RESEARCH ARTICLE

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Protective and Preventive Role of Folic Acid Against Ivermectin-Induced Renal Toxicity

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In addition to its widespread use as an antiparasitic in human and animal health, ivermectin (IVR) has been extensively investigated for its therapeutic use against the disease during the Covid-19 pandemic and its positive effects have been reported. Folic acid (FA) plays an important role in the protection of the nervous system, the formation of healthy red blood cells, the synthesis and repair of DNA and other genetic materials, and the division process of cells. As a small molecular weight compound, FA is freely filtered by the glomerulus. The aim of this study was to investigate the protective and preventive role of FA against IVR-induced renal toxicity. In this study, biomarkers that cause kidney toxicity, apoptosis and lipid peroxidation were evaluated. In the IVR+FA group, compared to the control group; urea, creatinine, malondialdehyde, interleukin-17A, nuclear factor kappa-B, Tumor necrosis factor-α, caspase-3, Bcl-2–associated X protein and kidney injury molecule-1 values were found higher, while glutathione, superoxide dismutase, catalase, glutathione peroxidase, B cell lymphoma-2 and aquaporin-2 values were found lower. Increases were observed in biomarkers causing kidney toxicity, apoptosis and lipid peroxidation in the IVR group. These results showed that FA protects against IVR nephrotoxicity.

Key Words: *Apoptosis, folic acid, ınflammation, ivermectin, nephrotoxicity*

Folik Asidin İvermektin Kaynaklı Böbrek Toksisitesine Karşı Koruyucu ve Önleyici Rolü

İvermektin (IVR), insan ve hayvan sağlığında antiparaziter olarak yaygın bir şekilde kullanılmasının yanında Covid-19 pandemisi sırasında hastalığa karşı terapötik kullanımı açısından da kapsamlı bir şekilde araştırılmış ve olumlu etkileri bildirilmiştir. Folik asit (FA), sinir sisteminin korunmasında, sağlıklı kırmızı kan hücrelerinin oluşumunda, DNA ve diğer genetik materyallerin sentezinde ve onarımında ve hücrelerin bölünme sürecinde önemli rol oynar. Küçük molekül ağırlıklı bir bileşik olan FA, glomerulus tarafından serbestçe filtrelenir. Bu çalışmanın amacı FA' nın IVR kaynaklı böbrek toksisitesine karşı koruyucu ve önleyici rolünü araştırmaktır. Bu çalışmada böbrek toksisitesine, apoptoza ve lipid peroksidasyonuna neden olan biyobelirteçler değerlendirilmiştir. IVR+FA grubunda, kontrol grubuyla karşılaştırıldığında; Üre, kreatinin, malondialdehit, interlökin-17A, nükleer faktör kappa-B, tümör nekroz faktör-α, kaspaz-3, Bcl-2 ile ilişkili X protein ve böbrek hasarı molekülü-1 değerleri daha yüksek bulunurken, glutatyon, süperoksit dismutaz, katalaz, glutatyon peroksidaz, B hücreli lenfoma-2 ve aquaporin-2 değerleri daha düşük bulundu. IVR grubunda böbrek toksisitesi, apoptozis ve lipid peroksidasyonuna neden olan biyobelirteçlerde artışlar gözlendi. Bu sonuçlar FA'nın IVR nefrotoksisitesine karşı koruyucu olduğunu gösterdi.

Anahtar Kelimeler: *Apoptozis, folik asit, inflamasyon, ivermectin, nefrotoksisite*

Introduction

Ivermectin (IVR) is a macrocyclic lactone isolated from *Streptomyces avermitilis* and is widely used against parasitic diseases caused by parasitic nematodes and arthropods in domestic and wild animals (1). In humans, it is considered the first choice drug for the treatment of onchocerciasis, strongyloidiasis, ascariasis, trichinosis and enterobiasis (2). Adverse effects of IVR in mammals include abdominal pain, nausea, tachycardia, asthma, edematous swelling, pruritus, back pain, eye problems, cardiac dysfunction, renal toxicity and liver disease (3). IVR, offers many potential effects to treat a range of diseases with its antimicrobial, antiviral and anti-cancer properties as a wonder drug. It is highly effective against a variety of microorganisms including some viruses. Therefore it may be helpful as a possible candidate in the treatment of a wide range of viruses including COVID-19 and other single-stranded RNA viruses (4). In a study conducted with IVR, it was observed that it also induced apoptosis. It has also been identified to have an effect on mitochondrial dysfunction as indicated by loss of mitochondrial membrane potential excessive disruption of mitochondrial Ca^{2+} and production of reactive oxygen species (ROS) (5). IVR reduces GSH levels and antioxidant enzyme activities such as SOD, CAT and GPx (6). The decrease in cellular defense system increases ROS levels, which lead to cellular damage by breaking down the structures of macromolecules such as membrane lipids, proteins and nucleic acids. Therefore an increase in MDA levels an indicator of lipid peroxidation, may be observed (7). As a result of all this it mediates acute kidney injury (8).

