Effect of Lycopene and Rosmarinic Acid on Gentamicin Induced Renal Cortical Oxidative Stress, Apoptosis, and Autophagy in Adult Male Albino Rat

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ABSTRACT

Gentamicin nephrotoxicity accounts for 10%–15% of all cases of acute renal failure. Several natural antioxidants were found to be effective against drug-induced toxicity. The possible protective effects of lycopene (Lyc) and rosmarinic acid (RA) alone or combined on gentamicin (Gen) induced renal cortical oxidative stress, apoptosis, and autophagy were evaluated. Sixty-three rats were randomly divided into seven groups named: control, group II received RA 50 mg/kg/day, group III received Lyc 4 mg/kg/day, group IV received Gen 100 mg/kg/day, group V (RA + Gen), group VI (Lyc + Gen), and group VII (RA + Lyc + Gen). At the end of the experiment, kidney functions were estimated then the kidneys were sampled for histopathological, immunohistochemistry, and biochemical studies. Administration of rosmarinic acid and lycopene decreased elevated serum creatinine, blood urea nitrogen, renal malondialdehyde and immunoexpression of the proapoptotic protein (Bax), autophagic marker protein (LC3/B), and inducible nitric oxide synthase (iNOS) induced by gentamicin. They increased reduced glutathione, glutathione peroxidase, superoxide dismutase, and immunoexpression of the antiapoptotic protein (Bcl2). They also improved the histopathological changes induced by gentamicin. The combination therapy of rosmarinic acid and lycopene shows better protective effects than the corresponding monotherapy. Anat Rec, 300:1137–1149, 2017. © 2016 Wiley Periodicals, Inc.

Key words: lycopene; rosmarinic acid; gentamicin; nephrotoxicity; Bax; Bcl2; LC3/B; antioxidants

INTRODUCTION

The kidneys are essential organs important for excretion of metabolic waste products as well as preserving chemical homeostasis amongst numerous other functions. The broad use of therapeutic drugs, natural products, environmental pollutants, and industrial chemicals during the last few decades has greatly increased the probability of kidney damage (Gaikwad et al., 2012). Aminoglycoside antibiotics have long been used as antibacterial therapy. Gentamicin is an aminoglycoside antibiotic derived from micomonospora purpurea. It is a bactericidal against most of the life threatening gram-negative bacteria (Gopal et al., 2013). Gentamicin is still the only effective therapeutic antibiotic against pseudomonas, proteus, and serratia that are resistant to other

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antibiotics (Balakumar et al., 2008). It has been reported that treatment of patients with aminoglycosides for more than 7 days gives rise to signs of nephrotoxicity in 30% of cases treated (Ali, 2003). Nephrotoxicity due to gentamicin is responsible for 10%–15% of all cases of acute renal failure (Rincon et al., 2004). In humans, a dose above 2 mg/mL of gentamicin results in nephrotoxicity. Gentamicin stimulates generation of superoxide anions, hydroxyl radicals and hydrogen peroxide after its concentration in the renal proximal tubular cells (Morales et al., 2010). Some investigators showed that gentamicin works as an iron chelator and that iron–gentamicin combination is an efficient stimulator for free radical formation (Yanagida et al., 2004). The mechanisms underlying gentamicin nephrotoxicity are not completely clear. However, oxidative stress, inflammation, increased monocyte-macrophages infiltration and apoptosis may be involved (Geleilete et al., 2002; Lee et al., 2013; Lee et al., 2013). Apoptosis and autophagy are two variants of programmed cell death. In different experiments of kidney damage, apoptosis has been elicited as a potential mechanism leading to tubular cell death (Bae et al., 2011). Autophagy imparts a nutritional reusing by eliminating unwanted or dysfunctional cellular components. However, metabolic disturbance and cellular death can be resulted from dysregulation of autophagy (Su et al., 2013).

It has been estimated that counterbalancing the reactive oxygen species and reinforcing the endogenous antioxidants defenses by using natural antioxidants safeguards against toxicity promoted by drugs (Brewer, 2011). Rosemary (*Rosmarinus officinalis*) is usually applied as spice and flavoring factor in food processing. It is formed of dried leaves and flowers amounts to an especially good source of biologically active phytochemicals as it consists of a diversity of phenolic components involving carnosol, carnosic acid, rosmarinic acid, rosmanol, 7-methyl-epirosemanol, isorosmanol, rosmadial, and caffeic acid (Genena et al., 2008). It has many biological activities, including antioxidative (Bakirel et al., 2008; Lee et al., 2008), antiinflammatory, antiapoptotic, and antitumor (Lee et al., 2008; Venkatachalam et al., 2013).

Carotenoids belong to fat-soluble pigments present in tomatoes and in another red fruits and vegetables (Tapiero et al., 2004). Lycopene is one of tomato carotenoids that has been received a special interest owing to its highly effective antioxidant with free radical scavenging capacity. Lycopene appears to be prophylactic against proteins, lipids and DNA oxidation. Its preventive effect was studied with many nephrotoxic agents and its protective capability was established (Palabiyik et al., 2013).

Although, some previous studies showed that concurrent administration of lycopene or rosmarinic acid prevents gentamicin toxicity (Karahan et al., 2005; Tavafi and Ahmadvand, 2011). But, almost many of those studies concentrated fundamentally on serum biochemical changes provoked by the effects of lycopene and rosmarinic acid on gentamicin induced nephrotoxicity without an accurate specification of the histological alterations and the immunohistochemical expressions of apoptotic and oxidative markers which took place in gentamicin nephrotoxicity. Therefore, this study was carried out to estimate the potential effects of lycopene and rosmarinic acid as preventive agents individually or in combination against gentamicin-evoked cortical oxidative stress, apoptosis and autophagy in rats by the use of histopathological, immunohistochemical and biochemical analysis in an attempt to verify the mechanism of the protective effect of both drugs and to verify if the combination therapy has better effects.

MATERIALS AND METHODS

Animals

Sixty-three healthy adult male albino Wistar rats weighing 200–250 g were used in the present study. Polypropylene cages were used to house the rats under standard lightening in a temperature-controlled room (25°C ± 2°C). Rats had *ad libitum* access to food and tap water. After 2 week of acclimatization to the laboratory environment, the experimental procedures described below began. The animal procedures were performed according to the national guidelines for animal care and were approved by the local Institutional Animal Ethical Committee of Faculty of Medicine, Tanta University, Egypt.

Experimental Design

The rats were randomly divided into seven main groups. Control group included 21 rats while the other groups contained seven rats per each as follows:

Group I (Control group) was further subdivided into three equal subgroups:

***Subgroup* (*i*)**: Received daily injection of normal saline intraperitoneally (i.p.), the diluting vehicle for gentamicin, for 12 days.

***Subgroup* (*ii*)**: Received 0.5 mL of corn oil, the diluting vehicle for lycopene via oral gavage for 12 days.

***Subgroup* (*iii*)**: Received dimethyl sulfoxide (DMSO, Sigma–Aldrich Chemical Co. St. Louis, MO, USA), the diluting vehicle for rosmarinic acid diluted with saline (5% DMSO) by oral gavage for 12 days.

Group II (RA group): Received rosmarinic acid (Sigma–Aldrich Chemical Co. St. Louis, MO, USA) at a dose of 50 mg/kg/day dissolved in DMSO by oral gavage for 12 days (Makino et al., 2002).

Group III (Lyc group): Received lycopene (Sigma–Aldrich Chemical Co. St. Louis, MO, USA) at a dose of 4 mg/kg/day dissolved in corn oil via oral gavage for 12 days. This dose was selected based on previous studies (Karahan et al., 2005).

Group IV (Gen group): Injected intraperitoneally with gentamicin (Sigma–Aldrich Chemical Co. St. Louis, MO, USA) at a dose of 100 mg/kg/day for 12 days. Gentamicin was dissolved in normal saline before injection to induce experimental nephrotoxicity (Karahan et al., 2005).

