Research Article

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Investigation of the protective effect of rosmarinic acid in rats given high dose gentamicin

Yüksek doz gentamisin verilen sıçanlarda rosmarinik asidin koruyucu etkisinin araştırılması

Hülya Çetin, Damla Gündüz, Hülya Aybek

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Abstract

Purpose: Gentamicin is an antibiotic, used to treat infections caused by microorganisms. Endogenous antioxidants are strengthened by using antioxidant protectors against the toxic effects caused by drugs. Rosmarinic acid has antioxidative, anti-inflammatory, antiapoptotic, and antitumoral effects. In our study, we aimed to investigate the protective effect, immunohistochemical changes, oxidative markers, histopathological changes and inflammation related Ifi44 expression of rosmarinic acid against gentamicin induced nephrotoxicity in rats.

Materials and methods: In our study, 32 rats randomly divided into four groups. These groups are consisted of control group, gentamicin group received gentamicin 100 mg/kg/day, gentamicin + rosmarinic group acid received gentamicin 100 mg/kg/day and rosmarinic acid 50 mg/kg/day, and rosmarinic acid group received rosmarinic acid 50 mg/kg/day. At the end of the study, histopathological, immunohistochemical, and biochemical changes in kidney tissues evaluated.

Results: Rosmarinic acid reduced creatine, urea, blood urea nitrogen, and total oxidative stress in blood serum. The toxic effect of gentamicin caused severe histopathological changes in the kidneys. A slight decrease in histopathological changes observed in the gentamicin + rosmarinic acid group. Antiproliferative Ifi44 expression was higher in the gentamicin group and gentamicin + rosmarinic acid group.

Conclusion: As a result of the application of rosmarinic acid, a decrease in oxygen radicals and an increase in antioxidant levels observed. When used in combination with gentamicin and rosmarinic acid, the protective effect of rosmarinic acid was partially observed, but it could not provide full protection.

Key words: Gentamicin, rosmarinic acid, Ifi44, rat.

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Öz

Amaç: Gentamisin, mikroorganizmaların neden olduğu enfeksiyonları tedavi etmek için kullanılan bir antibiyotiktir. İlaçların neden olduğu toksik etkilere karşı antioksidan koruyucular kullanılarak endojen antioksidanlar güçlendirilir. Rosmarinik asit antioksidan, antienflamatuar, antiapoptotik ve antitümör etkilere sahiptir. Çalışmamızda rosmarinik asidin sıçanlarda gentamisin ile indüklenen nefrotoksisiteye karşı koruyucu etkisini, immünohistokimyasal değişiklikleri, oksidatif belirteçleri, histopatolojik değişiklikleri ve enflamasyona bağlı Ifi44 ekspresyonunu araştırmayı amaçladık.

Gereç ve yöntem: Çalışmamızda 32 rat rastgele 4 gruba ayrıldı. Bu gruplar kontrol grubu, gentamisin grubu 100 mg/kg/gün gentamisin verildi, gentamisin + rosmarinik asit grubu gentamisin 100 mg/kg/gün ve rosmarinik asit 50 mg/kg/gün verildi, rosmarinik asit grubu rosmarinik asit 50 mg/kg/gün verildi. Çalışma sonunda böbrek dokularındaki histopatolojik, immünohistokimyasal ve biyokimyasal değişiklikler değerlendirildi.

Bulgular: Rosmarinik asit, kan serumunda kreatin, üre, kan üre nitrojeni ve toplam oksidatif stresi azaltmıştır. Gentamisinin toksik etkisi böbreklerde ciddi histopatolojik değişikliklere neden olmuştur. Gentamisin + rosmarinik asit grubunda histopatolojik değişikliklerde hafif bir azalma gözlendi. Antiproliferatif Ifi44 ekspresyonu gentamisin grubunda ve gentamisin + rosmarinik asit grubunda daha yüksekti.

Sonuç: Rosmarinik asit uygulaması sonucunda oksijen radikallerinde azalma ve antioksidan düzeylerinde artış gözlendi. Gentamisin ve rosmarinik asit birlikte kullanıldığında rosmarinik asidin koruyucu etkisi kısmen gözlenmiştir ancak tam koruma sağlayamamıştır.

Anahtar kelimeler: Gentamisin, rosmarinik asit, Ifi44, sıçan.