Folic acid (FA) is also known as vitamin B9 (9). FA is necessary for the production of purines and pyrimidines the precursors of DNA (10). Plants and bacteria can synthesize folate but humans and other animals cannot synthesize folic acid. Therefore folic acid is an essential nutrient. FA can be obtained from egg yolk, animal liver, leafy vegetables and yeast. FA is usually absorbed in the small intestine and converted intracellularly to tetrahydrofolate by dihydrofolate reductase (11). Due to its indispensable role in the synthesis of RNA and DNA molecules, it can cause megaloblastic anemia and neural tube defects in the fetus (12). FA, regulates a number of disorders due to its antiapoptotic and antioxidative properties (13).

This study was conducted to investigate the effects of FA against IVR-induced nephropathy in rats.

Materials and Methods

Research and Publication Ethics: This studay was approved by the Necmettin Erbakan Üniversitesi Animal Experiments Local Ethics Committee (28.11.2024, 2024–097). All experiments were performed in accordance with the European Directive 2010/63/EU for animal experiments. In addition, all animal-related procedures used in this study were performed in accordance with ARRIVE guidelines.

Chemicals: IVR, (Ivomec®, 1% sterile solution 50ml) Boehringer Ingelheim (Istanbul, Türkiye) purchased. FA, (Folbiol®, 5mg folic acid 50 tablets) Menari (Istanbul, Türkiye) purchased. The IVR dose was determined based on a study reported by Qureshi, S. (14). The FA dose was determined based on a study reported by Fakouri et al. (15). FA was dissolved in distilled water.

Experimental Procedures: This study employed 28 Wistar rats (weighing between 230 and 250g and aged between 12 and 14 weeks) as subjects. Rats were obtained from the Necmettin Erbakan Üniversitesi KONUDAM Experimental Medicine Application and Research Center (Konya, Türkiye).

Rats were randomly divided into four groups, with each group consisting of seven animals.

1. (Control): A solution of normal saline was orally administered once daily for 7 days.

2. Ivermectin (IVR): A single dose of 15 mg/kg/day ivermectin was administered intraperitoneally to rats on the first day.

3. Folic Acid (FA): For a 7-day period, 10 mg/kg of a was orally administered once daily.

4. Ivermectin + Folic Acid (IVR+FA): A single dose of 15 mg/kg/day ivermectin was administered intraperitoneally on day 1 and 10 mg/kg/day folic acid was administered orally for 7 days.

IVR was administered to the rats in the groups intraperitoneally once on the first day. FA was administered orally for 7 days and the experiment was terminated after 7 days. The experimental procedure and administration of the active substance were conducted in accordance with previously reported methods (17, 18).

All animals were sacrificed 24 h following their final treatment. Animals were sacrificed by decapitation under sevoflurane (Sevorane liquid 100%, Abbott Laboratory, Istanbul, Türkiye) anesthesia. Blood samples were collected and transferred into clean dry test tubes without anticoagulant. Thereafter, serum was separated by centrifugation at 1,000 x g for 10 min at 4ºC for determination of urea and creatinine. The kidneys were isolated immediately from the animals and washed with ice-cold physiological saline, and stored at -20ºC for biochemical analysis.

Determination of Kidney Function Analysis: The Serum Urea And Creatinine Levels Were Measured using a commercial kit (Diasis Diagnostic Systems, İstanbul, Türkiye)

Determination of Lipid Peroxidation and Antioxidant Enzymes Analysis: The MDA level was determined according to the method of Placer et al. (16). The antioxidant status in kidney tissues was assessed by analyzing SOD, CAT, GPx, and GSH levels. The supernatants used for the analysis of SOD, CAT, and GPx activities, as well as GSH levels, were obtained similarly as for lipid peroxidation. CAT activity was measured according to the method of Aebi (19), SOD activity according to the method of Sun et al. (20), GPx activity according to the method developed by Lawrence and Burk (21), GSH levels according to the method developed Sedlak and Lindsay (22). The total protein content of the tissues required for the calculation of enzyme activities was determined by the method of Lowry et al. (23).

Real Time PCR (RT-PCR): The effects of IVR and FA applications on the kidney tissues obtained at the end of the experiment on the relative mRNA transcript levels of the gene regions listed in Table 1 were examined by the qRT-PCR method. Total RNA isolation from the tissues was performed using the QIAzol Lysis Reagent (79306; Qiagen) kit. OneScript Plus cDNA Synthesis Kit (ABM, G236, Richmond, Canada) was used for cDNA synthesis from the isolated total RNAs. The prepared cDNAs were mixed with primer sequences and BlasTaq™ 2X qPCR MasterMix (ABM, G891, Richmond, Canada) and reacted. The reaction was carried out with the Rotor-Gene Q (Qiagen) device with the determined time and temperature cycles by the manufacturer's instructions. After the cycles were completed, gene expressions were normalized to β-Actin and evaluated by the $2^{-\Delta\Delta CT}$ method (16).