Group V (RA + Gen): Received rosmarinic acid (50 mg/kg/day), 1 hr before gentamicin injection for 12 days.

Group VI (Lyc + Gen): Received lycopene (4 mg/kg/day), 1 hr before gentamicin injection for 12 days.

Group VII (RA + Lyc + Gen): Received rosmarinic acid (50 mg/kg/day) and lycopene (4 mg/kg/day), 1 hr before gentamicin injection for 12 days.

At the end of experiments, twenty-four hours after the last drug regime, the body weight of each rat was estimated. The rats were anesthetized with intraperitoneal injection of

sodium pentobarbital (35 mg/kg body weight).The heart was exposed after incision of the chest wall then 5 mL of intracardiac blood was drawn and serum was separated to estimate blood urea nitrogen (BUN) and serum creatinine. Then the rats were sacrificed and kidneys were removed and perfused with a fixative solution (2%paraformaldehyde and 2% glutaraldehyde solution in 0.1 M phosphate buffer pH 7.2 and then weighed and sampled for histopathological studies. Values of reduced glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and malondialdehyde (MDA) were then estimated in renal tissues.

Assessment of Nephrotoxicity

Levels of BUN and serum creatinine were estimated by using standard laboratory techniques to assess nephrotoxicity and results were expressed as mg/dL (Reddy et al., 2012).

Assessment of Renal Oxidative Stress

Cold isotonic saline was used to wash the left kidney from each animal after its dissection. Mincing and homogenization of the kidney were then done in chilled 1.15% KCl. The homogenate was used for estimating renal MDA, reduced GSH, GSH-Px, and SOD activities.

Spectrophotometric method was used to analysis the tissue MDA level as an indicator of lipid peroxidation (Buyuklu et al., 2014a). This procedure was applied to get a spectrophotometric estimation of the color generated during the reaction of thiobarbituric acid (TBA) with MDA at 535 nm. The MDA value was represented as nmol/g-tissue protein. Antioxidant activity was checked by estimating reduced GSH, GSH-Px and SOD. The concentration of reduced GSH was estimated at 412 nm by spectrophotometer and results were expressed as μmol/g tissue protein (Reddy et al., 2012). The GSH-Px enzyme activity analysis was measured at 340 nm by spectrophotometer and expressed as U/g-tissue protein (Buyuklu et al., 2014a). Xanthine/xanthine oxidase assay was used to estimate SOD (Units/mg-tissue protein) by measuring the amount of reduced nitroblue tetrazolium, with one unit of SOD specified as the amount of protein that inhibits the rate of NBT reduction by 50% (Buyuklu et al., 2014a).

Histological and Immunohistochemical Examination

The right kidney from each animal was cut longitudinally into two halves and was kept in 10% of neutral buffered formalin for 24 hr. Then it was processed and embedded in paraffin wax and sections of 4 μm thickness were taken using a microtome. These sections were stained with hematoxylin and eosin (H&E) and were investigated under light microscope, to reveal histological alterations (Gamble, 2008). The histopathological findings in the sections were graded as grade 0, no change; grade 1, mild usually single-cell necrosis in scattered tubules; grade 2, moderate with more than one cell in scattered tubules; and grade 3, marked which exhibiting total necrosis in almost every power field (Farombi and Ekor, 2006).

Four μm sections of kidney were immunohistochemically stained to estimate immunoexpression of Bax (proapoptotic protein), Bcl2 (antiapoptotic protein), light chain 3B (LC3/B) (a marker for autophagy), and inducible nitric oxide synthase (*i*NOS). Sections were incubated with a monoclonal antibody against Bax and Bcl2 (Dako, Carpinteria California, USA); in a dilution of 1:200, a polyclonal anti LC3/B antibody (Biotechnologies Corp, Canada) in a dilution of 1:400 and a polyclonal anti *i*NOS antibody (Thermo Fisher Scientific, Massachusetts, USA) in a dilution of 1:100 using the streptavidin-peroxidase method according to the manufacturer's protocol. Cells that showed brown precipitation were considered positive for Bax, Bcl2, LC3/B, and *i*NOS expressions. Negative controls were done using the same protocol but without applying the primary antibody.

Quantitative Morphometric Measurement

Leica Qwin 500 C Image analyzer computer system (Leica Imaging System LTD., Cambridge, England) in (Central Research Lab, Tanta Faculty of Medicine, Egypt) was used to obtain the morphometric data in the current work. Ten non-overlapping fields in slides of each animal in each group were examined to estimate:

1. **The percentage area of Bcl2 and Bax immunoreactions at 400× magnification**: The image analyzer was used to measure the area of Bcl2 and Bax and was expressed in relation to the area of the measuring frame of a known area (estimate area%/20 μm^2 frame).
2. **The mean of optical density of LC3/B and iNOS reactions at magnification of 400×**: It was measured using the color detect menu and in relation to a standard measuring frame.

Statistical Analysis

Analyses of all data were performed using the software Statistical Package for Social Sciences version 17 (SPSS Inc, Chicago, IL, USA). The data were presented as the mean ± SD (standard deviation). Mann–Whitney U-test was used for the statistical analysis of the histological scores. Comparisons between two groups in all other data were analyzed by unpaired Student "t" test. However, the difference among the groups was assessed with One-way analysis of variance (ANOVA) followed by Post Hoc Tukey's test. Probability of chance (P value) < 0.05 was considered statistically significant.

RESULTS

During the study period (12 days), no death rate was recorded. In addition, we did not find any statistical differences between the subgroups of group I (Control group) in all the studied parameters. Therefore, to facilitate the presentation of our results, we choose to present the relation between the subgroup (i) and the other treated groups only (we referred to it in tables and figures by "control group").

Estimation of Body and Kidney Weights

There was significant ($P < 0.05$) decrease of the body weight in the group administrated gentamicin compared with control group and compared with groups

TABLE 1. Body and kidney weights of rats in the different studied groups

Parameter	Group I (Control)	Group II (RA)	Group III (Lyc)	Group IV (Gen)	Group V (RA + Gen)	Group VI (Lyc + Gen)	Group VII (RA + Lyc +Gen)	*F* value
Body weight (g)	235.9 ± 10.77	232.1 ± 13.22	229.4 ± 9.45	198.3 ± 11.87 [a]*P*	224.3 ± 12.77 [b]*P*	225.3 ± 11.73 [b]*P*	228.17 ± 12.42 [b]*P*	8.73
Kidney weight (g)	0.82 ± 0.031	0.81 ± 0.021	0.78 ± 0.019	0.54 ± 0.043 [a]*P*	0.75 ± 0.031 [b]*P*	0.73 ± 0.034 [b]*P*	0.78 ± 0.042 [b]*P*	9.34

Data is expressed as mean ± SD, *P* value = probability of chance, tested by using Student "t" test and two-way ANOVA followed by Tukey's post-hoc test at $P < 0.05$.
[a]*P* ($P < 0.05$) vs. the control group (Group I).
[b]*P* ($P < 0.05$) vs. Gen group (group IV).

administrated rosmarinic acid and lycopene alone or combined. However, other groups showed no significant differences in body weight when compared with control group (Table 1).

Gentamicin administrated group showed significant ($P < 0.05$) decrease in kidney weight compared with control group and compared with groups administrated rosmarinic acid and lycopene alone or combined. On the other hand, other groups showed no significant differences in kidney weight when compared with control group (Table 1).

Biochemical Results

Influence of rosmarinic acid and lycopene on serum creatinine and BUN. Influence of rosmarinic acid and lycopene either individually or combined on serum creatinine and BUN was shown in Figure 1. Gentamicin treated group showed significant increase in serum creatinine and BUN compared with control group. Treatment of gentamicin group with rosmarinic acid, lycopene, or combination of rosmarinic acid and lycopene significantly ($P < 0.05$) decrease gentamicin-evoked rising in serum creatinine and BUN levels. More improvement in serum levels of creatinine and BUN was recorded in the group treated with both rosmarinic acid and lycopene.

