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ARAŞTIRMA / RESEARCH

Effectiveness of melatonin in preventing vancomycin-induced nephrotoxicity: an experimental study

Vankomisin kaynaklı nefrotoksisiteyi önlemede melatoninin etkinliği: deneysel bir çalışma

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Abstract

Purpose: The aim of the study explores probable toxic effects of vancomycin on kidney and analysis of the probable protective effects of melatonin.

Materials and Methods: In this study, rats were randomly divided into 4 groups: the control group; the melatonin (10 mg/kg/day) group; the vancomycin-treated (200 mg/kg) group; and the vancomycin (200 mg/kg) + melatonin (10 mg/kg/day) group. Rats in the treatment group were given two doses of vancomycin a day with an interval of seven consecutive days and melatonin (10 mg/kg/day) once daily for seven consecutive days. The experiment was continued for 15 days. In each group, seven rats were grouped together. 15 days after the experiment, the rats were sacrificed under anesthesia and among all groups. Kidney tissues were collected and processed for further TNF- expression analysis, as well as histological analyses such as hematoxylin and eosin (H&E), Masson's tricrom, and Periodic acid schiff (PAS) staining to assess pathological severity. In addition, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to evaluate apoptosis.

Results: While vancomycin upregulated TNF-α expression, melatonin reduced levels of TNF-α immunoreactivity intensity and clearly improved pathological severity in rat kidneys. Further, melatonin significantly inhibited vancomycin-induced TUNEL-positive cell numbers.

Conclusion: Melatonin has protective activity against vancomycin-induced pro-inflammatory and proapoptotic

Öz

Amaç: Bu çalışmanın amacı, vankomisinin böbrek üzerindeki olası toksik etkilerinin belirlenmesi ve melatoninin olası koruyucu etkilerini araştırmaktır.

Gereç ve Yöntem: Bu çalışmada, sıçanlar rastgele 4 gruba ayrıldı: kontrol grubu, melatonin (10 mg/kg/gün) grubu, vankomisin uygulanan (200 mg/kg) grup ve vankomisin (200 mg/kg) +melatonin (10 mg/kg/gün) grubu. Tedavi grubundaki sıçanlara art arda yedi gün boyunca günde iki kez vankomisin ve ardından yedi gün boyunca günde bir kez melatonin (10 mg/kg/gün) verildi. Her grupta yedi sıçan yer aldı ve deneye 15 gün devam edildi. Deneyden 15 gün sonra, sıçanlar anestezi altında sakrifiye edildi. Sıçanlara ait böbrek dokuları alındı ve patolojik şiddeti değerlendirmek TNF-α ekspresyon analizi, hematoksilin ve eozin (H&E), Masson's tricrom ve Periodic acid schiff (PAS) gibi histolojik analizler yapıldı. Ek olarak, apoptozu değerlendirmek için Terminal deoksinükleotidil transferaz dUTP nick-end etiketlemesi (TUNEL) yöntemi uygulandı.

Bulgular: Vankomisin, TNF-α ekspresyonunu yükseltirken; melatonin, TNF-α immünoreaktivite yoğunluğunu azalttı ve sıçanların böbreklerinde açıkça patolojik şiddeti iyileştirdi. Ayrıca melatonin, vankomisinin neden olduğu TUNEL pozitif hücre sayılarını önemli ölçüde inhibe etti.

Sonuç: Bulgularımız, melatoninin organ koruma süresi boyunca böbreklerde vankomisinin neden olduğu proinflamatuar ve proapoptotik etkilere karşı koruyucu ve böbrek fonksiyonlarını iyileştirici etkiye sahip olduğunu gösterdi.

