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Research Article / Araştırma Makalesi

Nephroprotective Effect of Resveratrol Against Methotrexate-Induced Renal Toxicity in Female Rats

Dişi Sıçanlarda Resveratrol'ün Metotreksat ile İndüklenen Renal Toksisiteye Karşı Nefroprotektif Etkisi

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Abstract: The study purposed to appraise the nephroprotective effects of resveratrol-(RES) in relation to methotrexate-(MTX)-induced renal toxicity in female rats. The animals were allocated into three groups with six in each group: control, MTX:(15 mg/kg, only a dose, i.p), MTX+RES group: (15 mg/kg MTX, only a dose, i.p + 20 mg/kg RES, only a dose daily, oral gavage, 7 days). The nephroprotective efficacy was interpreted by measuring biochemical parameters such as serum renal function markers (uric acid, BUN and creatinine), total oxidant (TOS) and antioxidant status (TAS) in renal homogenates. Moreover, the effect of RES on kidneys was appraised by histopathological and immunohistochemical analyzes. In MTX-induced rats, RES treatment exhibited its nephroprotective effects with a significant increase in renal TAS as well as a significant decrease in serum BUN and renal TOS levels. In parallel with the biochemical data, it was observed that RES had a protective effect in the histological staining findings. Immunohistochemically, it was determined that TNF-α, one of the indicators of systemic inflammatory response, decreased with RES-treatment. The findings of the study show that RES administration 1 hour before MTX injection to rats has a curative effect on renal damage.

Keywords: Female rats, Methotrexate, Renal toxicity, Resveratrol.

Öz: Reaktif oksijen türlerinin (ROS) güçlü bir temizleyicisi olan resveratrolün (RES), böbrek hastalıkları da dahil olmak üzere çeşitli metabolik bozukluklara karşı koruyucu etkisi olduğu bildirilmektedir. Bu çalışma, dişi sıçanlarda metotreksat (MTX) ile indüklenen renal toksisitede resveratrolün nefroprotektif etkilerini değerlendirmeyi amaçlamaktadır. Çalışma, her grupta altı adet rat olacak şekilde üç gruba ayrıldı: Kontrol, MTX: (15 mg/kg, tek doz, i.p), MTX + RES grubu: (15 mg/kg MTX, tek doz, i.p + 20 mg/ kg RES, günde tek doz, oral gavaj, 7 gün). Serum renal fonksiyon belirteçleri (ürik asit, BUN ve kreatinin), renal homojenatlarda toplam oksidan (TOS) ve antioksidan durumu (TAS) gibi biyokimyasal parametreler ölçülerek nefroprotektif etkinlik yorumlandı. Ayrıca RES'in böbrekler üzerindeki etkisi histopatolojik ve immünohistokimyasal analizlerle değerlendirildi. MTX ile indüklenen ratlarda RES tedavisi, serum BUN, kreatinin ve renal TOS düzeylerinde anlamlı azalmanın yanında renal TAS'ta anlamlı bir artışla nefro-koruyucu etkilerini gösterdi. Histolojik boyama bulgularında da biyokimyasal verilere paralel olarak, RES'in koruyucu etkisinin olduğu gözlendi. İmmünhistokimyasal olarak sistemik inflamatuar yanıtın göstergelerinden biri olan TNF-α'nın RES tedavisi ile azaldığı belirlendi. Araştırmanın bulguları, ratlara MTX enjeksiyonundan 1 saat önce RES uygulamasının böbrek hasarı üzerinde iyileştirici etkisi olduğunu göstermektedir.

Anahtar Kelimeler: Böbrek toksisitesi, Dişi rat, Metotreksat, Resveratrol.

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Introduction

The kidneys perform a significant role in maintaining homeostasis, metabolism and excretion of toxins and drugs/drug metabolites (Perazella, 2009). Therefore, it is the most important organ in terms of drug toxicity. Excessive consumption of a wide variety of therapeutic agents, including antibiotics, nonsteroidal anti-inflammatory drugs, and anticancer agents, causes kidney damage and failure through tubular and glomerular damage (Elmansy et al., 2021). Drug-induced nephrotoxicity depends on their molecular properties, metabolites, and tendency to crystallize and precipitate in tubular lumens (Kwiatkowska et al., 2021).

