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Thymoquinone Prevents Valproic Acid-Induced Nephrotoxicity in Rat Kidney

Sebile Azırak [1*], Deniz Taştemir Korkmaz[2], Sedat Bilgiç[1], Meltem Özgöçmen[3] , Mehmet Kaya Özer[4]

[1] *Vocational School of Health Services, University of Adıyaman, Adıyaman, Turkey*
[2] *Department of Medical Biology, Faculty of Medicine, University of Adıyaman, Adıyaman, Turkey*
[3]*Department of Histology and Embryology, Faculty of Medicine, University of Suleyman Demirel, Isparta, Turkey*
[4]*Department of Pharmacology, Faculty of Medicine, University of Adıyaman, Adıyaman, Turkey*

*Corresponding author : sazirak@.adiyaman.edu.tr
Orcid No: https://orcid.org/0000-0001-9040-6773

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Abstract: Valproic acid (VA), widely used as an antiepileptic, causes structural and functional kidney disorders. Whether thymoquinone (TQ) has a beneficial effect on VA-induced nephrotoxicity has been investigated. Twenty-one male Spraque Dawley rats were grouped into control, VA, and VA + TQ groups (n=7 for per group). VA (500 mg/kg/day) and TQ (50 mg/kg/day) were applied to the rats orally for 14 days. They were euthanized on the 15th day of the treatment. The cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) gene expression levels, biochemical parameters, total antioxidant/oxidant statuses (TAS/TOS), oxidative stress index (OSI), histological and immunohistochemical analysis were performed to evaluate kidney toxicity. In the VA + TQ group, COX-1 expression levels increased, while COX-2 expression levels decreased. While the creatinine (Cr) and blood urea nitrogen (BUN) levels, production of caspase-3 (CAS-3) and NADPH oxidase-4 (NOX-4) were increased in the VA-treated group, they were decreased in VA + TQ group. Treatment with TQ against VA administration decreased TOS and OSI levels while increasing TAS. TQ protects the kidney against the toxic effects of VA.

Keywords: Apoptosis, COX-1, COX-2, Oxidative stress, Thymoquinone, Valproic acid

1. Introduction

Valproic acid (VA), an antiepileptic agent, has been used for the treatment of different types of seizure and psychiatric disorders for over 30 years. Although it is used as a safe drug in the treatment of diseases, high doses and long-term use bring some adverse effects e.g. weight gain, liver toxicity, gastrointestinal problems, pancreatitis, thrombocytopenia, encephalopathy, hematological abnormalities, and renal injury (Knights and Finlay 2014; Gezginci-Oktayoglu et al. 2016; Heidari et al. 2018). The renal injury in human and animal models caused by VA has been shown by previous studies but the mechanisms remain unclear yet (Altunbaşak et al. 2001; Endo et al. 2010; Gezginci-Oktayoglu et al. 2016; Heidari et al. 2018).

Toxic intermediates like VA can cause renal injury as a consequence of the formation of reactive oxygen species (ROS), inflammation, and fibrosis, and can disrupt the structure of the kidney (Chang and Abbott 2006; Sher et al. 2015). Studies have shown that VA causes oxidative stress, inflammation, carnitine and mitochondrial deficiencies, and fibrosis in kidney tissue (Hamed 2017). In particular, renal dysfunction and mitochondrial damage decrease ATP production and increase oxidative stress, impair apoptosis, and cause necrosis by leading to microvascular loss and fibrosis, thereby risking kidney function (Eirin et al. 2017). Oxidative stress in patients with acute renal failure is associated with an imbalance between the antioxidant system and ROS production (Ragheb et al. 2009).

The kidneys have important roles in the regulation of water, electrolyte, nitrogen, and acid-base balances to maintain the body's homeostasis (Faria et al. 2019). The kidney has abundant blood flow and a large capillary surface area. Due to the kidney's role as an excretory pathway and detoxifying effect of many drugs, it is vulnerable to drugs, and 20% of total acute renal failure cases are drug-related (Fanos and Cataldi 2002). Kidney dysfunction, which is often associated with drug use, can lead to accumulation and clinical toxicity of antiepileptic drugs, prolonging the elimination period (Asconapé 2014). The mechanisms of kidney injury include a wide network of signaling pathways driven by the interaction of ROS, apoptotic factors, and inflammatory cytokines/chemokines (El Sabbahy and Vaidya 2011). VA treatment induces the formation of ROS (Tung and Winn 2011). Increasing levels of ROS which are

also important secondary messengers in cellular signaling lead to the oxidation of DNA, proteins, and lipids that cause cellular damage in the kidney (Irazabal and Torres 2020).