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effects in kidneys during organ preservation time and improves kidney function.
Keywords:. Kidney damage, apoptosis, pro-inflammatory cytokines, melatonin, vancomycin

Anahtar kelimeler: Böbrek hasarı, apopitoz, pro-inflamatuar sitokinler, melatonin, vankomisin

INTRODUCTION

Vancomycin is the most effective antibiotic for treating gram-positive bacteria like Staphylococcus epidermidis and methicillin-resistant Staphylococcus aureus (MRSA) infections[1,2]. Also, these bacteria can evolve resistance to this antibiotic. Although it has potential side effects such as nephrotoxicity, a high dose of vancomycin (every 8–12 h, 15–20 mg/kg body weight) is recommended in the literature[3-5]. The molecular mechanism of nephrotoxicity induced by vancomycin is not fully understood, but it is thought to play a role in the pathogenesis of kidney injury by triggering apoptosis, oxidative stress, and inflammation[6,7]. As a result, research into the mechanism of vancomycin-induced nephrotoxicity is required in order to determine how to effectively overcome this antibiotic adverse effect. Protecting the kidney from vancomycin-induced damage is very important to benefit from the positive effects. Therefore, recent studies research the oxidative effects of vancomycin induced kidney damage[8].

Melatonin (N-acetyl-5-methoxytryptamine) which is released from the pineal gland as an endocrine hormone, is also synthesized at numerous extra pineal sites[9], as a potential useful agent in the treatment of several diseases and conditions. Melatonin has been considered as a potentional endogenous free radical scavenger due to its protective effects against mitochondrial damage and tissue injury by scavengering scavenging RNS (reactive nitrogen species) or ROS (reactive oxygen species) in vitro and in vivo[10]. In addition to protecting cells and tissues from radical damage as a strong antioxidan[11], melatonin reduces NF-ϰB by inhibiting proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α, during hepatic fibrosis progression[12].

Also, apoptotic cell death is known to be triggered by oxidative stress, which induces the caspase system[13]. Except for anti-inflammatory effects, melatonin is known to have anti-apoptotic effects by affecting pro-or anti-apoptotic proteins such as Bax and Bcl-2[14]. The use of mouse-derived mesenchymal stem cells in chronic kidney disease, has resulted in accelerated senescence as a treatment strategy, melatonin administration in these stem cell-based treatments has been shown to protect cells against senescence[15]. In experimental models, it has also been shown that, administration of melatonin prevents structural and functional injuries in the kidney[16,17,18]. Moreover, melatonin is potentially helpful in the treatment of various diseases including diabetes, cerebrovascular diseases, cancer, and cognitive decline associated with aging[19]. We hypothesize that melatonin drives the observed increase in pro-inflammatory cytokines in vancomycin-induced kidney damage. Here, we show that melatonin treatment of vancomycin-induced kidney damage regulates apoptotic cell death and reduces pro-inflammatory cytokin levels. The molecular mechanism and downstream effector of melatonin's therapeutic effects have not been explored yet. The current study sought to investigate the degenerative effects of vancomycin on the kidney as well as the protective effects of melatonin against vancomycin administration for the first time in the literature. Importantly, melatonin could offer a novel therapy for patients with intractable kidney damage caused by vancomycin.

MATERIALS AND METHODS

Experiment procedure

The experimental protocol for experiments on animals was approved by the Erciyes University Experimental Animals Ethics Committee (Decision number: 21/221) and all experiments were performed according to the animal ethics protocols regulated by the committee. All experimental procedures were performed at the Experimental and Clinical Research Center of Erciyes University. Subjects were kept in well-ventilated polypropylene cages with tap water and food ad libitum under controlled laboratory conditions with a normal temperature (25 ± 2 °C) and normal light/dark cycle to acclimatize for two weeks. The 28 healthy female adult Wistar albino rats were divided into four groups, each containing seven rats, and the experimental procedure was continued for 15 days. A normal diet and water were given to the control group. The melatonin-treated group received

melatonin (10 mg/kg/day) intraperitoneally (i.p.) once daily for seven consecutive days. The Vancomycin treated group received vancomycin (200 mg/kg) i.p. two times a day with an interval of 12 hours for seven consecutive days. The vancomycin and melatonin treated group received vancomycin once a day with an interval of 12 hours at a concentration of 200 mg/kg for seven consecutive days[20] and then melatonin (10 mg/kg/day) was administered once daily for seven consecutive days started on day 8th day. Kidney tissue samples were collected under general anesthesia with xylazine (10 mg/kg) and ketamine (50 mg/kg) at the end of the application period.