Influence of rosmarinic acid and lycopene on oxidative stress parameters. Gentamicin administrated group showed significant increase in MDA level compared with control group. The same group also showed significant decrease in the levels of GSH, GSH-Px, and SOD compared with control group. Treatment of gentamicin group with rosmarinic acid, lycopene, or combination of rosmarinic acid and lycopene significantly ($P < 0.05$) ameliorated the increase in MDA level and the decrease in the levels of GSH, GSH-Px, and SOD (Fig. 2).

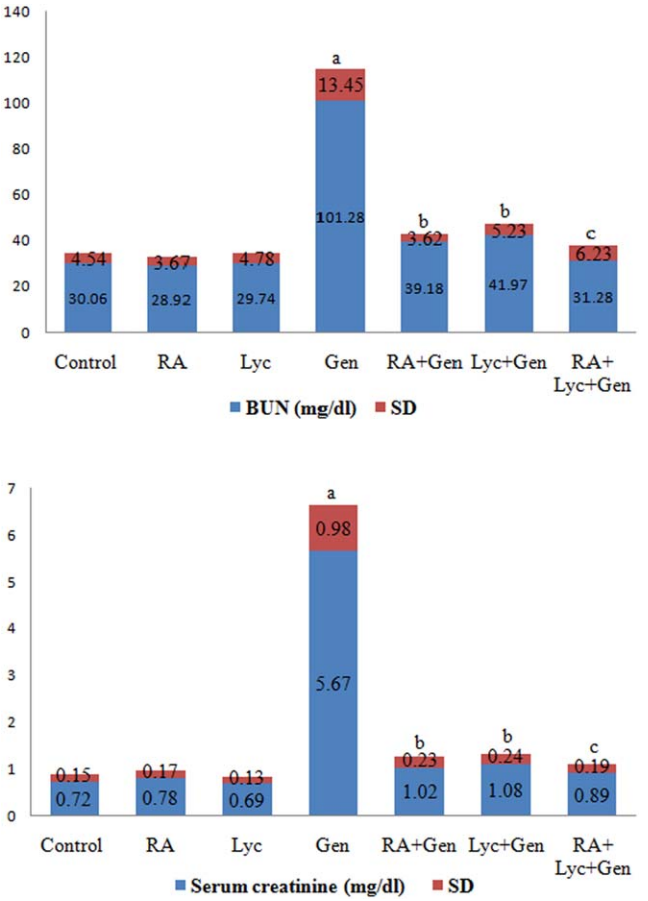

Fig. 1. Concentrations of creatinine and BUN in the examined groups. Student "t" test and two-way ANOVA followed by Tukey's post-hoc test were used. [a]$P < 0.05$ vs. control group; [b]$P < 0.05$ vs. Gen group (group IV); [c]$P(P < 0.05)$ vs. group V and VI. Data is expressed as mean ± SD.

Histological Results

Examinations of H&E-stained sections of control, rosmarinic acid, and lycopene groups were similar and showed normal histological architecture of renal cortex. Multiple glomeruli encompassed by Bowman's capsule were seen. Pyramidal cells with rounded nuclei and eosinophilic cytoplasm lined the proximal convoluted tubules (PCT). However, cuboidal cells with less eosinophilic cytoplasm lined the distal convoluted tubules (DCT) (Fig. 3).

In gentamicin group, shrunken glomeruli and dilatation of the capsular space were seen in Malpighian corpuscles. Degenerative changes as vacuolation with loss of the lining epithelium were observed in surrounding tubules. In addition, interruption of the tubular wall was occasionally noticed. Outspread necrosis with pyknosis of the nuclei was also recorded in several tubules (Fig. 4A,B). Interpretation of the histological score between gentamicin treated rat and normal rat showed a highly significant variation (2.87 ± 0.34, $P < 0.001$). In

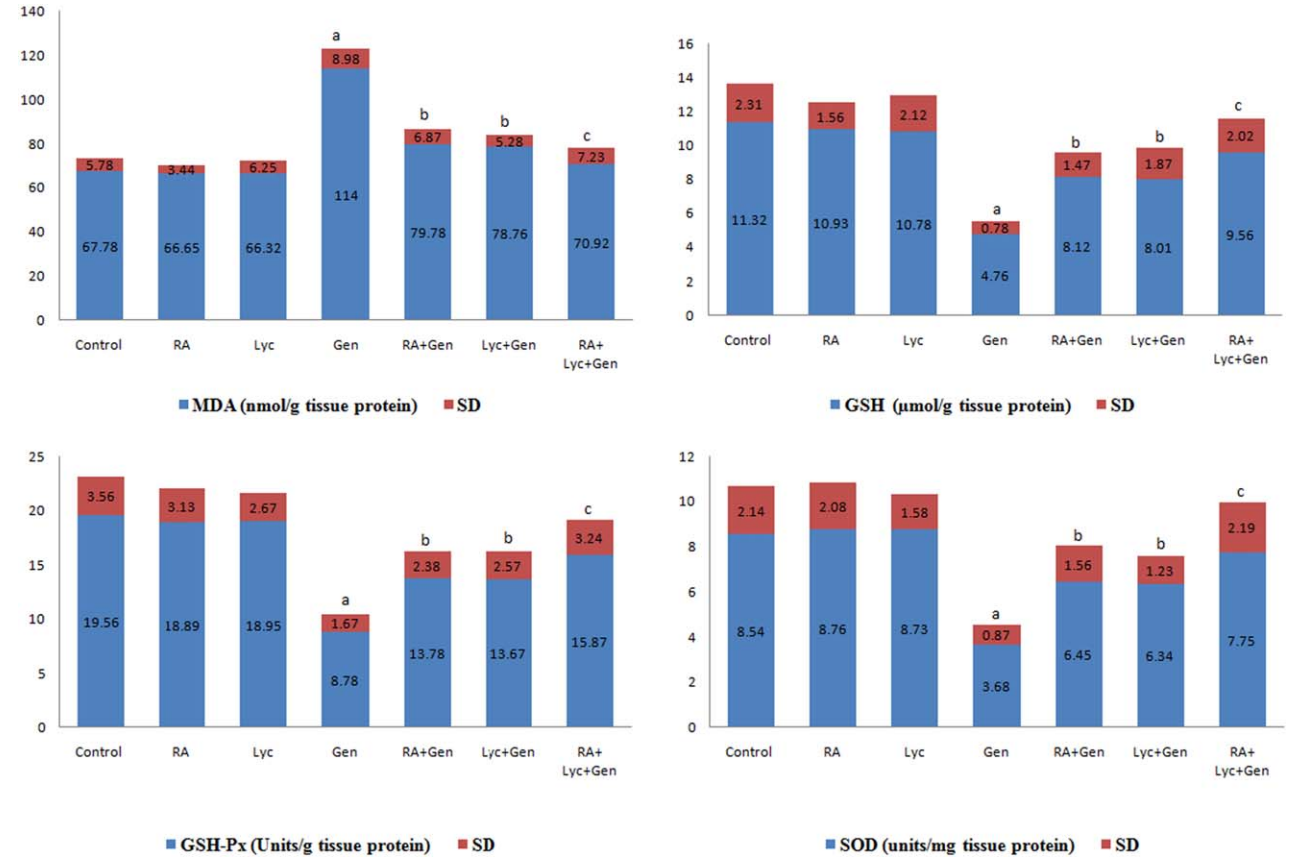

Fig. 2. Measurements of MDA, reduced GSH, GSH-Px and SOD values in the examined groups. Student "t" test and two-way ANOVA followed by Tukey's post-hoc test were used. $^{a}P < 0.05$ vs. control group; $^{b}P < 0.05$ vs. Gen group (group IV); $^{c}P (P < 0.05)$ vs. group V and VI. Data is expressed as mean ± SD.