Previous studies indicate that prototypical inflammatory cytokines e.g. TNF-α, interleukin 1β (IL-1β), IL-6, and prostaglandins (PGs), especially PGE 2, play an important role among the diverse inflammatory mediators that lead to epilepsy (Cole-Edwards and Bazan 2005; Vezzani et al. 2008). Cyclooxygenase (COX) is the main target of nonsteroidal anti-inflammatory drugs (NSAIDs), of which VA is one of them, and is a rate-limiting enzyme in PG synthesis (Takemiya et al. 2007; Blumenfeld et al. 2012). Neuroinflammation has a critical role in brain disorders, and COX is one of the major drug targets for reducing neuroinflammation (Dhir 2019). There are at least two COX isoenzymes: COX-1 and COX-2. COX-1 is a builder and produces PGs that protect the stomach and kidneys from damage (Vane and Botting 1998). COX-1 maintains the homeostatic functions of the body and is constitutively expressed in a variety of cells and tissues such as parenchymal cells of some organs, e.g. kidneys, endocrine glands, and neurons observed in the neuroendocrine system, and reproductive system (Zidar et al. 2009; Dhir 2019). COX-2 produces PGs that contribute to pain and swelling of inflammation (Vane and Botting 1998). In addition, COX-2 is a subtype that is upregulated by stimuli such as pain, inflammation, or cancer proliferation (Dhir 2019). PGs produced by COX-1 are responsible for the regulation of renal blood flow, mucous membrane protection, and homeostasis, while COX-2 is responsible for high prostanoid production at sites of disease and inflammation (Oksuz et al. 2016).

Nephrotoxicity is associated with mortality and morbidity, and the use of natural products has a very important place in investigating drug-induced nephrotoxicity and eliminating the side effects of the drug. Therefore, in this study, thymoquinone (TQ), a natural antioxidant, was used against nephrotoxicity caused by VA. TQ, as the most abundant component of the essential oil of black cumin (*N. sativa*) seeds, has properties such as antimicrobial, anti-nociceptive, anti-epileptic, hypoglycemic, hypolipidemic, and bronchodilator properties (Tavakkoli et al. 2017). TQ exhibits a variety of pharmacological activities including nephroprotection, gastroprotection, anti-hepatocellular carcinoma, cardioprotection, neuroprotection, anti-allergy, retinal protection, bladder protection, reproductive system protection, and respiratory protection (Talebi et al. 2021; Tastemir Korkmaz et al. 2021).

In this study, we aimed to investigate whether the antioxidant and anti-inflammatory features of TQ have ameliorative effects against oxidative stress-induced damage of VA in kidney cells.

2. Materials and Method

2.1. Study groups and experimental design

Twenty-one 3-4 month-old and 250-300 g weighted Sprague Dawley albino male rats were obtained from Fırat University Experimental Research Center in Elazığ/Turkey. They were kept in polycarbonate cages with free access to food and water at 24°C, 42% ± 5% relative humidity, and a 12/12 hour light/dark cycle. They were randomly separated into 3 groups: Control (n=7), VA (n=7), and VA + TQ (n=7). Nothing was applied to the rats in the control group. The VA group received daily doses of VA (500 mg/kg) for 14 days whereas TQ (50 mg/kg) was given in addition to VA (500 mg/kg) to the VA + TQ group for 14 days orally (Atta et al. 2017; Barrett et al. 2017). When the study was completed, all rats were sacrificed through cervical dislocation under anesthesia. Venous blood samples were centrifuged at 5000 x g after collection and serums were obtained for biochemical analysis. Besides, the kidneys were taken for genetic, histological, and immunohistochemical analysis, and both the serums and kidneys were stored at -80 °C until use. This study was approved by Animal Experiments Local Ethics Committee (Protocol no 2017/135).

2.2. Biochemical Analysis

2.2.1. Serum Levels of Creatinine (Cr) and Blood Urea Nitrogen (BUN)

Serum Cr and BUN levels were measured to assess kidney function. For this purpose, Roche Diagnostics kits (Mannheim, Germany) and Hitachi automatic biochemical analyzer 7060 c (Japan) were used according to the picric acid method (Junge et al. 2004). Cr and BUN levels were analyzed as mg/dL serum.

2.2.2. Serum levels of Total Antioxidant/Total Oxidant Statuses (TAS/TOS) and Oxidative Stress Index (OSI)

Serum obtained with the automated colorimetric kit Erel (REL Assay Diagnostics, Gaziantep, Turkey) was used to determine the total antioxidant/total oxidant statuses (TAS/TOS). The method applied by Bilgic et al. (2017) was used to determine TAS and TOS levels. Oxidative stress index (OSI) was calculated according to the formula OSI = TOS / TAS (Gul et al. 2017).