Histological analysis

For the light microscopy examinations, kidney tissue samples of rats from all groups were fixed in 10% formaldehyde for histological analysis. Following the washing and dehydration, the kidney tissues were embedded in paraffin. Then these tissue blocks were sectioned at a 5 μm thickness. Thereafter, the staining with hematoxylin and eosin (H&E), Masson's tricrom and Periodic acid schiff (PAS), histopathological changes in the kidney tissue sections of the control and experimental groups were monitored. Also, the apoptotic cells were analysed in the vancomycin treated groups to compare with the control and melatonin treated groups.

Immunohistochemistry analysis

Tissue paraffin sections were stained for TNF-α immunoreactivity in the kidney using the avidin-biotin-peroxidase method (Thermo Scientific, Waltham, MA), as recommended by the manufacturer. After incubation for 5 minutes in distilled water at room temperature, sections were washed in PBS and blocked with 3% hydrogen peroxide for 10 min at room temperature. Then, sections were kept in the microwave at 95 oC for 20 minutes for antigen recovery in 0.01 M sodium citrate buffer (pH 6.0). Sections were washed in PBS after being incubated overnight at 4 oC with primary antibody TNF-α antibody diluted 1:50 (sc.25280; Santa Cruz Biotechnology, Santa Cruz, CA). Following the application of biotinylated secondary antibodies for 15 min at room temperature, slides were washed in PBS again, and stained with diaminobenzidine (DAB). In addition to that, counterstaining with hematoxylin was followed. TNF-α positive cells were observed to stain brown after dehydration and covering. The TNF-α immunoreactivity intensities for each section were calculated as the mean immunoreactivity intensity in ten random microscopic fields using Image J software (National Institutes of Health, Bethesda, MD).

Apoptosis analysis

TUNEL (Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling) as an in situ cell death detection kit (ApopTag; Millipore, Berlin, Germany) was used to detect apoptosis in kidney tissue. After deparaffinization and rehydration, the slides were treated with digoxigenin-dUTP in the presence of TdT (terminal deoxynucleotidyl transferase) for 1 h at 37°C. An Anti-digoxigenin fluorescence unconjugated antibody was used to visualize TUNEL-positive cells. Finally, the association of TUNEL-positive cells with apoptosis in the experimental groups was evaluated by identifying apoptotic cells morphologically. Cells were counted in ten randomly selected fields, and the apoptotic cell numbers were calculated by using ImageJ software (ImageJ) at the same magnification.

Statistical analysis

The means ± SEM (standard error of the mean) of all data were analyzed by using GraphPad Prism software version 9.0 (GraphPad Inc., San Diego, CA). One-way ANOVA with Bonferroni analysis was performed to evaluate the statistical significance of differences between the control and experimental groups in terms of TNF-α variables in the study. Kruskal-Wallis tests were used for comparisons of more than two groups, Dunn's test was performed for multiple comparisons for TUNEL results. P-values less than 0.05 were considered statistically significant.

RESULTS

The impact of vancomycin and melatonin on the function and structure of the kidney was evaluated histologically. Kidney tissues of subjects in the control and melatonin groups exhibited normal histology in the functional units such as tubules and glomerulus (Figure 1). However, degenerative changes such as abnormal necrosis, swelling of tubules, lymphocyte infiltration, and vacuolation in tubular epithelium were seen in the vancomycin treated group. In the melatonin-treated group,

damage was comparatively less because of the fact that the glomerulus was not severely disturbed and the tubules were not swollen.

Staining with masson trichrome was used to determine collagen deposition (Figure 1). Normal connective tissue was observed in the kidney sections of the control group and melatonin treated group. It was observed that collagen fibers around the stroma area increased in the vancomycin treated group compared to the control group. Almost no fibrosis was detected in the kidney tissue of subjects in the vancomycin+melatonin treated group. While normal proximal tubules such as prominent brush border with continued basement membrane were observed in PAS stained kidney tissue sections of the control group, severe desquamation in tubules and brush border of epithelium were seen in the vancomycin treated group. Moreover, no difference was detected between the kidney tissues of the vancomycin+melatonin treated group and the control group in terms of tubular damage.