Histopathological Analyzes: At the end of the experiment, kidney tissues taken from rats were kept in %10 buffered formalin solution for fixative purposes. Tissues that underwent routine paraffin follow-up procedure were embedded in paraffin as the last stage. 5 µm sections were taken from paraffin blocks and stained with Hematoxylin and Eosin (H&E) to see

histopathological changes. Images from stained preparations were evaluated using a binocular Olympus Cx43 light microscope (Olympus Inc., Tokyo, Japan) and an EP50 camera (Olympus Inc., Tokyo, Japan).

Statistical Analysis: The data obtained at the end of the study were analyzed using IBM SPSS software (Version 20.0). Data were presented as mean ± standard deviation (SD). Since the number of samples in the study was less than 50, the Shapiro-Wilk test was used to determine whether the data showed a normal distribution. As a result of the test, p>0.05 was obtained for the markers. After this value, ANOVA and Tukey tests were performed for statistical evaluation of the data. Statistical significance was determined at $p<0.05$, $p<0.01$, $p<0.001$.

Results

Effects of IVR and FA on renal functions: Serum urea and creatinine levels were evaluated as markers of renal function. Their levels were significantly high in IVR-treated animals compared with the control group ($p<0.001$). In rats with IVR-induced nephrotoxicity, FA treatment caused significantly low serum urea and creatinine levels compared with those treated with IVR alone ($p<0.001$). However, FA did not alter renal function markers as shown in Figure 1.

Effects of IVR and FA administration on oxidant and antioxidants: Oxidative stress was assessed by measuring renal SOD, CAT and GPx activity Figure 2. These antioxidant enzymes were significantly ($p<0.001$) low in the kidney tissue of IVR administered rats compared to the control group. Treatment with FA significantly high the activities of these enzymes compared to the IVR administered group ($p<0.001$).

IVR administered animals had significantly low renal GSH contents compared to the control group ($p<0.001$). However, treatment with FA significantly high the level of renal GSH contents compared to the IVR administered group ($p<0.001$, Figure 2).

MDA was evaluated as an indicator of lipid peroxidation. IVR administered animals had significantly high renal MDA contents compared to the control group ($p<0.001$). However, treatment with FA significantly low renal MDA levels compared to the IVR administered group ($p<0.001$, Figure 2).

Effects of IVR and FA administration on renal TNF-α, IL-17A and NF-κB levels: TNF-α, IL-17A and NF-κb levels were evaluated as inflammation markers. Their levels were significantly higher in IVR administered animals compared to the control group ($p<0.001$). FA treatment significantly low the elevation of TNF-α, and NF-κB levels compared to the IVR administered group ($p<0.05$, Figure 3).

Effects of IVR and FA administration on renal Caspase-3, Bax activity and Bcl-2 levels: Caspase-3 and Bax activity were evaluated as apoptosis markers. Bcl-2 level was evaluated as pro- and anti-apoptotic markers. Caspase-3 and Bax activity levels were significantly higher in IVR administered animals compared to the control group ($p<0.001$). Bcl-2 level was significantly lower ($p<0.001$). On the contrary, treatment with FA significantly low the high in Caspase-3 and Bax activity levels ($p<0.01$) compared to the IVR administered group and significantly high the Bcl-2 level ($p<0.05$, Figure 4).

Effects of IVR and FA application on KIM-1 and AQP-2 levels: Hihg levels of KIM-1 and low levels of AQP-2 have been reported as biomarkers for nephrotoxicity In animals treated with IVR, KIM-1 level was significantly higher ($p<0.001$) and AQP-2 level was significantly lower ($p<0.05$) compared to the control group. During FA treatment, KIM-1 level was significantly lower ($p<0.01$) and AQP-2 level was significantly higher ($p<0.05$, Figure 5).

Light Microscopy Findings: Histopathological changes in H&E stained sections of kidney tissues taken from rats are given in Figure 6. Kidney tissues in the control and folic acid groups had similar results. In both groups, it was seen that the tubules and renal corpuscles localized in the cortex layers of the kidney tissue had normal histological structures. On the other hand, pathological changes were remarkable in the ivermectin administered group. Shrinkage and atrophy glomeruli and enlarged Bowman spaces were observed in this group. When the tubules were examined, exfoliation in epithelial cells and degenerative changes in places and enlargement in tubule structures were observed. In addition, there were areas of interstitial mononuclear cell infiltration, widespread local hemorrhages and vascular congestion. In the IVR+FA subgroup, improvement in pathological changes was recorded in the kidney tissue compared to the IVR group. Atrophic glomeruli, epithelial cell exfoliation and vascular congestion were seen in rare areas.