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Hülya Çetin, Prof. Pamukkale University, Faculty of Medicine, Department of Histology and Embryology, Denizli, Türkiye, e-mail: hcsorkun@pau.edu.tr (https://orcid.org/0000-0001-8731-0631) (Corresponding Author)

Damla Gündüz, PhD. Malatya Turgut Özal University, Faculty of Medicine, Department of Histology and Embryology, Malatya, Türkiye, e-mail: damla.gunduz@ozal.edu.tr (https://orcid.org/0000-0003-4838-6574)

Hülya Aybek, Prof. Pamukkale University, Faculty of Medicine, Department of Medical Biochemistry, Denizli, Türkiye, e-mail: haybek@pau.edu.tr (https://orcid.org/0000-0002-0635-4251)

Introduction

Gentamicin (GM) is widely used to treat infections caused by gram-negative and gram-positive microorganisms and is an aminoglycoside antibiotic. Clinicians stated that using this drug potentially causes nephrotoxicity and histopathological lesions are seen in 30% of patients. 90% of the GM used for therapeutic purposes retains its structure without being metabolized by the liver and is excreted by the renal tubules and especially the proximal convoluted tubules. [1, 2]. While extensive necrosis was observed with GM, significant apoptosis was observed in epithelial cells of rats in which a clinical dose was given, and proximal convoluted tubules were seen without necrosis [3].

Although the mechanism of GM-induced nephrotoxicity cannot be fully explained, it is suggested that it is caused by oxidative stress, inflammation, increased monocyte and macrophage infiltration, and apoptosis [4-7]. It is stated that reactive oxygen species can be balanced by strengthening endogenous antioxidants by using natural antioxidant preservatives against the toxic effects caused by drugs [8]. Rosmarinic acid (RA) is one of the most important and well-known polyphenolic antioxidants, abundant in various medicinal plants of the Lamiaceae family and historically used in traditional Chinese medicine. In various studies, it has been reported that rosmarinic acid has antioxidative, anti-inflammatory, antibacterial (both gram-positive and gram-negative bacteria), antiviral, antiapoptotic, antihyperglycemic, antitumoral (against various types of carcinoma, colorectal, pancreas, breast, lung, ovary, melanoma, etc.), analgesic, cardioprotective, neuroprotective and hepatoprotective effects [9-17].

Interferons (IFN) involved in inflammation are important cytokines. [18]. Microtubule-associated protein 44 (Ifi44) has been reported to be antiproliferative. Ifi44, also called interferon-α inducible protein 44 or p44 because it assembles to form microtubular structures, is a member of the Type I IFN-α inducible gene family. The functions of ifi44 include participation in microtubule formation, promotion of apoptosis, inhibition of proliferation, and participation in the autoimmune response. [19, 20]. It has therefore been suggested that Ifi44 may be associated with the inflammation involved in GM-induced nephrotoxicity [21]. In our study, we aimed to investigate the protective effect of RA against GM-induced nephrotoxicity in rats in terms of oxidative markers, histopathological changes, and immunohistochemically the expression of Ifi44, which is claimed to be associated with inflammation.

Materials and methods

In our study 32 wistar albino adult male rat weighing 230-250 gr was used. Rats were hosted in at room temperature (25°C) and in standard lighting (12 hours dark/luminous) cages. Tap water and pellet feed was provided as standard for feeding the animals. The procedures applied to the rats were carried out per the provisions of all national and international laws that are a party to Pamukkale University Animal Experiments Ethics Committee Directive.

Our study was approved by Pamukkale University Animal Experiments Ethics Committee (PAUHADYEK-2018/21) and was carried out with the support of Pamukkale University Scientific Research Projects Coordination Unit (project no: 2019SABE014).

After the rats were randomly divided into 4 groups (8 in each group) injections were made at the same times every day for 12 days. Rosmarinic acid (sigma-Aldrich Chemical Co.St. Louis, MO, USA) and gentamicin sulfate (GOLBIO, Gold Biotechnology; cat. G-400, CAS. 1405-41-0) were injected into rats prepared by mixing with physiological saline every day. Control group (C): Only 0.5 mL saline was injected intraperitoneally. GM group (G): It was dissolved in physiological saline corresponding to the dose of GM sulfate 100 mg/kg and injected intraperitoneally [22]. GM+RA group (GR): It was dissolved in physiological saline corresponding to the dose of GM sulfate 100 mg/kg and RA 50 mg/kg and injected intraperitoneally (RA was administered intraperitoneally one hour before GM sulfate administration) [23]. RA group (R): It was dissolved in physiological saline corresponding to the dose of RA 50 mg/kg and it was injected intraperitoneally [24].

On the 13th day, the body weights of the anesthetized rats were taken and recorded. The thorax was opened and the blood obtained by intracardiac puncture was collected in yellow biochemistry tubes and centrifuged. Urea,

Blood Urea Nitrogen (BUN), creatinine, Total Antioxidant Status (TAS), and Total Oxidant Status (TOS) (Rel Assay Kit Diagnostics, Gaziantep, Turkey) levels were determined from serum. Kidneys were removed, weighed, and fixed in 10% formaldehyde. 5-micron sections were taken from tissue that embedded paraffin. These sections were stained histochemically with hematoxylin and eosin (H+E) and periodic acid schiff (PAS) stains (Histomed BS-0046. Lot.092016.001). Staining was carried out according to the procedure specified in the PAS dye kit. The histopathological findings were evaluated in these sections.