Methotrexate (4-amino-10-methylfolic acid, MTX), a folic acid antagonist, blocks purine and pyrimidine synthesis by inhibiting several key enzymes, which is responsible for some toxicities as well as its efficacy in cancer therapy (Kremer, 2004; Braun and Rau, 2009). MTX is properly utilized in the treatment of various malignant and non-malignant diseases such as neoplastic diseases, psoriasis, rheumatoid arthritis and lupus erythematosus (Chan and Cronstein, 2013; Bedoui et al., 2019). The adverse effects of MTX often limit its therapeutic applications (Khan et al., 2012; Shah et al., 2016). MTX increases the formation of reactive oxygen species (ROS) and pro-inflammatory cytokines through various mechanisms (Abdel-Raheem and Khedr, 2014; Ju et al., 2020; Kaundal et al., 2021; Hobl et al., 2011). Based on MTX-induced nephrotoxicity studies, renal damage is thought to occur either through precipitation of MTX and its metabolites or through the direct toxic effect of MTX on the renal tubules (Widemann and Adamson, 2006). MTX-induced nephrotoxicity can be reduced by the use of ingredients with anti-oxidant and anti-inflammatory potential (Abouelela et al., 2020; Drishya et al., 2022).

Resveratrol (trans-3,4',5-trihydroxystilbene; (RES)), a polyphenolic compound and natural non-flavonoid antioxidant, is a phytoalexin produced in response to stress in certain plants such as grapes, peanuts, and cranberries (Fremont 2000; Yu et al., 2002). Studies have found that RES is well tolerated at therapeutic doses up to 5 g/day, by evaluating safety and potential mechanisms of activity following multiple dose administration (Nunes et al., 2009; Brown et al., 2010; Calamini et al., 2010). Published studies have shown that RES as a natural phenolic compound and a phytoestrogen is beneficial in the prevention and treatment of cardiovascular diseases, liver disorders, diabetes, cancer, obesity, pain, inflammation, tissue damage, and neurodegeneration. (Baur and Sinclair, 2006; Yeung et al., 2019). It has been shown in animal models that resveratrol can ameliorate various kidney injuries such as diabetic nephropathy, drug-induced injury, and ischemia-reperfusion injury through its antioxidant effect (Kitada and Koya, 2013; Wang et al., 2017). Different inflammatory molecules, especially Tumor necrosis factor-α (TNF-α), one of the proinflammatory cytokines, play a specific role in the development of nephropathy (Navarro and Mora-Fernández, 2006). This study is intended to biochemically and histopathologically examine the potential protective effects of RES on blood and renal tissue against oxidative damage induced by acute MTX exposure.

Materials and Methods

Experimental protocol

Female Wistar Albino rats (weighing between 240-360 g) were purchased from Burdur Mehmet Akif Ersoy University Experimental Animal Production and Experimental Research Center, used in the experiment. The animals were maintained in climate-controlled rooms (25 °C; 55% humidity) with diurnal lighting (12:12-h light:dark photoperiod). The rats had access to standard rodent chow and tap water ad libitum throughout the whole study. All animal use and accompanying procedures were in accordance with the animal research guidelines of the National Institutes of Health and were Burdur Mehmet Akif Ersoy University Animal

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Experiments Local Ethics Committee-approved (Ethical approval number: 17.03.2021-742).

In our study, 3 different experimental groups, each consisting of 6 female rats, were formed. Group-1, named as the control group, was treated with a single dose of 0.9% saline (1 mL/kg) intraperitoneal injection (i.p) on the 1st day. Group-2 was given only a single dose of 15 mg/kg i.p MTX (Kocaman and Çolakoğlu, 2013); Group-3 was given RES (Yuluğ et al., 2013) at a dose of 20 mg/kg 1 h before MTX (a single dose of 15 mg/kg i.p) administration by oral gavage. Group-3 was administered RES at the same time for 7 days. 24 hours after the last administration, on the 8th experimental day, the animals were euthanized by surgical anesthesia of 10% ketamine HCl (Ketalar® Eczacıbaşı, İstanbul) and 2% xylazine (Alfazin amp) administered intraperitonealy in groups. Until the end of the experiment, 2 rats from group-3 died, and the data of the study were evaluated accordingly. After anesthesia, blood and kidney tissue samples were taken. The blood samples were centrifuged at 5000 rpm for 10 minutes and serum samples were obtained to analyze the kidney function tests. One of the kidney tissues of each sample was taken to be homogenized for biochemical analysis, while the other kidney was placed in 10% formaldehyde solution for histopathological studies.