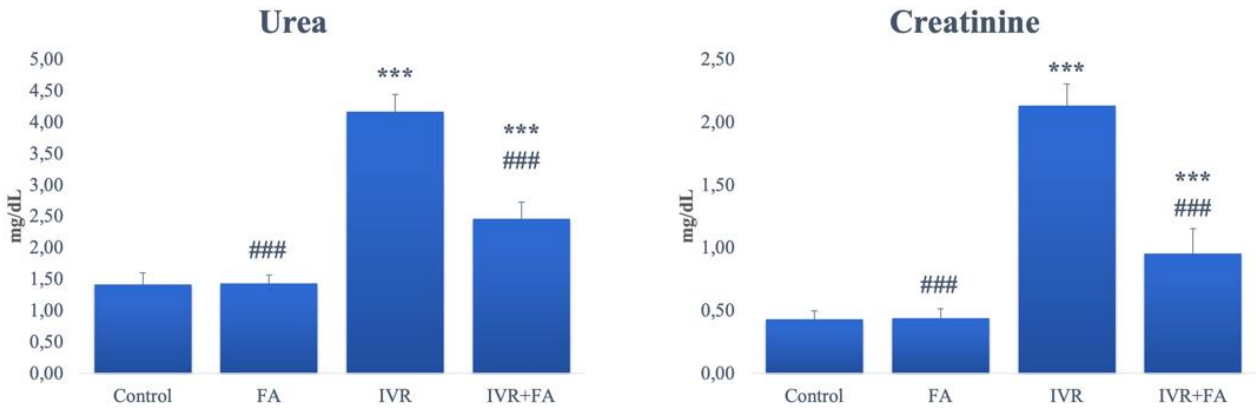

Figure 1. Effects of IVR and FA on serum urea and serum creatinine levels. Values are given as mean ± SD. Control vs others: ***p<0.001; IVR vs others: ###p<0.001

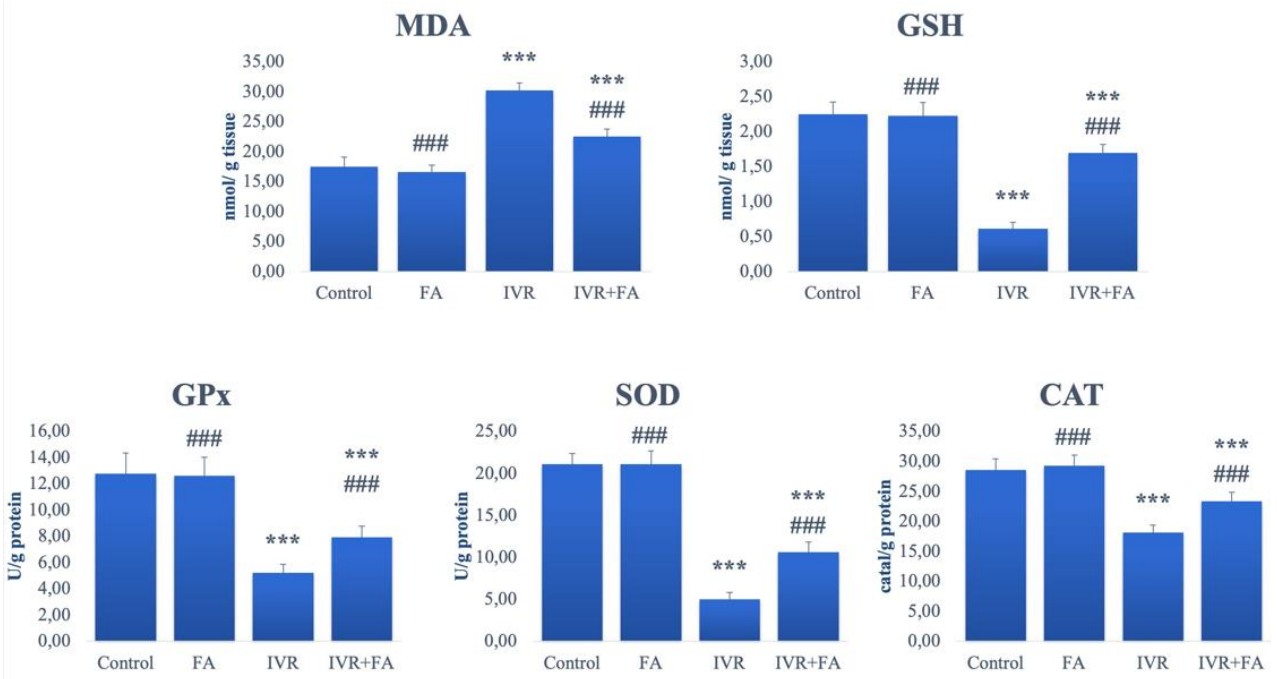

Figure 2. Effects of IVR and FA on MDA, and GSH levels and SOD, CAT, and GPx activities and in rat kidney tissues. Values are given as mean ± SD. Control vs others: ***p<0.001; IVR vs others: ###p<0.001