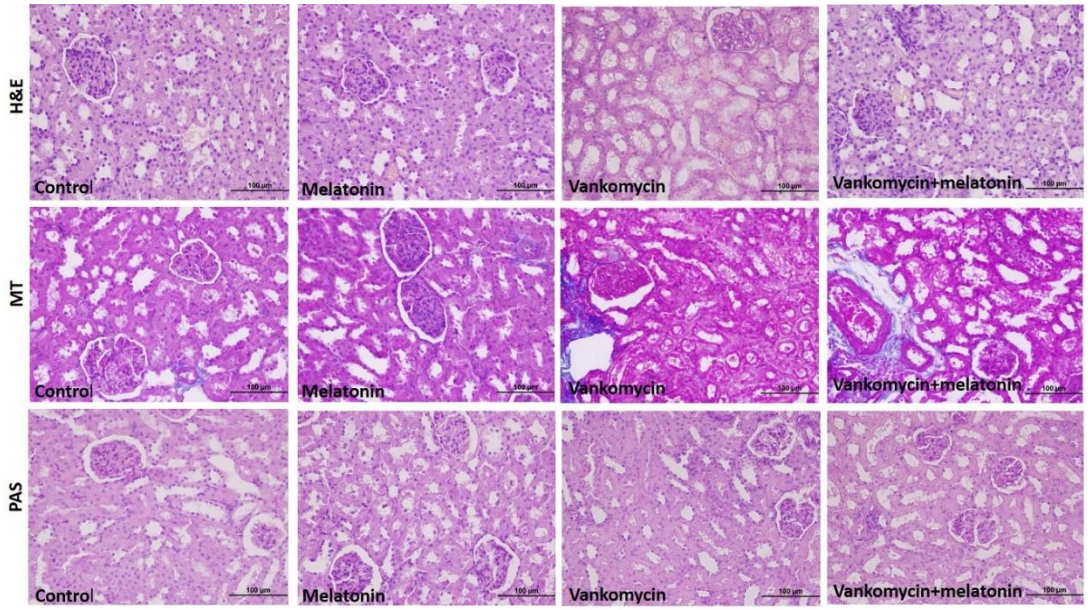

Figure 1. Light microscopic images of the renal cortex (hematoxylin-eosin, magnification X40).

Normal glomerulus and tubules were observed both in control group and melatonin treated group, besides no cytoplasmic granulation was found in tubular. Necrosis, interstitial inflammatory infiltration and cytoplasmic granulation in tubular cells together with vacuolisation in tubular epithelium cells and tubular swelling were seen in vancomycin treated group. Less necrotic area and less tubular swelling were observed in the melatonin+vancomycin treated group. The sections staining with masson trichrome was no detected fibrosis in the kidney tissue of all experimental groups (Masson trichrome. magnification X40). Clearly visible normal brush borders were seen in proximal tubules of both control group and melatonin treated group in PAS staining sections. Besides, the basement membrane of the proximal and distal tubules and the parietal leaf of Bowman's capsule were in normal structure. Tubular epithelial cells were seen to seperate from each other or from the basement membrane, and debris accumulations in the tubular lumen were shown in vancomycin treated group. In additon, numerous atypical proximal tubules which lost their brush borders were observed. The basement membrane of the proximal and distal tubules and the parietal leaf of Bowman's capsule were seen in normal structure in melatonin+vancomycin treated group (PAS, magnification X40).