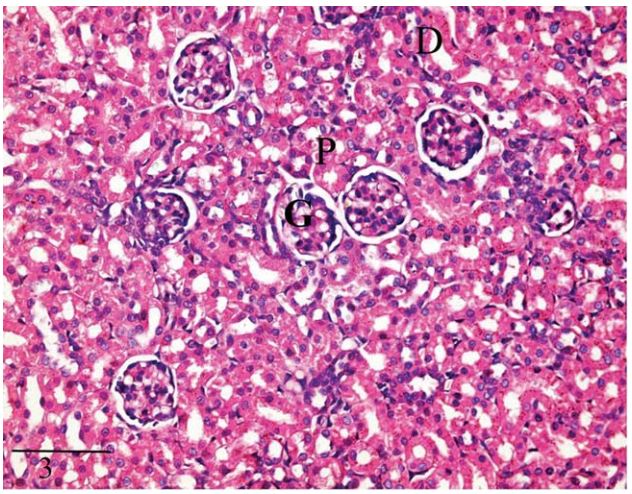

Fig. 3. Normal structure of the cortical tissue appears in the control group with multiple glomeruli of normal cellularity surrounded by Bowman's capsule (G). PCTs (P) is lined by deeply acidophilic cells and DCTs (D) is lined by acidophilic cubical cells with wider lumen (Hematoxylin & eosin stain, Bar: 40 µm).

addition, interstitial hemorrhage (2.79 ± 0.26, $P < 0.001$) and edema were significantly appeared with infiltrations by mononuclear cells (Fig. 4C,D).

Administration of rosmarinic acid, lycopene, or combined rosmarinic acid and lycopene with gentamicin, ameliorated the histological changes induced by gentamicin. The renal cortex appeared with histological architecture nearly similar to control group (Fig. 5A–C). Moreover, there were more amelioration in the histopathological changes in the group treated with rosmarinic acid and lycopene when compared with the groups treated by either rosmarinic acid or lycopene alone. Statistical analysis of animals administrated rosmarinic acid and lycopene either alone or combined demonstrated significant ($P < 0.05$) decrease in the histopathologic score recorded in gentamicin group (Fig. 6).

Results of Immunohistochemistry

Immunohistochemical reaction of Bax antigen. The cortex of the kidney manifested no immunostaining reaction for Bax in the control, rosmarinic acid and lycopene groups. While, scarce dispersed cells showed weak reaction in their cytoplasm (Fig. 7A). Gentamicin group, showed strong positive reaction over through most of the

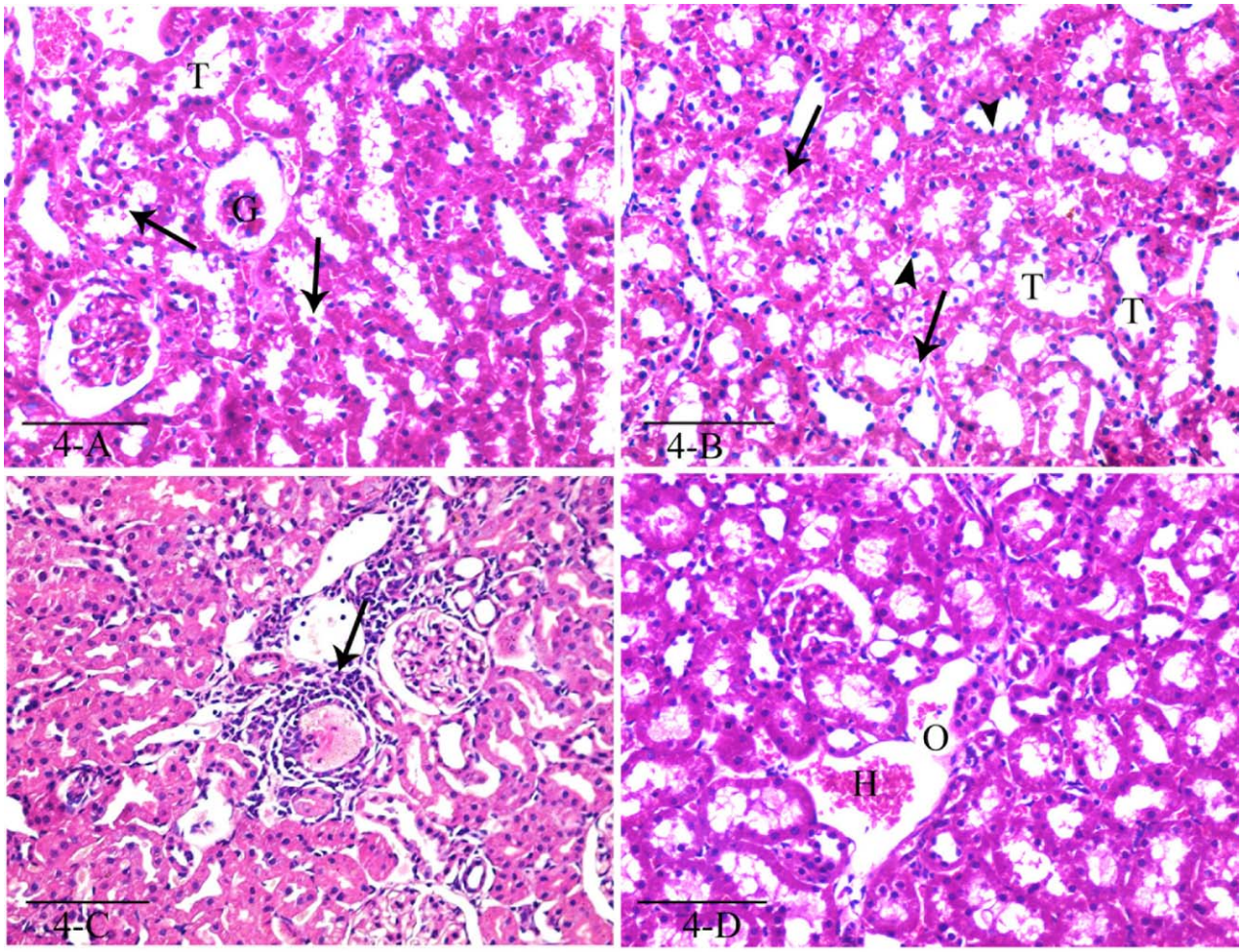

Fig. 4. Cortical sections from gentamicin group (A) showing abnormal shrunken glomeruli (G), dilatation of the tubules (T) and loss lining epithelium (→). (B) Showing dilated tubules (T), vacuolation (→), pykynosis and shedding of epithelial lining (➤). (C) Showing infiltrations by mononuclear cells (→). (D) Showing hemorrhage (H) and edema (O) in-between the tubules (Hematoxylin & eosin stain, Bar: 40 μm).

renal cortical cells (Fig. 7B). Gentamicin animals received rosmarinic acid, lycopene, or rosmarinic acid and lycopene in combination manifested weak reaction (Fig. 7C–E).Very weak reaction for Bax was observed in the group treated with both rosmarinic acid and lycopene (Fig. 7E). Statistical analysis of morphometric data revealed significant increase in the area percentage of Bax positive cells in gentamicin group in comparison with control animals group. While, gentamicin rats received rosmarinic acid or lycopene individually or combined with each other demonstrated significant ($P < 0.05$) decrease in the area percentage of Bax compared with gentamicin-administrated rats (Table 2**).**

Immunohistochemical reaction of Bcl2. Examination of cortical sections from control rats manifested moderate to marked reactivity of Bcl2 in the cytoplasm of the cortical cells (Fig. 8A). Bcl2 immunoexpression in cortical tissue of gentamicin group appeared with weak reaction when compared with control rats (Fig. 8B). Gentamicin rats received rosmarinic acid, lycopene, or rosmarinic acid and lycopene in combination manifested strong reaction of Bcl2 when compared with rats received gentamicin (Fig. 8C–E). In addition, stronger immunostaining reaction for Bcl2 was observed in the group treated with both rosmarinic acid and lycopene (Fig. 8E). Statistical analysis of morphometric data revealed significant decrease in the area percentage of Bcl2 positive cells in cortical tissue of gentamicin group compared with that of control group. While, gentamicin rats received rosmarinic acid or lycopene individually or combined with each other manifested significant ($P < 0.05$) increase in the area percentage of Bcl2 in comparison with gentamicin administrated rats (Table 2).