Ifi44 (Invitrogen, PA5-96967, Lot: R93772) expression was evaluated immunohistochemically. Sections were incubated after deparaffinization. After washing in PBS, incubated in serum blocking solution (Thermo Fisher Scientific, TP 125UB, Fremont, USA) at room temperature. Primary antibody (Ifi44) diluted 1:100 was dropped on the sections and left overnight. Slides were treated with secondary antibodies (Thermo Fisher Scientific, TP 125UB, Fremont, USA). The slides were washed with PBS and incubated with HRP-streptavidin working buffer for 10 min. Sections were treated with DAP. Counterstaining was done with hematoxylin.

Sections which dyed both with histochemically and immunohistochemically methods were investigated under the light microscope (Olympus Microscope BX51).

Immunohistochemical evaluation

Photographs were obtained at X400 magnification from immunohistochemically stained sections of kidney tissue of each rat. Immune-reacting cells were counted in 10 different areas using the Image J program. The mean number of immune-reacting cells belonging to the groups was obtained.

Biochemical analysis

Urea, BUN, and creatinine levels were measured by spectrophotometric method immediately after obtaining the serum. The serum was kept at − 70°C until TAS and TOS levels were measured.

The principle of TOS measurement is based on the conversion of the ferrous ion chelator complex of the oxidants in the sample to ferric ion, which reacts with the chromogen in an acidic environment and causes an increase in absorbance. The increase in absorbance observed spectrophotometrically is directly proportional to the oxidant molecules in the sample. TOS was studied from the obtained sera by means of a commercial kit (Rel Assay Diagnostic, Lot.RL0024). The color intensity, which can be measured spectrophotometrically with an ELISA reader, is related to the total amount of oxidant molecules present in the sample. Results are expressed as μmol H_2O_2 Equiv/L. The principle of TAS measurement is based on the fact that all antioxidants in the sample convert the blue-green 2,2'-azino-bis ABTS (3-ethylbenzothiazoline-6-sulfonic acid) radical into colorless reduced ABTS. The change in absorbance of the sample is proportional to its antioxidant level. TAS measurement was made with a commercial kit (Rel Assay Diagnostic, Lot. RL0017) and evaluated spectrophotometrically with an ELISA reader. Results are expressed per μmol Trolox Equiv/L.

Histopathological evaluation

The sections of the groups were coded to avoid prejudices and were evaluated blindly by two researchers. Semi-quantitative assessment of kidney tissue and severity of damage were examined according to previously published criteria [25].

(i) Glomerular damage (% of renal parenchyma involvement): none=0, less than 25% of all renal parenchymal tubules=1, 25-50% of glomeruli exhibit nonspecific damage characteristics=2, that 50-75% of glomeruli exhibit nonspecific damage characteristics=3 and that more than 75% of glomeruli exhibit nonspecific damage characteristics=4; (ii) Acute tubular necrosis (% of renal parenchyma involvement): none=0, less than 25% of all renal parenchymal tubules=1, 25-50% of all renal parenchymal tubules=2, 50-75% of all renal parenchymal tubules=3 and more than 75% of all renal parenchymal tubules=4; (iii) Tubulointerstitial inflammatory infiltrates: none=0, limited leukocytes within the interstitium=1, and leukocyte infiltrating interstitium and tubules epithelial cell=2, the scoring system used was done according to the following measurement: (A) none nephrotoxicity:

0-1, (B) mild nephrotoxicity: 2-4, (C) moderate nephrotoxicity: 5-7, (D) severe nephrotoxicity: 8-10 [25].

Basal membrane thickness was evaluated semiquantitatively in the sections where PAS staining was performed by two researchers.

Statistical analysis

Data was analyzed with SPSS package program. Continuous variables average+/- standard deviation and categorical variables were given as numbers and percentages when parametric test assumptions were provided. One-way Analysis of Varyans (post-Hoc Turkey test) in comparison of independent group differences; when parametric test assumptions were not provided has been done. Kruskal Wallis Varyans Analysis (post Hoc Bonferroni should correct Mann Whitney U test) used the comparison to independent group differences. Chi-square always was used in comparison to categorical variables. *P*<0.05 was considered statistically significant.

Results

The mean body weights of the G and GR groups treated with GM were significantly reduced. A slight increase was observed in the mean kidney weights of the same groups (Table 1). When the mean body weights of the G and GR groups were compared with the C group, there was a statistically significant decrease. When the mean weights of the kidneys were evaluated, the weight of the left kidney was higher in the G group. The mean left kidney weight of the G group was statistically significantly increased when compared to the R group (Table 1).