Preparation of renal tissue samples

Renal tissues of each group stored at -20 ºC were weighed separately after being brought to room temperature and diluted 10 times with 50 mM phosphate buffer (pH 7.4). The homogenization was completed by treatment with tissue shredder (Janke & Kuntel Ultraturrax T-25, Germany) and then sonicator (UW-2070 Bandeun Electronic, Germany). The samples were centrifuged at 10.000 rpm, 10 min. The renal supernatants were transferred to eppendorf tubes and used in further studies to determine the oxidant/antioxidant status.

Biochemical anaylsis

The biochemical parameters, a sign of kidney function such as uric acid, blood urea nitrogen (BUN) and creatinine (Cr) in serum were measured on an automatic clinical chemistry analyzer (Gesan chem 200, Italy) device in Veterinary Training Hospital of Burdur Mehmet Akif Ersoy University.

Total Oxidants and Antioxidants Status

TOS (Total Oxidant Status) and TAS (Total Antioxidant Status) parameters were studied by spectrophotometric method using Rel Assay Diagnostic kits (Mega Tip, Gaziantep, Turkey) and Biotek® (Epoch 2 Microplate Spectrophotometer) microplate reader in the renal supernatants obtained. TOS results were expressed in μmoL H_2O_2 equivalent/L (μmol H_2O_2 eq/L). TAS results of the samples were clarified as mmol Trolox equivalent/L (mmol Trolox eq/L). Establishing of OSI, which is an determinative parameter of oxidative stress level, the ratio of TOS to TAS was calculated using the following formula:

OSI (arbitrary unit)
= [((TOS, μmol/L))
/ (TAS, μmol Trolox equivalent/L)X 100]

Histopathological procedure

Renal tissues were removed from each rat and after cleaning they were washed in aqua over night. Than tissues were fixed in 10% neutral buffered formalin and dehydrated in 50–100% ethanol, made transparentin xylol. After all tissues buried in paraffin and were cut to 3–5 μ. At last they were stained with hematoxylin and eosin (H-E). The slides were examined using a light microscope (LeicaSM2000R, Germany) and photographed. Degeneration evelations were made acording to method of by Refaiy et al., (2011).

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Immunohistochemical procedure

The samples were stained with TNF-α primary ab (rabbit-anti-TNF-α antibody, Abcam, Cambridge-USA). Semi-quantitative evaluation method was used by Refaiy et al. (2011) to describe the observed staining intensities.

The staining score for H&E and IHC was evaluated as;
(-),0; none staining
(+), 1 mild staining
(++), 2 moderate staining
(+++), 3 intense staining.

Statistical Analysis

Statistical analyzes of the study were made using the IBM SPSS 20.0 program. All results are expressed as mean ± standard error. In histological analysis, Kruskal-Wallis test was used for semi-qualitative evaluation and non-parametric Mann-Whitney U test was used for pairwise comparisons. One-way ANOVA was used for intergroup comparison in biochemical analyses. The valuation of p below 0.05 were considered significant.

Results

Biochemical markers of renal function

The serum levels of uric acid was not significantly affected, but the BUN and Cr values in the MTX-induced female rats demostrated a significant increase in comparison with the control. RES admistration significantly decreased BUN parameter (Table 1), ($p<0.05$).

Table 1. Biochemical parameters in the serum.

	Uric acid (mmol/dL)		BUN (mg/dL)		Creatinine (mg/dL)	
Groups	Mean ±SD	P	Mean ±SD	P	Mean ±SD	P
Control	0.58±0.64		17.13±2.48	**p<0.000	0.27±0.04	
MTX	0.80±0.44		22.11±0.63	*p<0.000	0.33±0.02	*p=0.021
MTX+RES	1.28±0.60		20.09±0.66	*p=0.036 **p=0.008	0.30±0.03	

MTX - Methotrexate; RES - Resveratrol. Values are presented as means±SD. The relationships between groups and results of biochemical markers are assessed by one-way ANOVA. *p: Comparison with the control, **p: Comparison with the MTX.

Table 2. TOS, TAS and OSI values of renal tissues.