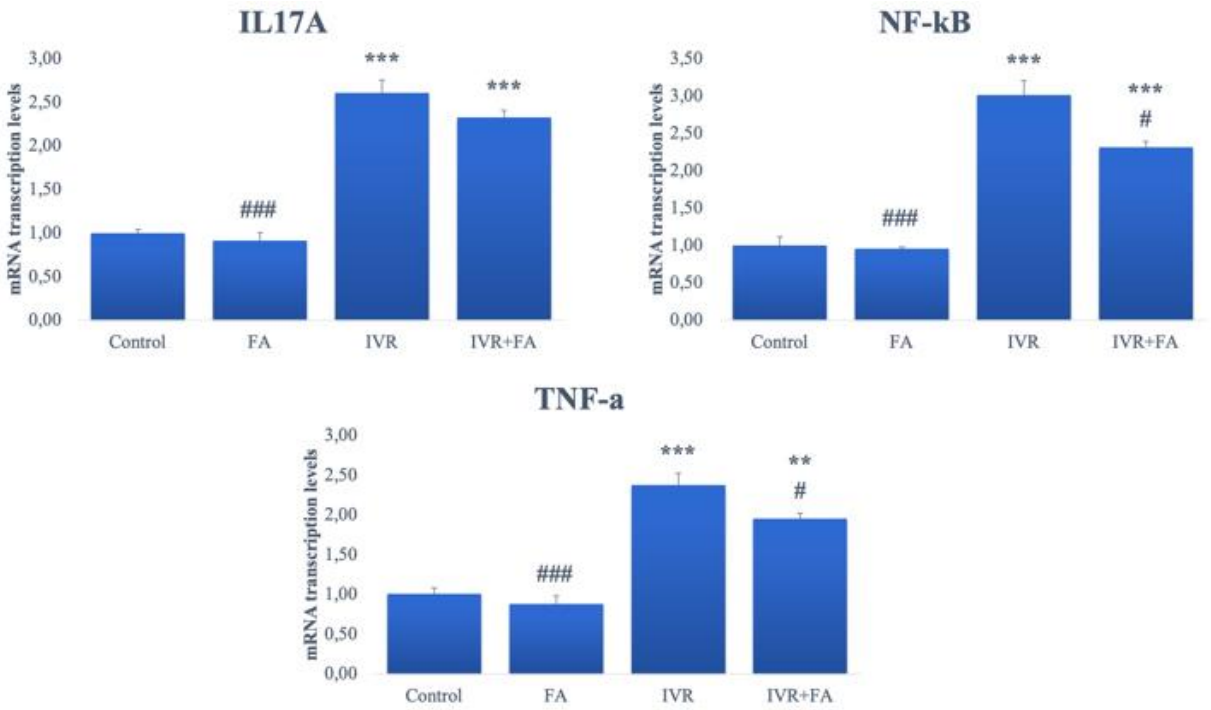

Figure 3. Effects of IVR and FA on IL17A, NF-κB and TNF-α mRNA transcription levels in rat kidney tissues. Values are given as mean ± SD. Control vs others: ***p*<0.01, ****p*<0.001; IVR vs others: #*p*<0.05, ###*p*<0.001