Table 1. Mean body and kidney weights of rats in different study groups

Groups	Group C	Group R	Group GR	Group G
Body weight (gr)	228.08±22.04	225.02±42.70	177.17±9.69 ^{k}p	175.07±12.87 ^{k}p
Right kidney weight (gr)	0.88±0.12	0.93±0.20	1.02±0.11	1.08±0.06
Left kidney weight (gr)	0.88±0.15	0.88±0.17	1.05±0.18	1.09±0.05 ^{ʁ}p

^{k}p (p<0.05); statistically significant with group C, ^{ʁ}p (p<0.05); statistically significant with group R

Mild tubulointerstitial inflammation was observed in the renal cortex of two rats in the C group. Histological changes were not observed in the kidney tissue of the remaining six C group rats (Figure 1).

In all of the rats which administered GM; glomerular damage, tubular necrosis, and moderate nephrotoxicity as a result of tubulointerstitial inflammation scoring were determined. In five rats in the GR group that applied RA together with GM, the nephrotoxicity level was mild. Moderate nephrotoxicity was observed in the remaining three rats.

Two of the R-group rats had mild tubulointerstitial inflammation. No histological changes were noted in the kidneys of the remaining rats belonging to group R (Figure 1) (Table 2).

When the kidney damage of the G group was compared statistically with both C and R groups, it was highly significant (p<0.001). The kidney damage of the GR group was also statistically significant when compared with the C group and R group (p<0.001). There was no statistical difference between the G and GR groups in terms of kidney damage (p=0.070). When the kidney damage of group C and group R was compared, it was not statistically significant (p=0.928).

In the histological sections of the G and GR groups, there were loss, flattening, necrosis, and vacuolization in the epithelial cells of the tubules. Shrinkage of the glomerular structure of the Malpighi corpuscle, dilatation of the capsular cavity, and extensive tubulointerstitial inflammation areas were observed (Figure 1).