Immunohistochemical reaction of LC3/B. The cortex of the kidney showed weak immunostaining reaction for LC3/B in the control, rosmarinic acid and lycopene groups (Fig. 9A). Animals received gentamicin showed marked expression of LC3/B activity in the cortical tissue (Fig. 9B). However, gentamicin group administrated rosmarinic acid, lycopene or rosmarinic acid and lycopene in combination showed weak expression of LC3/B activity close to that observed in control rats (Fig.

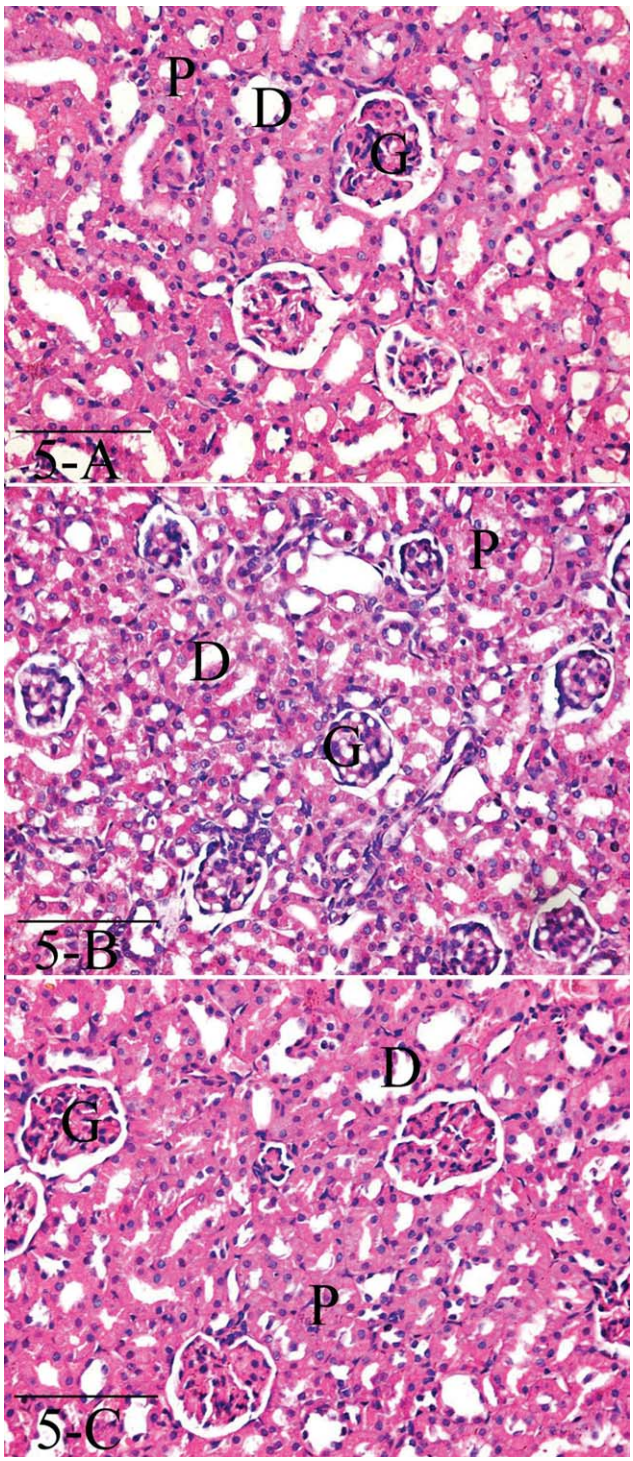

Fig. 5. Cortical sections showing to a great extend normal histological structure with normal glomeruli (G) PCT (P) and DCT(D). (A) Gen + RA group, (B) Gen + Lyc group and (C) Gen + RA + Lyc group (Hematoxylin & eosin stain, Bar: 40 μm).

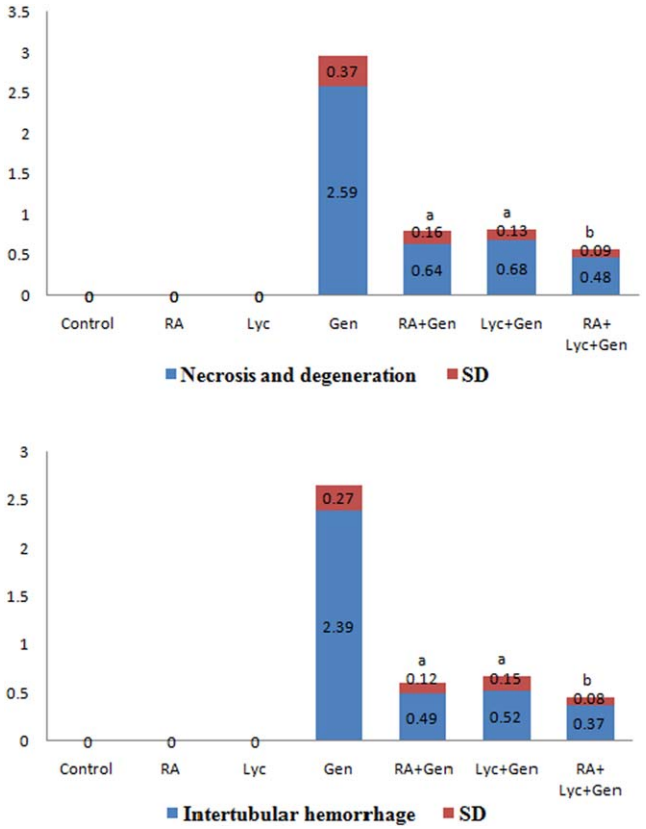

Fig. 6. Hisopathological score in the examined groups. Mann-Whitney U-test was used. $^{a}P < 0.05$ vs. control group; $^{b}P < 0.05$ vs. Gen (group IV).

9C–E). Combined treatment demonstrated weaker reaction of LC3/B activity as compared with parallel group (Fig. 9E). Statistical analysis of gentamicin group demonstrated significant increase in optical density of LC3/B compared with control one. While, gentamicin rats received rosmarinic acid or lycopene individually or combined with each other demonstrated significant ($P < 0.05$) decrease in the optical density of LC3/B in comparison with gentamicin administrated rats (Table 2).

Immunohistochemical reaction of iNOS. The cortex of the kidney showed weak expression of iNOS reaction in the control, rosmarinic acid and lycopene groups (Fig. 10A). In gentamicin administrated rats strong immunoreaction for iNOS was shown mostly in cortical tubules and was scarcely noticed in the glomeruli (Fig. 10B). Gentamicin group administrated rosmarinic acid, lycopene, or rosmarinic acid and lycopene in combination showed weak expression of iNOS activity (Fig. 10C–E). Statistical analysis of gentamicin group demonstrated significant increase in optical density of iNOS compared with control rats. While, gentamicin rats received rosmarinic acid or lycopene individually or combined with each other demonstrated significant ($P < 0.05$) decrease in the optical density of iNOS in comparison with gentamicin administrated rats (Table 2).