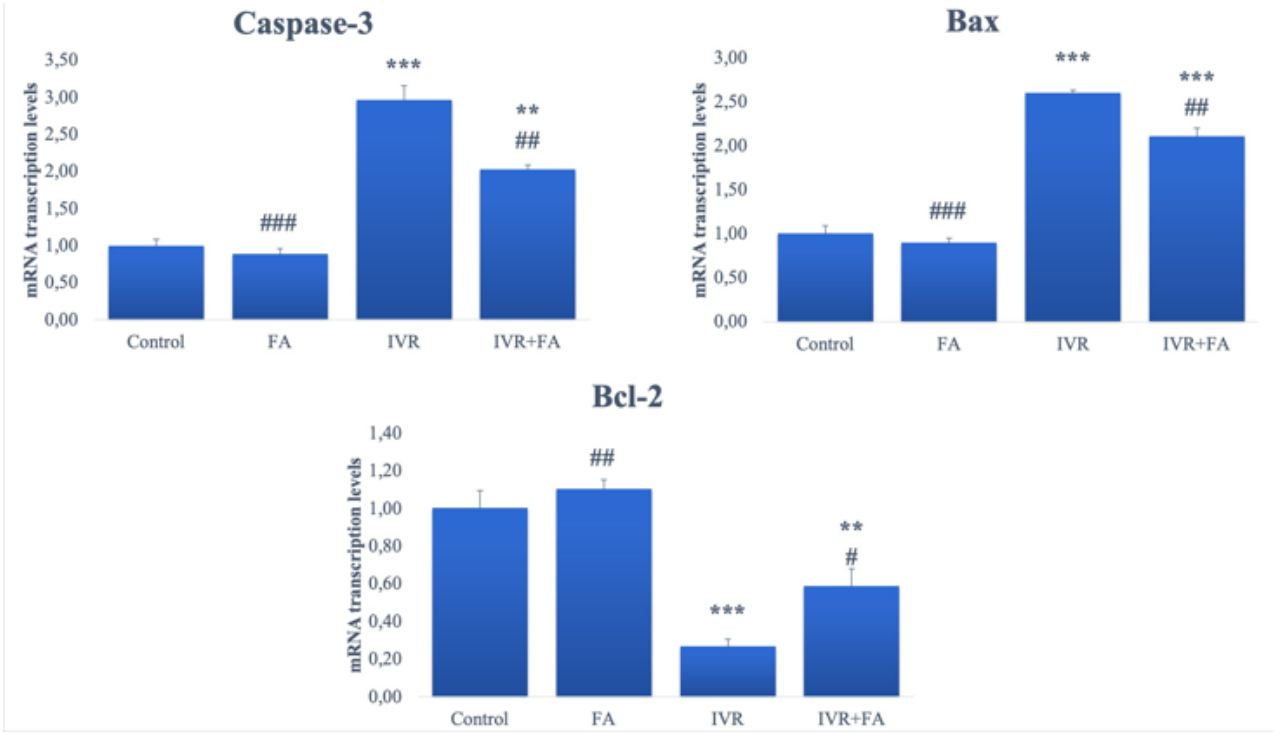

Figure 4. Effects of IVR and FA on Caspase-3, Bax, and Bcl-2 mRNA transcription levels in rat kidney tissues. Values are given as mean ± SD. Control vs others: ***p*<0.01, ****p*<0.001; IVR vs others: #*p*<0.05, ##0.01, ###*p*<0.001

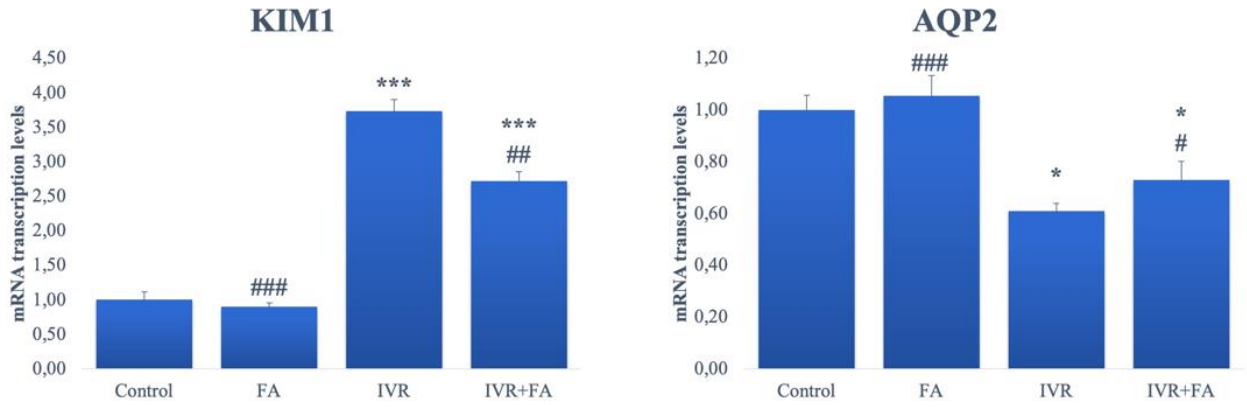

Figure 5. Effects of IVR and FA on KIM1 and AQP2 mRNA transcription levels in rat kidney tissues. Values are given as mean ± SD. Control vs others: *p<0.05, ***p<0.001; IVR vs others: #p<0.05, ##0.01, ###p<0.001

