA) level via the TBA 0.67% color reaction. Results were expressed as nmol/mg protein. Renal NO levels were measured using the Griess diazotization reaction after conversion of nitrate to nitrite by nitrate reductase in supernatant. The concentration of nitrite in the sample was determined from a sodium nitrite ($NaNO_2$) standard curve. GPx activity was measured with the GSH peroxidase kit (Randox Labs, Crumlin, U.K).

4.8. Statistical analysis

Data are expressed as Mean ±SD and all statistical comparisons were made by one-way ANOVA test followed by Tukey's *post hoc* analysis. *p*-value less than 0.05 was considered statistically significant.

Disclosure statement

The authors declare no conflict of interest related to this work.

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