DISCUSSION

Regardless of unwanted gentamicin nephrotoxicity, it still represents an efficient therapeutic agent against life threatening gram-negative infections insensitive to other antibiotics. Gentamicin has been broadly applied as a

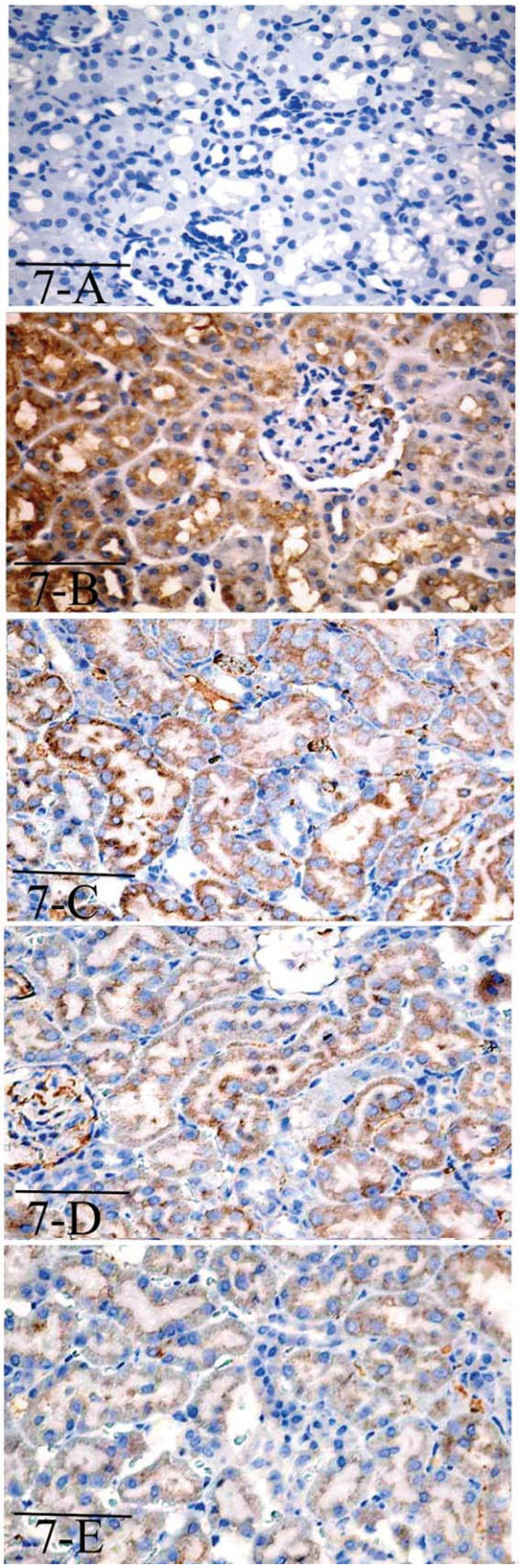

Fig. 7.

model to investigate acute renal failure in experimental animals. Several dosage schemes for gentamicin administration have been reported. Gentamicin injection at a dose of 100 mg/kg/day for 6 (Wiland and Szechcinski, 2003), 8 (Lakshmi et al., 2009), and for 12 days (Tavafi and Ahmadvand, 2011) were reported to cause marked nephrotoxicity.

Various natural agents have been used to attenuate drugs toxicity. In this research, we studied the preventive effect of rosmarinic acid and lycopene against gentamicin-caused nephrotoxicity.

In gentamicin-induced nephrotoxicity higher serum concentrations of creatinine, urea, and BUN have been suggested as considerable indicators for renal injury. The capability of the kidney to filter creatinine is diminished in gentamicin-induced nephrotoxicity as a result of decreased glomerular filtration rate (Srinivasan et al., 2009). In the current work, serum creatinine levels and BUN were significantly increased in gentamicin group compared with control group. These results are coordinated with other earlier researches (Alarifi et al., 2012; Bekheet et al., 2013). Significant reduction in serum creatinine and BUN was observed in gentamicin rats treated with rosmarinic acid, lycopene or combined rosmarinic acid and lycopene as compared with gentamicin group. The present results support their capacity to safeguard the kidneys from damage. The results of the present work are fairly harmonious with earlier works demonstrating normalization of serum urea, creatinine, BUN as well as creatinine clearance in gentamicin-induced nephrotoxicity received lycopene (Karahan et al., 2005) or rosamarinic acid (Tavafi and Ahmadvand, 2011; Azab et al., 2014).

Body and kidney weights in the present study, were significantly decreased in gentamicin treated rats. This reduction is probably a result of acidosis and increased catabolism resulted from acute renal failure, which leads to anorexia and diminished food ingestion (Ali et al., 2008). This result is in contrast to some other studies which demonstrated that the weight of kidney in gentamicin-treated rats was elevated as a result of the edema resulted from the acute tubular necrosis occurred (Erdem et al., 2000). Body and kidney weights were returned to normal values in gentamicin rats treated with rosmarinic acid and lycopene individually or in combination.

Gentamicin-treated rats revealed a significant variation in histopathological results when compared with control group. The kidney tubules showed vacuolation, necrosis with pyknosis of the nuclei and loss of the epithelial lining with interrupted wall of the tubules. These results could be explained by the concentration of gentamicin in the proximal tubules as the aminoglycosides are transported into the epithelial cells of the renal proximal tubules and remain for a long time, which lead to nephrotoxicity (Nagai and Takano, 2004; Alarifi et al., 2012). Proximal tubules are the main places of

Fig. 7. (A) Showing cortical tissue from control rat with no immunoexpression of Bax activity. (B) Showing marked immunoexpression of Bax activity in both cortical glomeruli and tubules of gentamicin group. (C, D, and E) Showing weak immunoexpression of Bax activity in groups administrated Gen + RA, Gen + Lyc, and Gen + RA + Lyc, respectively (Bax, immunostaining, Bar: 20 μm).

TABLE 2. The mean area % of Bax and Bcl2, the mean optical density of LC3/B and iNOS in the different groups studied.

Parameter	Group I (Control)	Group II (RA)	Group III (Lyc)	Group IV (Gen)	Group V (RA + Gen)	Group VI (Lyc + Gen)	Group VII (RA + Lyc +Gen)	*F* value
Bax (area %)	0.07 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	23.03 ± 2.23 [a]*P*	1.47 ± 0.37 [b]*P*	1.34 ± 0.28 [b]*P*	0.62 ± 0.14 [c]*P*	5.79
Bcl2 (area %)	42.78 ± 2.47	41.06 ± 2.58	40.38 ± 3.73	13.37 ± 1.39 [a]*P*	36.92 ± 1.89 [b]*P*	35.76 ± 2.34 [b]*P*	39.87 ± 2.39 [c]*P*	6.67
LC3/B (Optical density)	0.072 ± 0.014	0.061 ± 0.013	0.057 ± 0.012	1.12 ± 0.21 [a]*P*	0.13 ± 0.03 [b]*P*	0.083 ± 0.02 [b]*P*	0.059 ± 0.013 [c]*P*	65.5
*i*NOS (Optical density)	0.378 ± 0.064	0.367 ± 0.073	0.345 ± 0.083	1.232 ± 0.213 [a]*P*	0.546 ± 0.124 [b]*P*	0.589 ± 0.105 [b]*P*	0.398 ± 0.083 [c]*P*	25.8

Data is expressed as mean ± SD, *P* value = probability of chance, tested by using Student "t" test and two-way ANOVA followed by Tukey's post-hoc test at $P < 0.05$.
[a]*P* ($P < 0.05$) vs. the control group (Group I).
[b]*P* ($P < 0.05$) vs. Gen group (group IV).
[c]*P* ($P < 0.05$) vs. group V and VI.

reabsorption and active passage as they display a very extensive apical endocytic apparatus involving the proteins megalin and cubilin. This leads to a high rise of gentamicin in the lining epithelium of these tubules which modifies the function of various organelles and processes that are pivotal for cell viability (Nagai et al., 2006). Marked improvement in the histological changes was recorded in renal tissues of gentamicin rats pre-administrated rosmarinic acid, lycopene or combined rosmarinic acid and lycopene compared with gentamicin group. These results are in accordance with earlier works with rosmarinic acid (Tavafi and Ahmadvand, 2011; Azab et al., 2014) and lycopene (Karahan et al., 2005).

Many researchers have correlated oxidative stress as a likely cause for gentamicin-induced renal damage or nephrotoxicity. Morales et al. (2010) documented that gentamicin leads to augmentation of superoxide anions production that enhance the output of H_2O_2 in renal cortical mitochondria. H_2O_2 react with Fe^{2+} forming a reactive and unstable hydroxyl radical. Accumulation of free oxygen radicals leads to beginning of protective mechanism by renal cells using different antioxidant enzymes such as glutathione peroxidase and superoxide dismutase. Decreased activity of one or more antioxidant systems by the direct toxic influence of gentamicin results in a significant elevation in lipid peroxidation and augments the oxidative injury making the prognosis more severe.