	TOS (μmol H_2O_2 eq/L)		TAS (mmol Trolox eq/L)		OSI (AU)	
Groups	Mean ±SD	P	Mean ±SD	P	Mean ±SD	P
Control	43.43±4.10	** p=0.001	0.97±0.04	**p=0.015	4.47±0.40	** p=0.005
MTX	98.35±11.94	* p=0.001	0.77±0.09	*p=0.015	12.94±2.58	*p=0.005
MTX+RES	28.53±0.00	*p=0.004 ** p=0.001	1.17±0.07	*p=0.005 ** p<0.001	2.44±0.15	*p<0.001 **p=0.002

MTX - Methotrexate; RES - Resveratrol. Data are presented as means±SD. One way ANOVA was used for comparison between groups.

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Table 3. Grading histological structural changes according to groups.

	Group 1 Control (n=6)	Group 2 MTX (n= 6)	Group 3 MTX + RES (n= 4)
Glomerules Degeneration and Vacuolization	-	++	+
Tubular Dilatation and Degeneration	-/+	++	+/-
Enlargements in Bowman Capsules	-/+	++	+/-
Mononuclear Cell Infiltration	-	+++	+++/++

Oxidative stress parameters in the renal homogenates

TOS, an indicator of the formation of ROS, increased significantly in group-2 compared to control, but decreased in group-3 (p=0.001 and 0.004, respectively). It was observed that RES therapy significantly decreased the TOS level compare with group-2 (Table 2), (p=0.001). TAS, an indicator of antioxidant capacity, was significantly decreased in group-2 and increased in the group-3 compared to the control (p=0.015, and 0.005, respectively). It was observed that RES therapy significantly increased the TAS level compare with group-2 (Table 2), (p<0.001). It was clearly seen that the OSI increased significantly in the group-2 compared to the control (p=0.005), and decreased significantly in the RES-treated group (p<0.001). It is seen that RES treatment significantly reduces the OSI level when compared to group-2. (p=0.002), (Table 2).

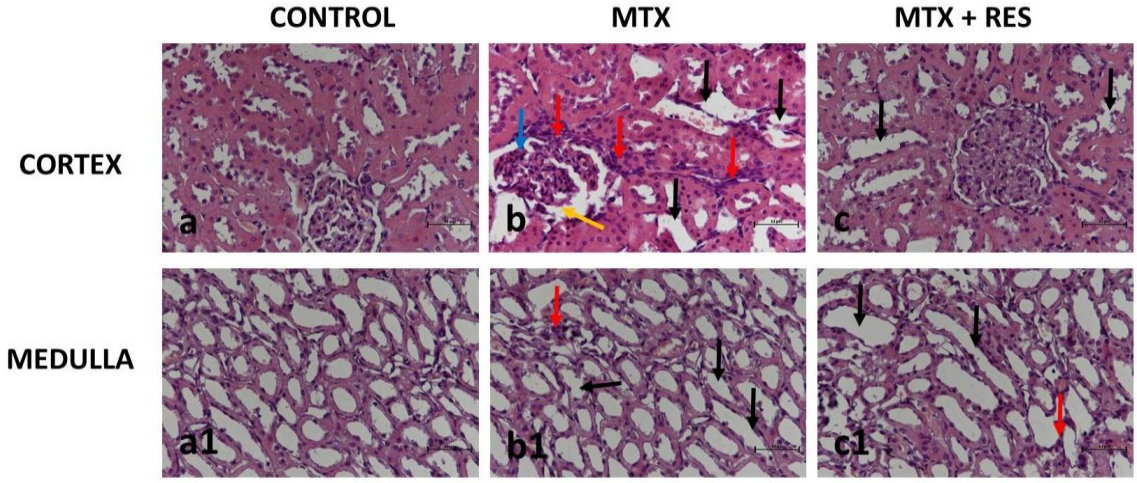

Figure 1. Control group: Kidney tissues were normal in control and there was very little tubular dilatation, too. MTX and MTX+RES group: Glomerules vacuolization (yellow arrow), glomerules degeneration (blue arrow), tubular dilatations and degeneration (black arrow), mononuclear cell infiltrations (red arrow), (a,b,c; kortexs - a1,b1,c1; medulla, H-E x400).

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Histopathological findings of kidney

The findings observed under the light microscope are given in Table 3. Histopathology of renal tissues were normal appearance in group-1, but also there was very little tubular dilatation, too (Table 3, Fig.1; a-a1), ($p>0.05$). The histopathological changes were observed significantly in group-2, which were remarkabled; degeneration of glomerules and vacuolization, tubular degeneration, tubular dilatation in the most of distal and proximal tubules, enlargements in Bowman capsules and mononuclear cell infiltration in the intertubular and perivascular fields (Table 3, Fig.1; b-b1). These histopathological findings were mostly observed in group-2 compared to group-3 (Table 3, Fig.1; c-c1), ($p<0.05$).