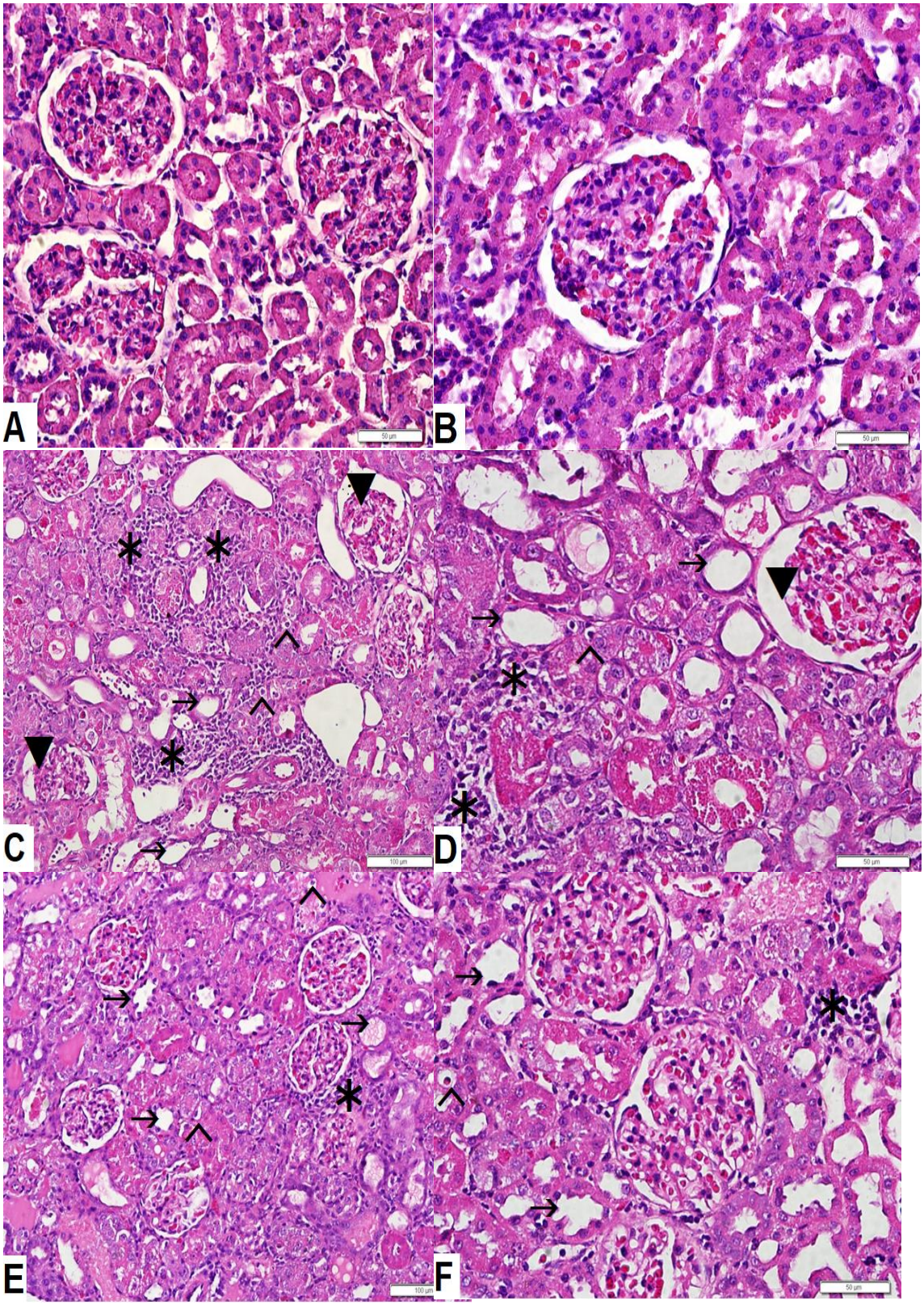

Figure 1. Microphotographs of the renal cortex of group C (A) and group R (B) (H&E X40). In rat kidney cortex treated with gentamicin (group G) (C X20, D X40) and gentamicin+rosmarinic acid (group GR) (E X20, F X40) intense areas of inflammation (*), vacuolized cells (^), dilated tubule and epithelial loss (→), glomerular damage and dilated Bowman's capsule (▲) (H&E)

Table 2. Histological changes in kidneys of rats in all groups

Rat no	Glomerular damage	Tubular necrosis	Tubulointerstitial inflammation	Total score	Scoring scale
C1	0	0	0	0	A
C2	0	0	0	0	A
C3	0	0	0	0	A
C4	0	0	0	0	A
C5	0	0	1	1	A
C6	0	0	0	0	A
C7	0	0	0	0	A
C8	0	0	1	0	A
G1	2	2	1	5	C
G2	2	2	1	5	C
G3	2	2	1	5	C
G4	2	2	1	5	C
G5	2	2	1	5	C
G6	2	2	1	5	C
G7	2	2	1	5	C
G8	2	2	1	5	C
GR1	1	1	1	3	B
GR2	1	2	1	4	B
GR3	1	2	1	4	B
GR4	2	2	1	5	C
GR5	1	1	1	3	B
GR6	2	2	1	5	C
GR7	2	2	1	5	C
GR8	1	1	1	3	B
R1	0	0	0	0	A
R2	0	0	1	1	A
R3	0	0	0	0	A
R4	0	0	0	0	A
R5	0	0	1	1	A
R6	0	0	0	0	A
R7	0	0	0	0	A
R8	0	0	0	0	A

No nephrotoxicity: (0-1)=A, mild nephrotoxicity: (2-4)=B, moderate nephrotoxicity: (5-7)=C, severe nephrotoxicity: (8-10)=D

When the sections of the C group and R group were examined, the brush border of the proximal tubules could be observed. However, the brush border structure of kidney tubules in the G and GR groups was lost. Especially in the G group, both glomerular and tubular basement membrane thickening was observed (Figure 2).

When we examined immunohistochemically, it was observed that the expression of Ifi44, which has an antiproliferative effect, was quite intense in the kidney tissues of the G and GR groups compared to the C and R groups (Figure 3). It was observed that the rate of Ifi-44 immunoreactivity in the G group was statistically higher than the other groups (C, GR, R) (p<0.001). The rate of Ifi-44 immunoreactivity in the GR group was statistically higher than in both the C and R groups (p<0.001). No statistically significant difference was found between group C and group R in terms of Ifi-44 immunoreactivity (p=0.954).