Because reactive oxygen species have extremely short half-lives, they are difficult to be measured directly. Therefore, in the present study we assessed thiobarbituric acid reactive substances, which measures malondialdehyde present in the sample (Buyuklu et al., 2014a). Gentamicin treated group in this study, showed significant increase in MDA level compared with control group. This group also showed significant decrease in the levels of GSH, GSH-Px, and SOD compared with control group. These changes in biochemical measurements were consistent with the histological results, indicating that oxidative stress performs an essential part in gentamicin-produced nephrotoxicity and clarifying the causes of outspread and massive tubular necrosis manifested through the cortical tissue. Pre administration of rosmarinic acid is helpful in interrupting this vicious circle by elevating the levels of SOD, GSH, GSH-Px and decreasing the level of MDA. This preventive action of rosmarinic acid may be due to its high protective capability against reactive oxygen and nitrogen species (Bakirel et al., 2008; Lee et al., 2008) as well as through stimulation of endogenous antioxidant defense system (Tavafi and Ahmadvand, 2011). The same results were obtained in rats pre-administrated lycopene individually or combined with rosmarinic acid. Among the carotenoids, lycopene is more effective in protecting the cortical tubular cells from oxygen radical induced injury by preventing the generation of oxidized products (Tapiero et al., 2004; Palabiyik et al., 2013).

The nephrons' response to nephrotoxic agents is variable. Apoptosis and autophagy are two distinct form of programmed cell death (Su et al., 2013). Apoptosis performs a main role in the physiological processes of kidney growth and changes in renal diseases and drug-caused nephrotoxicity. It can be initiated through either intrinsic or extrinsic pathways (Servais et al., 2008). The intrinsic mitochondrial pathway is the main route for drug-induced nephrotoxicity. Injury of the mitochondria results in the release of caspase activators, such as cytochrome c and inhibitors of antiapoptotic responses (Morales et al., 2010). Proteins of Bcl-2 series are either pro- or antiapoptotic and serve as molecular regulators for the mitochondrial pathway. The pro-apoptotic proteins (Bax and Bak) undergo changes in their structure after exposure to death signals and modify the mitochondrial membrane structure causing the release of cytochrome c and pro-apoptotic factors (Lalier et al., 2007). Binding of Bcl-2 and Bcl-xL, antiapoptotic proteins to pro-apoptotic proteins suppress their stimulation and thus preserve the integrity of mitochondrial membrane (Chipuk et al., 2010). In the present study, the area percentage of Bax positive cells was significantly increased while; the area percentage of Bcl2 positive cells was significantly decreased in gentamicin treated rats compared with control rats. Apoptosis caused by gentamicin has been revealed in human renal tubular cells (Lee et al., 2013) and it causes proximal tubular cell death (Lopez

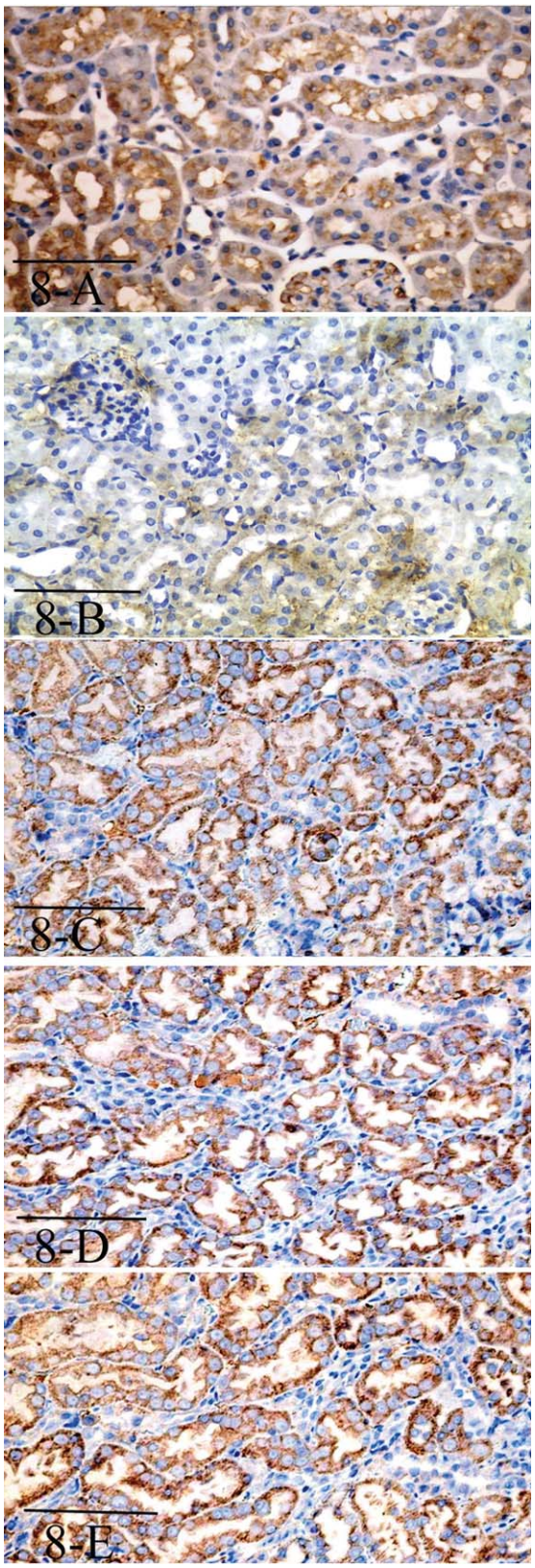

Fig. 8. (A) Showing cortical tissue from control rat with marked immunoexpression of Bcl2 activity. (B) Showing weak immunoexpression of Bcl2 activity in both cortical glomeruli and tubules of gentamicin group. (C, D, and E) Showing marked immunoexpression of Bcl2 activity in groups administrated Gen + RA, Gen + Lyc, and Gen + RA + Lyc, respectively (Bcl2, immunostaining, Bar: 20 μm).