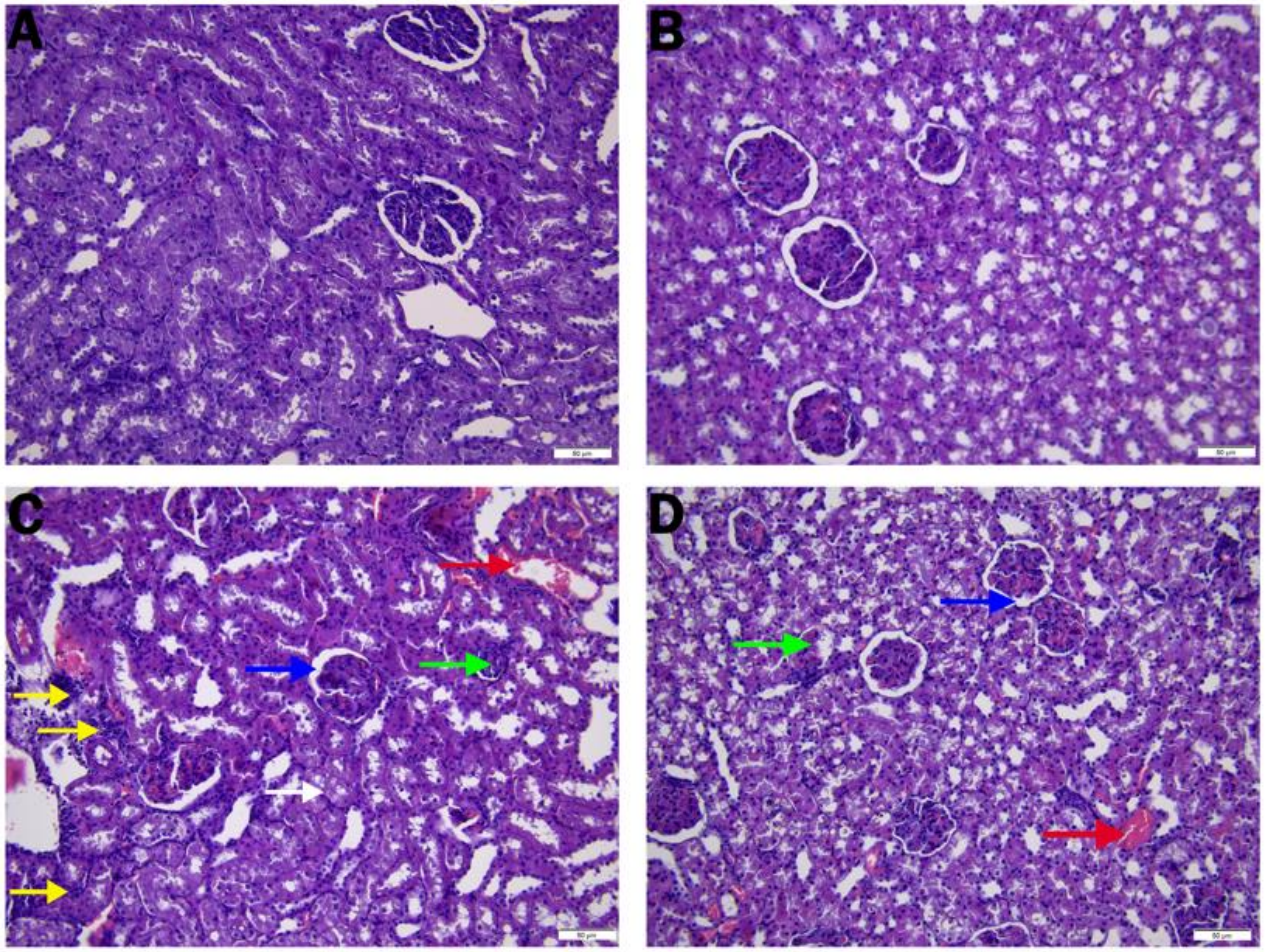

Figure 6. Photomicrograph (H&E, x200) of kidney tissue stained with hematoxylin and eosin from different groups. No histological changes are observed in the kidney tissues of the control (A) and FA (B) groups. Glomerular atrophy (green arrow), widened Bowman space (blue arrow), degenerative renal tubular epithelium (white arrow), areas of interstitial mononuclear cell infiltration (yellow arrow), widespread local hemorrhages and vascular congestion (red arrow) were seen in the IVR (C) group. Normal glomeruli, rare areas of atrophic glomeruli (green arrow), relatively widened Bowman space (blue arrow) and congestion in some interstitial vessels (red arrow) were observed in the IVR+FA (D) subgroup

Discussion

This study demonstrated the protective effects of FA against IVR-induced nephrotoxicity. IVR can be taken into the body by oral, subcutaneous or intramuscular routes and can reach the blood and organs such as liver and kidney for metabolism. Although it has been established as a safe and non-toxic drug for mammals it probably has some effects on the oxidative stress system of mammals, considering that it is a xenobiotic for the body. Since the xenobiotic is unknown to the host, it can be expected to cause adverse effects such as toxicity, allergic response, and cancer (17). Moreover, IVR induced oxidative stress in the tissue of North African catfish (24). Common adverse effects of IVR including headache, pruritus, myalgia, cough, dyspnea, nausea, vomiting, diarrhea, blurred vision, postural hypotension, and confusion have been reported in clinical studies. It can also cause nephropathy, psychiatric disorders, liver disorders and multiple organ dysfunction syndrome (25). In the present study, the properties of FA including antiinflammatory effects, reducing interleukin levels and antioxidant effects (26) were invastigated to reduce the nephrotoxic effects of IVR.

Tawfeek et al. (3) also reported nephrotoxic effects of IVR on the kidneys, which were similar to our current study. Abdellatefe et al. (27) reported that IVR caused hepatorenal toxicity and significantly increased renal biomarkers such as urea and creatinine compared to the control group.