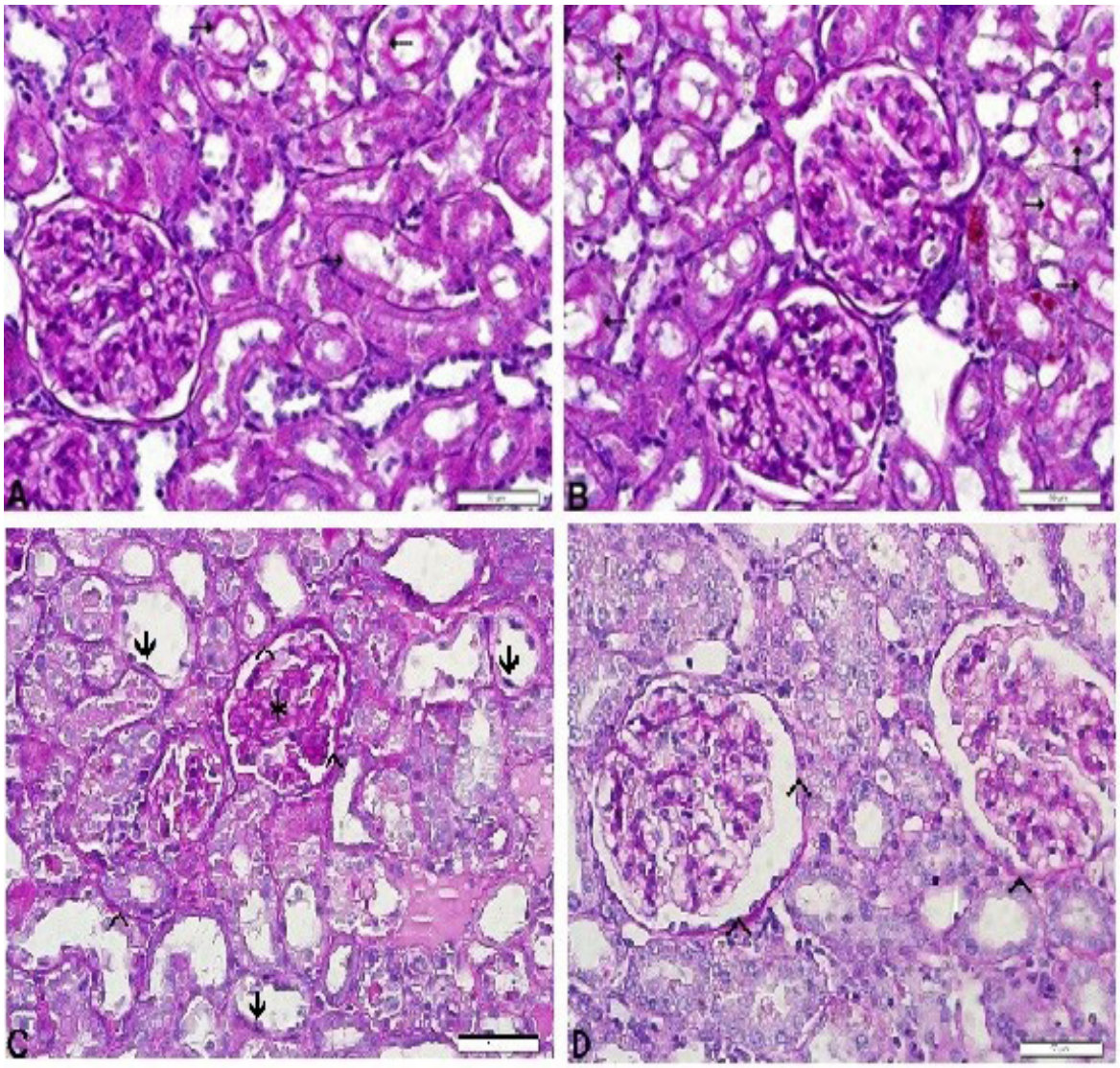

Figure 2. Normal tubule structures and brush border (→) in group C (A), and group R (B). Thickened basement membrane (^) and flattened tubule cells (↓), glomerular damage (*) in group G (C) and group GR (D) (PAS+ reaction) (X40)