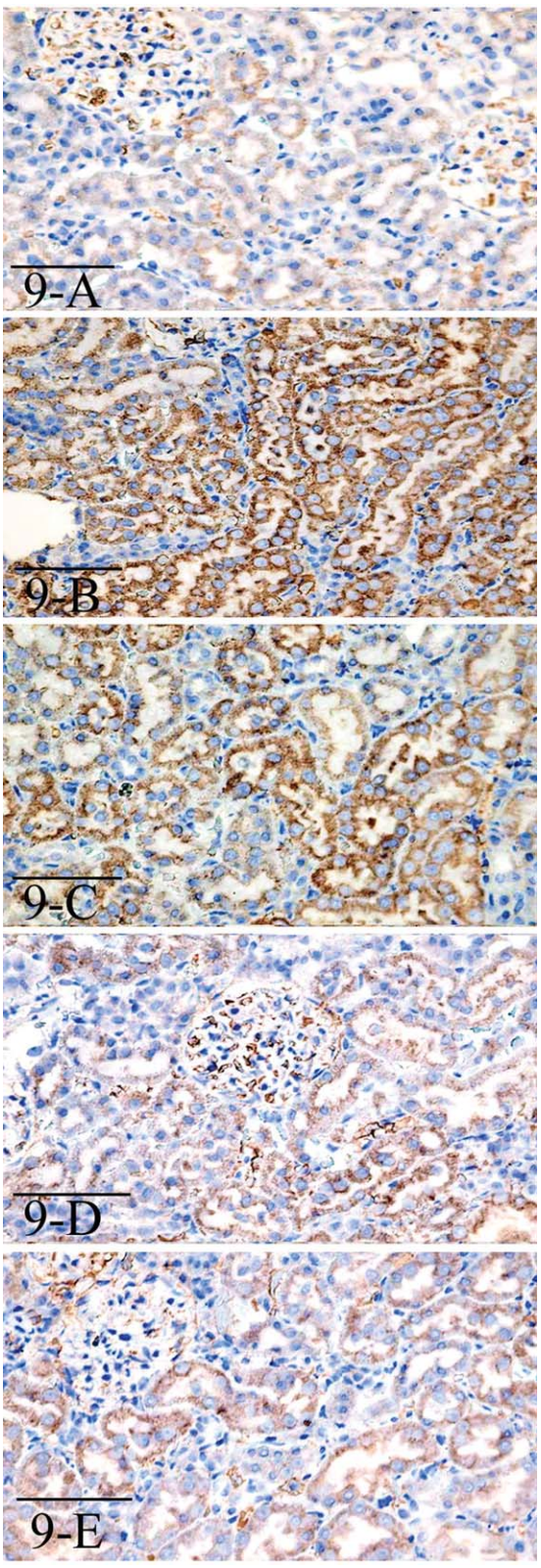

Fig. 9. (A) Showing cortical tissue from control rat with weak immunoexpression of LC3/B activity. (B) Showing cortical tissue from gentamicin administrated rat with marked immunoexpression of LC3/B activity. (C, D, and E) Showing weak immunoexpression of LC3/B activity in groups administrated Gen + RA, Gen + Lyc, and Gen + RA + Lyc, respectively (LC3/B, immunostaining, Bar: 20 μm).

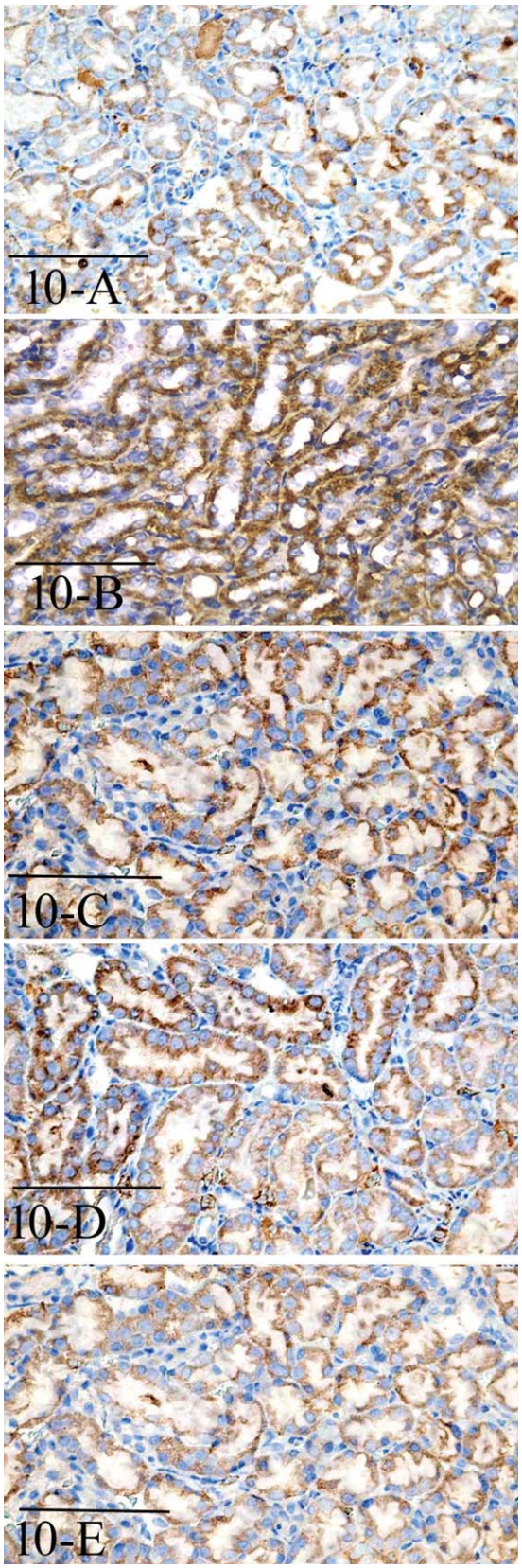

Fig. 10.

et al., 2011). This can be explained by the ability of gentamicin to bind with phospholipids of the cell membrane of renal tubules and subcellular organelles as mitochondria and lysosomes activating specific stress sensors and send death signals (Erdem et al., 2000; Morales et al., 2010(. Apoptotic cell death induced by gentamicin has been proposed to be due to reactive oxygen species (Morales et al., 2010). In the present work, the Bax activity was significantly deceased and Bcl2 activity was significantly increase in the group received gentamicin with rosmarinic acid when compared with gentamicin group and as a result apoptosis was inhibited. The inhibitory impact of rosmarinic acid on apoptosis are in accordance with the work of Domitrović et al. (2014) who assumed that oral administration of rosmarinic acid reduces the overexpression of cleaved caspase-3. Preadministration of lycopene either individually or combined with rosmarinic acid in the present work, had similar results gained from treatment with rosmarinic acid individually although, combined administration of rosmarinic acid and lycopene showed better results compared with their symmetric group. These results are in consistent with earlier published works concluding that lycopene inhibits apoptosis in contrast medium-caused renal damage (Buyuklu et al., 2014a).

Autophagy is normally a cell-survival pathway that provides homeostasis and nutritional recycling by getting rid of unwanted or dysfunctional cellular components. Whilst, uncontrolled autophagy causes metabolic disturbance and cell damage (Su et al., 2013). Previous studies showed that autophagy has been involved in nephrotoxin-induced acute renal damage (Funk and Schnellmann, 2012; Buyuklu et al., 2014a,b). In the present study, rats received gentamicin showed marked expression of LC3/B activity in the cortical tissue. LC3 is mostly used as an indicator of continuing autophagy as described by Rubinsztein et al.(2009). Gentamicin group pretreated with rosmarinic acid, lycopene, or rosmarinic acid and lycopene in combination showed weak expression of LC3/B activity demonstrating their importance in decreasing autophagic cell damage. Both apoptosis and autophagy have an essential role in renal cell damage induced by gentamicin as indicated by augmentation of the immunoexpression of LC3/B and Bax and suppression of Bcl2 immunoexpression.

Nitric oxide (NO) production by induction of the inducible NO synthase (iNOS) is an important for nonspecific host defense, helping to kill tumors and intracellular pathogens. However, cytotoxicity can result from its massive formation as reaction of NO with superoxide causes formation of peroxynitrite, which is a powerful cytotoxic agent (Trachtman et al., 2002; Förstermann and Sessa, 2012). Renal tissue content of *i*NOS increases in kidney injury and may be present in normal kidney tissue in scarce amounts (Manikandan et al., 2011; Buyuklu et al., 2014a,b). This data is in accordance with the result of the current work as

Fig. 10. (A) Showing cortical tissue from control rat with weak immunoexpression of iNOS activity. (B) Showing cortical tissue from gentamicin administrated rat with marked immunoexpression of iNOS activity. (C, D, and E) Showing weak immunoexpression of iNOS activity in groups treated with Gen + RA, Gen + Lyc, and Gen + RA + Lyc, respectively (iNOS, immunostaining, Bar: 20 μm).

statistical analysis of gentamicin group demonstrated significant increase in immunoexpression of iNOS compared with control rats. While, gentamicin rats received rosmarinic acid or lycopene individually or combined with each other demonstrated significant decrease in the immunoexpression of iNOS in comparison with gentamicin administrated rats.

The biochemical, histological, and immunohistochemical results obtained in the current study support the view that gentamicin has harmful effects on the renal tissue. Rosmarinic acid and lycopene alone or combined were shown to have beneficial effects versus gentamicin-caused renal damage. Such effects were accomplished by safeguarding against renal oxidative stress, autophagy, and apoptosis. The combined therapy of rosmarinic acid and lycopene showed more beneficial preventive effects than the corresponding monotherapies. Accordingly, if gentamicin is prescribed it is advisable to employ combined therapy of rosmarinic acid and lycopene as natural nephroprotective factors.

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