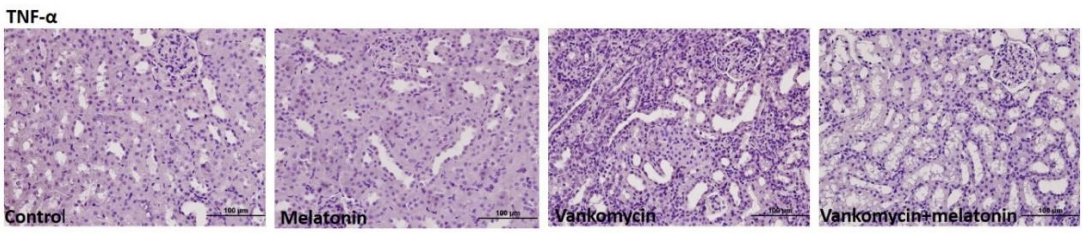

Figure 2. Melatonin reduces kidney inflammation caused by vancomycin in vivo.
Representative immunohistochemical staining of TNF-α.

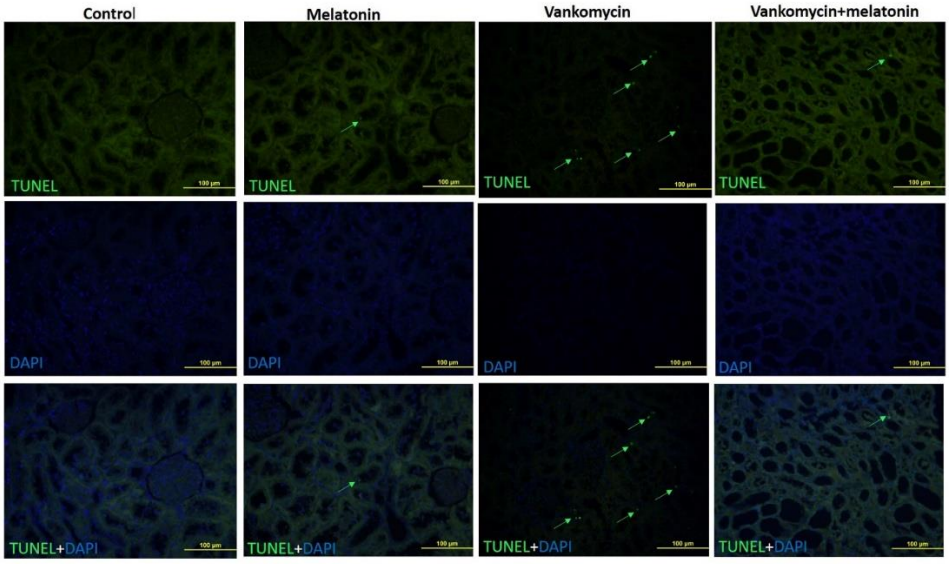

Figure 3. Antiapoptotic effect of melatonin treatment after vancomycin administration in vivo.
Representative images of apoptotic cells. The apoptotic cells were detected by TUNEL (green), and the nuclei were detected by DAPI (blue).

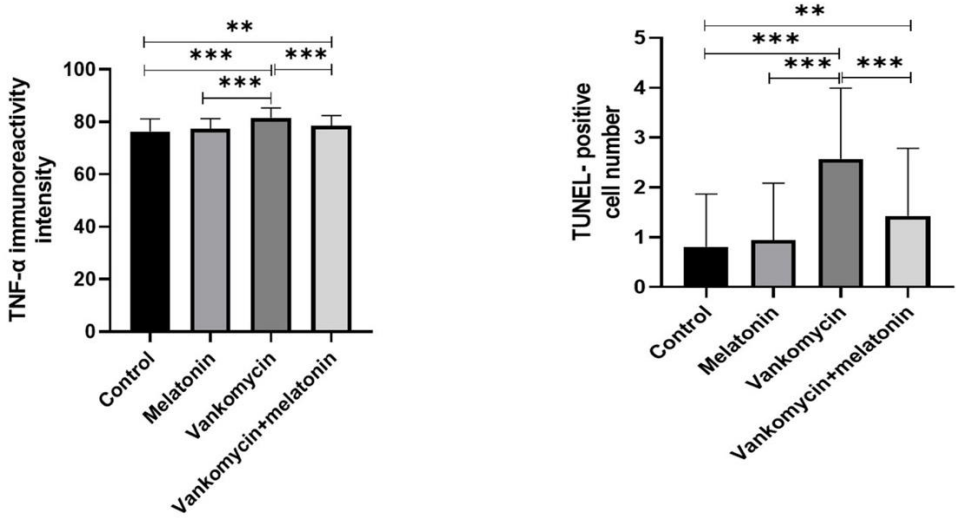

Figure 4. The graphs show immunoreactivity intensity result of TNF-α and TUNEL-positive cell number in kidney tissue of different experimental group.
Data are presented as mean ± SEM.*p < 0.05,**p < 0.01,***p < 0.001.