Immunohistochemical findings of kidney

A semi-quantitative assessment determined that the renal tissues of rats in group-1 had either very mild or no TNF-α staining. (Table 4, Fig.2; a), ($p<0.05$). However, an intense level of staining was seen in group-2 (Table 4, Fig. 2; b), while it was mild in group-3 (Table 4, Fig. 2; c).

Table 4. TNF-α staining**.**

	Control Group 1 (n=6)	MTX Group 2 (n=6)	MTX+RES Group 3 (n=4)
TNF-α	**-**	+/++	+/-

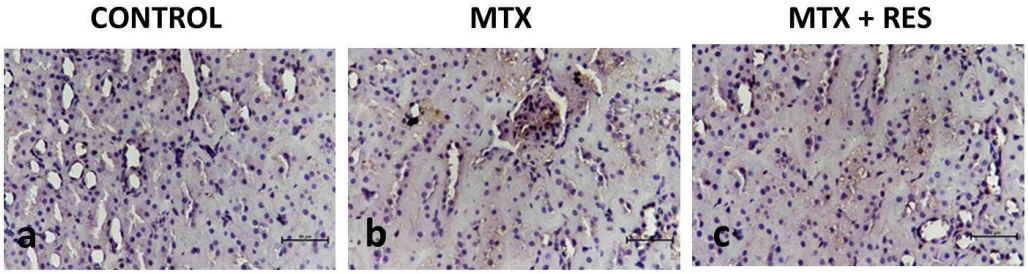

Figure 2. Control group a; staining was either very mild or nonexistent. MTX group b; showed mild/intense staining intensity compared with other groups. Immunohistological sections from MTX+RES group c; showed less staining intensity than group MTX b, (TNF- α immunstaining, ×400).

Discussion

The current study was conducted to evaluate whether RES can prevent or reduce MTX-induced renal injury by examining different biochemical and histopathological parameters related to renal function of female rats. Biochemical and histopathological findings clearly showed significant changes in renal function due to increased renal oxidative stress after MTX exposure. Available data suggest that RES can ameliorate MTX-induced renal damage by altering the levels of endogenous antioxidants.

Since the kidneys are responsible for the biotransformation and elimination of various toxins and drugs, they tend to generate free radicals that are involved in the pathogenesis of renal injury (Singh et al., 2003; Perazella, 2009). MTX administration is routinely applied in the treatment of malignant and non-malignant ailments and various systemic adverse effects are seen (Green and Chamberlain, 2009; Sotoudehmanesh et al., 2010; Gaies et al., 2012). Studies indicate that the administration of MTX produces functional and morphological changes in the kidney due to the direct toxic effects of the

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drug. MTX cytotoxicity and damage to the renal tubules are associated with the formation of free radicals and oxidative stress (Savran et al., 2017; Heidari et al., 2018).

The current study showed that while MTX caused an increment in serum BUN and Cr values, it did not cause a significant increment in uric acid values (Table 1). The findings are in line with the results of the investigation by Asci et al. (2017) reporting that MTX has a major role in the pathogenesis of renal dysfunction. Armağan et al. (2015) also detected biochemical results parallel to our study on MTX-induced renal dysfunction. Increased concentrations of serum urea, BUN, and Cr may be an indication of ROS generation via MTX in the kidney, resulting in kidney damage/deficiency (Abdel-Raheem and Khedr, 2014; Ahmed et al., 2015).