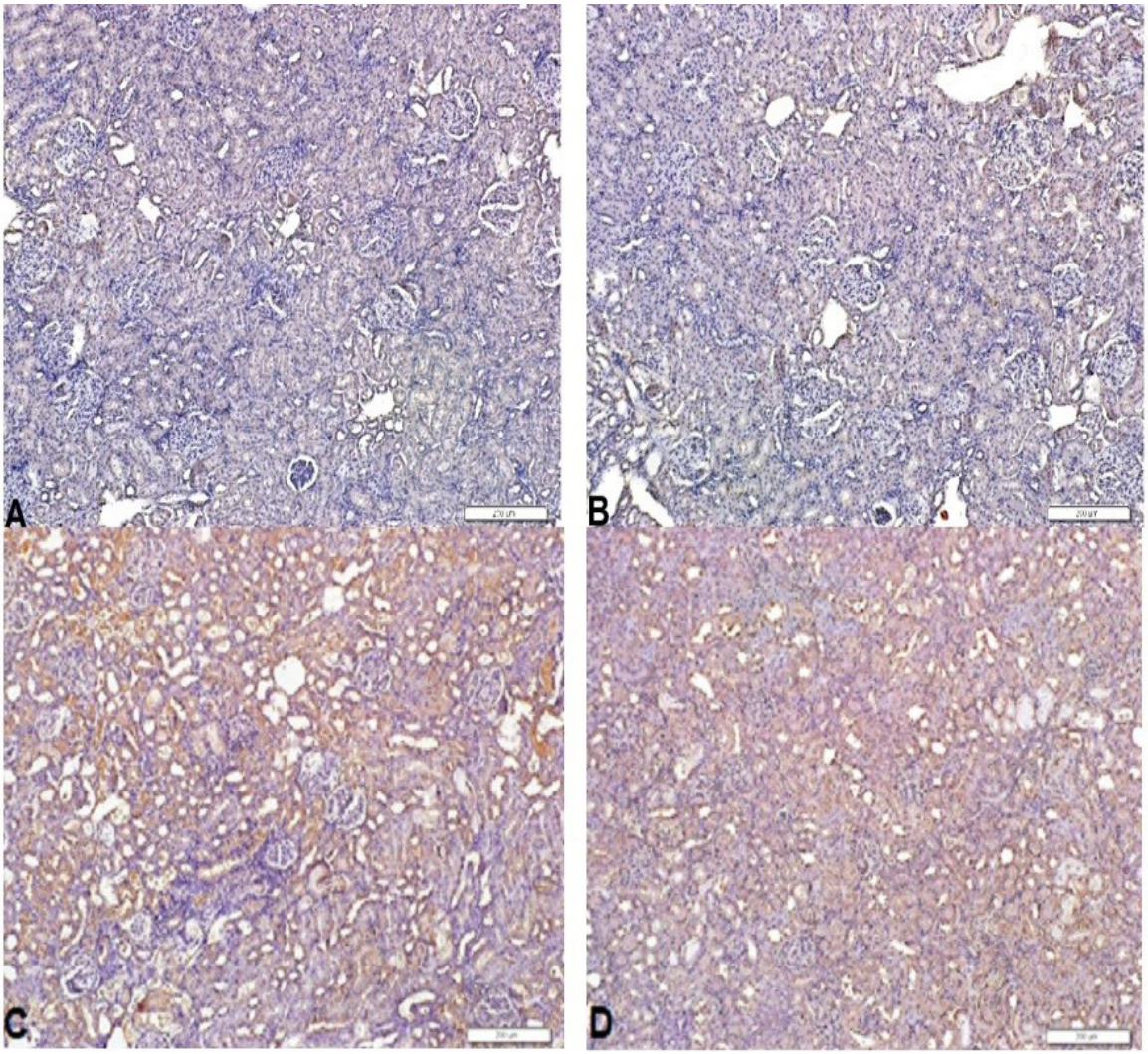

Figure 3. Immunohistochemical analysis of Ifi44 expression in kidney tissue. Low immunoreactivity is observed in A (C group) and B (R group), and high immunoreactivity in C (G group) and D (GR group) (X10)

Serum creatinine, urea, and BUN levels were higher in G and GR groups. Serum creatinine, urea, and BUN levels of the G and GR groups were statistically significantly higher when compared to C and R groups.

Serum TAS levels of G and GR groups were lower. However, the mean serum TAS level did not differ statistically between the groups.

It was observed that the mean serum TOS level was higher in the G and GR groups. Especially in the G group, the serum TOS level seemed to have increased significantly. Serum TOS level was significantly decreased in the GR group compared to the G group. However, the GR group was higher than the C and R groups. The serum TOS levels of the GM-treated (G and GR) groups were statistically significantly higher when compared with the C and R groups (Table 3).

Table 3. Biochemical values of the groups

Biochemical markers	Group C	Group R	Group G	Group GR
Creatinin (mg/dL)	0.35±0.05	0.31±0.09	1.36±0.50 ^{k}p, ^{ъ}p	1.16±0.32 ^{k}p, ^{ъ}p
Urea (mg/dL)	36.74±3.37	35.17±6.37	152.95±42.80 ^{k}p, ^{ъ}p	100.68±42.80 ^{k}p, ^{ъ}p
BUN (mg/dL)	17.17±1.57	16.44±2.98	71.48±20.00 ^{k}p, ^{ъ}p	47.05±20.04 ^{k}p, ^{ъ}p
TAS serum (µmol/L)	1.18±0.22	1.25±0.31	1.12±0.27	0.88±0.37
TOS serum (µmol/L)	44.39±13.67	34.28±7.99	146.63±80.47 ^{k}p, ^{ъ}p	68.78±9.96 ^{k}p, ^{ъ}p

^{k}p (p<0.05); statistically significant with group C, ^{ъ}p (p<0.05); statistically significant with group R

Discussion

Although its nephrotoxic effect is known, GM is still an antibiotic used against gram-negative bacterial infections. In order to examine the damage caused by GM, the results were evaluated experimentally by applying GM at different doses and times [21, 23, 26]. Studies have shown that both the body weight and kidney weight of rats given GM decreased compared to the C group [23]. It has been reported that the reason for this is acidosis and increased catabolism due to acute renal failure, loss of appetite, and decreased food intake [1]. In order to eliminate or partially reduce the damage of toxic substances that harm the body, natural agents with antioxidant effects are used. One of them is RA which is reported to have antioxidant, anti-inflammatory, antiapoptotic, and antitumoral effects [23].

In our study, mean body weight decreased in the G and GR groups and showed statistical significance when compared with the control group.