To clarify the possible protective effect of melatonin on vancomycin-induced kidney injury, TNF-α expression in kidney tissues was analyzed by immunohistochemical methods, and the results are shown in Figure 2. While the control and melatonin groups showed weak positive TNF-α expression in kidney tissue sections, the vancomycin group showed intensely positive TNF-α immunoreactivity in tubular

epithelial cells of kidney tissues compared to the control group. TNF-α immunoreactivity, on the other hand, decreased in the vancomycin+melatonin treated group compared to the vancomycin treated group (Figure 4) ($p<0.001$).

In order to detect the rate of cell death in the kidney tissue sections of each group quantitatively, TUNEL staining was preferred. After staining, TUNEL positive cells were counted, and the results are shown in Figure 3. The number of TUNEL-positive cells was significantly up-regulated in the vancomycin treated group compared with the control group ($p<0.05$). The TUNEL positive cell count was found to be significantly decreased in the vancomycin+melatonin treated group, compared to the vancomycin treated group (Figure 4) ($p<0.01$).

DISCUSSION

The present study shows that vancomycin causes inflammation and oxidative stress, which causes histological changes together with structural defects in the kidney. In addition, it was discovered that systemic melatonin treatment limits inflammation and pathological changes and supports functional capacity. To the best of our knowledge, this is the first study to detect melatonin's therapeutic activity on vancomycin-induced kidney injury from histological and immunohistochemical perspectives.

As an inhibitor of bacterial cell wall synthesis, vancomycin is an antibiotic extensively used to treat infections caused by methicillin resistant Staphylococcus epidermidis (MRSE) and Staphylococcus aureus (MRSA)[21]. Besides that, it is known to have potentially fatal side effects causing nephrotoxicity due to the excretion from the kidneys, which limits its administration and efficacy[2].

Although studies suggests that oxidative stress[22,23], inflammation, and apoptosis may play a role in the pathology of vancomycin induced renal toxicity[2], the exact mechanism of vancomycin-induced nephrotoxicity is not fully understood. In many countries, in addition to chemical drugs, the use of natural antioxidants, which reduce complications with fewer side effects, low toxicity, and affordable prices, are being investigated. By deactivating ROS, these antioxidants are used to protect against drug-induced toxicity, induce endogenous antioxidant production, and renovate homeostatic balance[24]. The alleviation of vancomycin-induced nephrotoxicity is dependent on enhancing the clinical success of vancomycin therapy. In this study, we examined vancomycin-induced acute renal injury and its related underlying mechanism. On the other hand, we examined vancomycin-induced apoptosis and its inhibition by melatonin, a natural antioxidant.

Melatonin is one of the hormones secreted by the pineal gland, which has immunomodulatory activity and an antioxidant ability to scavenge free radicals[25,26]. Besides being a chief hormone of the pineal gland, melatonin is a pleiotropic molecule that plays a role in regulation of the circadian system, seasonal reproduction, and regulation of sleep, exhibiting significant immunoregulatory, antiinflammatory, and antioxidant properties[11]. In addition, it has recently been shown to have a strong antioxidant capacity[27].

As in a similar study[13], interstitial inflammations were found in histopathological examinations of kidney tissues from subjects treated with vancomycin. This study support that vancomycin causes nephrotoxicity, by histopathology examination of kidney tissues. However, we observed various features related to renal injury, such as necrosis, leukocyte infiltration, and obliteration of renal tubules, which were significantly reversed with melatonin treatment. We detected that melatonin reduced the tubular necrosis and interstitial fibrosis due to vancomycin administration. This further confirms that melatonin has potent antioxidant and anti-inflammatory effects on oxyradicals. Under certain pathophysiological conditions, melatonin reduces the level of pro-inflammatory cytokines by diminishing free-radical-mediated damage; therefore, it contributes to the decrease of pro-inflammatory cytokines and the increase of anti-inflammatory cytokines[28].