Oxidative stress induces damage to DNA and cellular biomolecules, resulting in degradation of cellular redox homeostasis, cellular apoptosis, and abnormal activation of signaling pathways (Sesti et al., 2012). TOS, which is an indicator of oxidation capacity, increased in support of the oxidative damage caused by MTX admistration. TAS, which is an indicator of antioxidant capacity, increased, indicating that RES teratment creates an antioxidant effect. It has been shown that MTX can modify the activity and levels of some ingredient of the tissue antioxidant defense system, increment the production of free radicals, especially ROS, and the formation of lipid peroxidation (Abdel-Raheem and Khedr, 2014; Armagan et al., 2015; Kandemir et al., 2017; Asci et al., 2017). It is known that while RES is a weak antioxidant in vitro, it is a strong antioxidant in vivo due to nitric oxide synthesis and free radical scavenging effect (Bay Karabulut, 2008). The findings of the study suggest that RES ameliorates the adverse effects of MTX on TAS and TOS. Many previous studies are in line with present findings, as they indicate that RES can directly scavenge reactive oxygen species such as toxic hydroxyl and superoxide radicals in the kidneys (Yu et al., 2013; Zhang et al., 2014; Shahbazi et al., 2020). Although it has been shown in different studies that RES improves renal damage with its antioxidant properties, there are not enough studies on oxidative stress and antioxidant markers TOS, TAS and OSI in MTX-induced nephrotoxicity. TAS and TOS levels were evaluated in MTX-induced organ damage studies performed with antioxidant agents in different tissues (Gunyeli et al., 2021; Soylu Karapınar et al., 2017; Özgöçmen and Yeşilot, 2021). In addition, agents with different antioxidant properties such as vitamin E (Taghizadieh et al., 2014), quercetin (Erboga et al., 2015; Yuksel et al., 2017), silymarin and naringin (Kandemir et al., 2017), gallic acid (Asci et al., 2017) vitamin C (Savran et al., 2017), rutin (Tambağ et al., 2021) have been shown to have protective effects in renal damage caused by MTX.

In the present study, the preventive effect of RES towards MTX-induced oxidative stress-mediated renal disfunction was evaluated at the histopathological level. In the study by El-Sheikh et al., while significant glomerular damage, enlarged Bowman's space, tubular necrosis, leukocytic infiltration and hyaline eruptions and deterioration in kidney structure were observed in the histopathology of the kidney of the MTX-treated rats, normal findings similar to the control group were observed in the RES-treated group (El-Sheikh et al., 2016). Silan et al. showed that RES has a protective effect against gentamicin-induced nephrotoxicity histopathologically, lipid peroxidation and cellular damage. In the histopathology slides of the same study, less parietal cell hyperplasia, tubular vacuolization, and tubular necrosis were detected in resveratrol-treated rats compared to the gentamicin-treated group (Silan et al., 2007). Consistent with our patho-histological results, RES has been previously reported to have nephroprotective efficacy in other models of induced kidney injury in which it was involved (Yu et al., 2013; Akbel et al., 2018; Shahbazi et al., 2020). The authors of the studies concluded that the free radical scavenging property of resveratrol may be

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responsible for its nephroprotective effects against induced nephrotoxicity.

The primary pathological mechanism linking oxidative stress, inflammation, and progression of kidney disease is the initiation of kidney damage due to the inflammatory response resulting from the activities of intracellular and extracellular oxygen-derived radicals (Elmarakby and Sullivan, 2012; Tucker et al., 2015; Ker et al., 2020). Activation of proinflammatory cytokines and generation of inflammatory response are associated with MTX-induced renal toxicity (Çakır et al., 2015). It is known that TNF-α is an important proinflammatory cytokine and a pathogenic factor in renal damage (Baud and Ardaillou, 1995; Navarro and Mora-Fernández, 2006). In the current study, it was detected immunohistochemically that TNF-α, one of the systemic inflammatory response indicators, increased in the MTX group and decreased in the RES treatment group (Table 4, Fig.2). The nephroprotective effects of RES are not limited to amelioration of pathological renal fibrosis, but also include renal morbidities. RES shows protective effects mostly against various renal damage due to its antioxidant properties (Malhotra et al., 2015). The findings of Jang et al show that resveratrol exerts protective effects on aging kidneys by reducing oxidative stress, inflammation and fibrosis through Ang II suppression and MasR activation (Jang et al., 2018). In the study by Kandemir et al., (2017) it was declared that TNF-α expression in kidney tissue increased with MTX application. Studies showing that RES reduces kidney damage through modulation of oxidative stress and TNF-α-induced inflammation in rats are consistent with current study (Saldanha et al., 2013; El-Sheikh et al., 2017; Wang et al., 2020). These results are associated with the nephroprotective antioxidant effects of RES on MTX-induced nephrotoxicity.

Conclusion

The indications suggest that oxidative stress reasoned by aberrant ROS formation is responsible for the pathophysiology of MTX-induced nephrotoxicity. RES treatment ameliorates MTX nephrotoxicity in female rats by restoring kidney functions and inhibiting TNF- α with its free radical scavenging and natural antioxidant effects. As a dietary supplement, RES can be used with MTX therapy as it reduces nephrotoxic side effects. Therefore, RES supplementation as adjuvant therapy may be promising in alleviating the systemic adverse effects of drugs.

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