However, when the mean kidney weights were compared, an increase was observed in the G and GR groups, but statistical significance was observed only between the G group and the R group in the mean left kidney weight. When the mean kidney weights were compared, no statistical significance was observed between the C group and the other groups. In this respect, our study is compatible with the study stating that there may be an increase in kidney weight as a result of edema caused by acute tubular necrosis in rats given GM [27].

It has been suggested that increased serum creatinine, urea, and BUN values can be considered an indicator of nephrotoxicity in kidney damage due to GM exposure. In addition, there are different studies in which an increase in oxidative stress agents, monocyte-macrophage cell infiltration, inflammation, apoptosis, and brush border loss of tubular epithelial cells were observed as a result of experimental kidney damage caused by the application of GM at different doses and for different durations [23, 28-32]. In our study, it was observed that the

mean urea, creatinine, and BUN levels of the G and GR groups were high. It was observed that these values were lower in the GR group than in the G group. In this respect, our study supports the studies that apply RA treatment against the nephrotoxic effect of GM [23, 33, 34].

It was stated that oxidative stress is among the possible causes of renal damage in many studies [23, 35, 36]. GM causes increased superoxide anion production in renal cells. Accumulation of free oxygen radicals causes the initiation of protective mechanisms in renal cells. The reduction of one or more antioxidant systems leads to increase lipid peroxidation and more oxidative damage [23]. In our study, the serum TOS level of group G was considerably higher than the other groups.

Serum TOS level was lower in the R group than in the C group, and lower in the GR group than in the G group.

Serum TAS was found at the highest level in the R group. These values can be considered as an indicator of the antioxidative property of RA. As a matter of fact, our study was in agreement with studies reporting that it has an antioxidant effect by reducing RA oxidative stress [12, 37] and alleviates GM nephrotoxicity [33]. It was observed that the TAS level was lower in the GR group than in the other groups. This result has shown that the antioxidant properties of RA could not be seen in applied together with GM.

An increase in the biochemical values of the G group was observed, at the same time, it was observed that renal tubule and glomerular damage were quite high. Our findings were consistent with studies reporting proximal convoluted tubules, disruption of brush border and epithelial cell damage in their lumens, glomerulus damage, disruption of the Bowman capsule, and inflammation in animals given GM [23, 28-34]. In our study, kidney damage was statistically significantly higher in G and GR groups compared to C and R groups. Histological changes in the GR group were similar to the G group. Although it was not statistically significant in the GR group compared to the G group, the area of inflammation, glomeruli, and tubule damage was found to be less (p=0.070). According to these results in our study, we can say that the anti-inflammatory effect of RA was partially observed.

It was reported that GM causes damage through inflammation with excessive consumption of reactive oxygen species and causes an increase of the Ifi44 expression, which has an antiproliferative effect [19, 38]. RT-qPCR and western blot, and immunohistochemistry showed increased expression of Ifi44 in rat cochlear and kidney tissues following GM administration, suggesting that Ifi44 is associated with inflammations associated with GM-induced ototoxicity and nephrotoxicity [21]. In our study, Ifi44 expression was statistically significantly higher in our G and GR groups than in the C and R groups (p<0.001). The increase in the expression level of Ifi44 was also statistically significant in the G group compared to the GR group (p<0.001). These results at the expression level of Ifi44 showed that RA can`t fully demonstrate its antiapoptotic effect. It has showed that RA could not fully protect against the toxic effects of GM.

In our study also, it has been seen that RA which is used alone for protection purposes could not provide full protection against GM in parallel with the study stating that the combined treatment of RA and lycopene has a more beneficial preventive effect than RA treatment alone. The changes we recorded in the biochemical measurement were in agreement with the histological findings.

The biochemical results are parallel to the histological results and have revealed the importance of nephrotoxicity oxidative stress in the renal tissue caused by GM. The inability to detect TAS and TOS values in kidney tissue is one of the shortcomings of our study. The biochemical, histological, and immunohistochemical findings obtained as a result of our study showed the toxic effect of GM on renal tissue. As a result of RA application, a decrease in oxygen radicals and an increase in antioxidant levels were observed. However, when used together with GM, the protective effect of RA was observed partially, and its full protective effect could not be observed. We think it may be beneficial to apply it before encountering agents with toxic effects or at high doses in order to have protective effects. We are of the opinion that there is a need for comprehensive studies to investigate the effect of RA applied at different doses and times against toxic agents.

Conflict of interest: No conflict of interest was declared by the authors.

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Authors' contributions to the article

H.C. constructed the main idea and hypothesis of the study. H.C. developed the theory and arranged/edited the material and method section. H.A. contributed to the biochemical evaluation of the findings. D.G. contributed to the histological and immunohistochemical evaluation of the Results. The article written by H.C. In addition, all authors discussed the entire study and approved the final version.