In the present study, we showed that, vancomycin treatment leads to elevated expression of the TNF-α and this correlates with tissue damage. On the other hand, this increase in immunreactivity of inflammation biomarker, TNF-α due to vancomycin-induction significantly abrogated by melatonin. These results were supported by similar findings that were reported previously[29,30]. Melatonin administration resulted in a dramatic reduction in TNF-α expression in the current study, as evidenced by the recovery of renal tubule parenchyma.

Apoptosis, or programmed cell death, which occurs with the death receptor pathway (exogenous) or the chondrial pathway (endogenous), clears damaged

cells and maintains homeostasis. Accumulating evidence has shown that apoptosis may be one of the main factors leading to kidney injury[31,32,33]. Melatonin increases cell survival by reducing oxidative stress, endoplasmic reticulum stress, apoptosis, and mitochondrial fission by activating various signaling pathways[34]. Due to its antioxidant properties, melatonin is considered an antiapoptotic medication[35] and can modulate the apoptosis process[36]. The TUNEL assay was used for DNA fragmentation analysis wich presented cell degeneration through the presence or absence of the apoptosis cascade[37]. The present study revealed that quantitative data of the TUNEL assay was significantly reduced in the melatonin treated group in tubules, compared with the control group. This finding revealed that melatonin is one of the most robust biochemical components that can reduce DNA degradation independent of the apoptotic pathway. Although we reported a therapeutic effect of melotonin on vancomycin-induced kidney damage, our study still has some limitations. As a limitation of this study, the kidney function tests could be done, but we have not been able to provide enough urine samples from rats because of technical deficiency. Analyzing the long-term effect of melatonin on the degenerative effects of vancomycin on the kidney with more samples might be an interesting topic for future study.

This study has detected the potential protective effects of melatonin against vancomycin-induced renal injury, which is a main disadvantage of vancomycin treatment. Besides that, this study also identified the anti-inflammatory characteristics of melatonin. The antioxidant activity of melatonin is crucial in attenuating the effects of oxy radicals produced due to vancomycin treatment. Renoprotective effects of melatonin seem to be associated with decreased TNF-α expression. Moreover, it has also inhibited activation of the apoptotic process, which suppresses renal TUNEL expression. Although the protective effects of melatonin on renal damage caused by inflammatory disease are a current topic[38,39], there is limited evidence about the role of melatonin on vancomycin-related renal injury in the literature. According to our histological examination, we found impairments in the brush borders of renal tubules, swelling in glomeruli, tubular necrosis, peritubular capillary congestion, and an increase in the renal corpuscle area. Regarding our results, the cellular and extracellular injuries of the kidneys may be the result of inflammation triggered directly or indirectly by proinflammatory cytokines such as TNF-α. TUNEL activation is considered an unrecoverable final event before cell death, which is used as a marker of apoptosis.

In this experimental study, we also examined the possible apoptotic effects induced by the vancomycin on the kidney with TUNEL staining. In conclusion, melatonin showed beneficial effects in preventing vancomycin-induced nephrotoxicity. Nevertheless, the results of this study may pave the way for the search for optimal therapeutic agents against vancomycin-induced toxicity. Further evaluation is required to understand the role of melatonin in nephrotoxicity caused by vancomycin.

Yazar Katkıları: Çalışma konsepti/Tasarımı: ÖÖG; Veri toplama: ÖCM, DB; Veri analizi ve yorumlama: DB, ÖCM, MK; Yazı taslağı: ÖÖG; İçeriğin eleştirel incelenmesi: GÇ, DB, ÖCM; Son onay ve sorumluluk: ÖÖG, ÖCM, DB, MK, SSP, GC; Teknik ve malzeme desteği: DB, MK, ÖCM; Süpervizyon: ÖÖG; Fon sağlama (mevcut ise): yok.
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