Urea, when quantified in blood, is usually referred to as blood urea nitrogen (BUN) and is a product of protein metabolism. BUN is considered a non-protein nitrogenous (NPN) waste product. Amino acids obtained from protein breakdown undergo deamination to produce ammonia. Ammonia is then converted to urea by liver enzymes. Therefore, urea concentration depends on protein intake, the body's ability to catabolize protein, and adequate urea excretion by the renal system. Urea accounts for the majority (up to 80–90%) of NPN excreted by the body. The body's dependence on the renal system to excrete urea makes it a useful analyte for assessing renal function. Creatinine, also an NPN waste product, is produced from the breakdown of creatine and phosphocreatine and can also be used as an indicator of renal function (28). The effect of IVR was investigated biochemically and the results showed an increase in serum renal function markers as presented in Figure 1. Serum urea and creatinine levels high with IVR administration compared to the control group. Consumption of FA in parallel with IVR in rats protected against this deterioration. These findings also emphasized the effect of oral FA in improving metabolism and renal function. These results were consistent with that IVR + FA treated rats showed a significant low in serum urea, supporting the effect of FA as a scavenger of free radicals. Furthermore, some studies reported that rats treated with IVR for 15 days developed a significant high in urea and creatinine levels compared to the untreated group, thus confirming the low in renal glomerular filtration (29). High serum urea and creatinine levels as a result of IVR-induced kidney injury in the present study support these observations in previous studies.

Oxidative stress driven by high ROS contributes to the pathogenesis of numerous diseases by causing cellular damage, vascular permeability, tissue necrosis, and lipid peroxidation (30). High levels of MDA, a marker of lipid peroxidation, indicate high oxidative stress, while enzymatic antioxidants such as SOD, CAT, and GPx act as essential defense mechanisms by reducing ROS and detoxifying harmful compounds (31). Non-enzymatic antioxidants such as GSH further help neutralize oxidative damage (32). Strong antioxidant defenses protect tissues from toxic effects by scavenging free radicals (33). While IVR reduces SOD, CAT and GPx activities and non-enzymatic GSH levels (34), It was observed that it high MDA levels. FA given with IVR brought these levels closer to the levels of the control group Figure 2.

Xu et al. (35) reported the protective effect of therapeutic application with FA on the kidneys, which is consistent with our present study. Also, Jing and Chen (36) reported the protective effect of FA against kidney damage in their study on mice.

Proinflammatory cytokines and activation of the systemic inflammatory response are also involved in IVR-induced kidney injury**.** Inflammatory mediators such as TNF-α, IL-17A and NF-κB play an important role in the pathogenesis of IVR nephrotoxicity (37). These cytokines activate inflammatory cells (neutrophils, macrophages, monocytes, platelets, mastocytes), which emit large amounts of toxic ROS and lead to cellular damage through a variety of mechanisms, including peroxidation of membrane lipids and oxidative damage to DNA and proteins (38). In addition, it has been reported that TNF-α transcription is regulated by NF-κB. NF-κB-associated mediators, such as some cytokines and chemokines, play an important role in the promotion of various inflammatory diseases in many organs (39). It is also known that DNA damage caused by anticancer therapy activates NF-κB. Therefore, inhibition of NF-κB may be beneficial in reducing kidney damage. Therefore, inhibition of NF-κB may increase sensitivity to apoptosis caused by both chemotherapy drugs and radiation. This study showed that as a result of renal toxicity of IVR, pro-inflammatory cytokines TNF-α, IL-17A and NF-κB levels high compared to the control group Figure 3. In addition, IVR application higher KIM-1 compared to the control group, while AQP-2 lower compared to the control group. FA treatment had the opposite effect when compared to the IVR group Figure 5. Moreover, the nephroprotective effect of FA can be explained by its ability to inhibit the signaling pathway that causes excessive higher in markers such as TNF-α, IL-17A and NF- κB (40).

Apoptosis plays an important role in the development and maintenance of homeostasis in most multicellular organisms (41). Caspase is known as a group of cysteine proteases and is the main agent of apoptotic cell death. In general, Caspase-3 stands out

among caspases (42). This is because it is the primary agent that is activated by various death signals and the degradation of important cellular proteins. IVR causes apoptosis through purine and pyrimidine inhibition, leading to DNA defects. Caspase-dependent apoptotic signaling plays an important role in IVR-induced apoptotic damage (43). Similarly, another study observed higher apoptosis in kidney tissue after IVR administration. In our study, we used Liu et al. (44) In parallel with the results of the study, we found an higher in the expression of Caspase-3 and Bax in rats that received a single dose of IVR, while Bcl-2 expression was lower. In the FA + IVR application, we obtained the opposite results Figure 4. Thus, we observed that IVR induced apoptosis.

In the study of Almawla and Al-baggou (45), it was stated that IVR increased proapoptotic biomarkers compared to the control group. In the current study, proapoptotic biomarkers were found to be significantly higher in IVR application compared to the control group.

As a results FA supplementation alleviated IVR-induced renal dysfunctions and reduced oxidative stress. FA suppressed inflammation, oxidative stress and apoptosis associated with IVR toxicity and was therefore beneficial in reducing nephrotoxicity complications.

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