2.3. COX1 and COX2 Gene Expression Analysis

2.3.1. RNA Extraction and cDNA Preparation

Fresh frozen rat kidneys were processed according to the manufacturer's instructions under RNAse-free conditions using total RNA extraction solution (Bioneer) for RNA extraction and its purity was measured in a NanoDrop spectrophotometer (Denovix DS-11) at 260/230 nm and 260/280 nm, and stored at -80 °C. For qRT-PCR, the first 5 µg of total RNA was reverse transcripted using RT PreMix (Bioneer) using appropriate controls to ensure the absence of genomic DNA contamination.

2.3.2. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Assay

The GreenStar qRT-PCR PreMix (Bioneer) using the ExiCyclerTM96 qRT- PCR system (Bioneer) was used for detecting the expression levels of COX-1 and COX-2 genes. The GAPDH gene was amplified as an internal control. The primers used were taken from previous studies (Kis et al. 2003; Langnaese et al. 2008; Qiao et al. 2013). PCR

conditions were 95 °C for 1 min, followed by 45 cycles at 95 °C for 5 sec, and 55 °C for 40 seconds. The $2^{\wedge-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression.

2.4. Histopathological and Immunohistochemical Analysis

All kidney tissues preserved in 10% neutral formaldehyde were used for histological and immunohistochemical studies. Binocular light microscopy (ECLIPSE Ni-U, Nikon, Tokyo, Japan) was used to detect damage and healing rate in kidney tissues. Approximately 4-5 µm-thick tissue was obtained from paraffin-wax embedded kidney tissue with microtome stained with hematoxylin-eosin for detecting structural changes (vascular congestion, dilatation, and degeneration of proximal-distal tubules, glomerular degeneration, tubular dilatation, hemorrhagic areas, and mononuclear cell infiltration in the medulla) in all groups. The results were evaluated according to the scoring made by Abdel-Wahhab et al. (1999). The method mentioned by Savran et al. (2020) was used for detecting and scoring the activity of Caspase 3 (CAS-3) and NADPH oxidase-4 (NOX-4).

2.5. Statistical Analysis

Statistical Package for the Social Sciences (SPSS) 25.0 Software program was used for all comparisons. All data are presented as mean ± SEM. The normality was identified by the Shapiro-Wilk test. One-way ANOVA after LSD posthoc was used for the comparison of parametric values in genetic parameters. A semi-qualified evaluation of the histopathological scores was evaluated with the Mann Whitney U test to determine significant differences between groups. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Serum Levels of Oxidative Stress Biomarkers

Cr and BUN levels are shown in Table 1. They were increased in the VA group against the control and VA + TQ groups and these increases were found statistically significant ($p < 0.05$). Additionally, the levels of TOS and OSI were increased significantly in the VA group when compared with the control and VA + TQ groups ($p < 0.05$) (Table 1).

It was observed that TAS levels increased with TQ applied against VA, while TOS and OSI levels decreased ($p < 0.05$). TAS level was found to be significantly higher in the control group compared to the VA group ($p < 0.05$). While TAS level increased significantly in VA + TQ group compared to VA ($p < 0.05$), TOS level increased in the VA group when compared to the control and VA + TQ groups, and these differences were found statistically significant ($p < 0.05$). OSI level was found higher in the VA group than control and VA + TQ groups significantly ($p < 0.05$) (Table 1, Figure 1).

Table 1 Serum biochemical and renal tissue oxidative stress biomarkers of the experimental groups.

	STUDY GROUPS		
	Control	**VA**	**VA + TQ**
BUN (mg/dL)	30.600 ± 1.039^{b}	$56.00 \pm 5.263^{a,c}$	41.00 ± 4.313^{b}
Cr (mg/dL)	0.530 ± 0.018^{b}	$0.613 \pm 0.031^{a,c}$	0.548 ± 0.043^{b}
TOS (µmol/L)	3.42 ± 0.62^{b}	$6.41 \pm 0.42^{a,c}$	4.31 ± 0.56^{b}
TAS (mmol/L)	1.45 ± 0.15^{b}	$0.61 \pm 0.05^{a,c}$	1.15 ± 0.28^{b}
OSI (AU)	0.25 ± 0.06^{b}	$1.13 \pm 0.14^{a,c}$	0.57 ± 0.23^{b}

Each group represents the mean ± SEM for seven rats. a: Significant from Control; b: Significant from VA; c: Significant from VA + TQ. $p < 0.05$. Abbreviations: Cr, creatinine; BUN, blood urea nitrogen; TAS, total antioxidant status; TOS, total oxidant status; OSI, Oxidative stress index; VA: valproic acid; TQ: thymoquinone; VA: 500 mg/kg VA; VA + TQ: 500 mg/kg VA + 50 mg/kg TQ. AU: Arbitrary Units

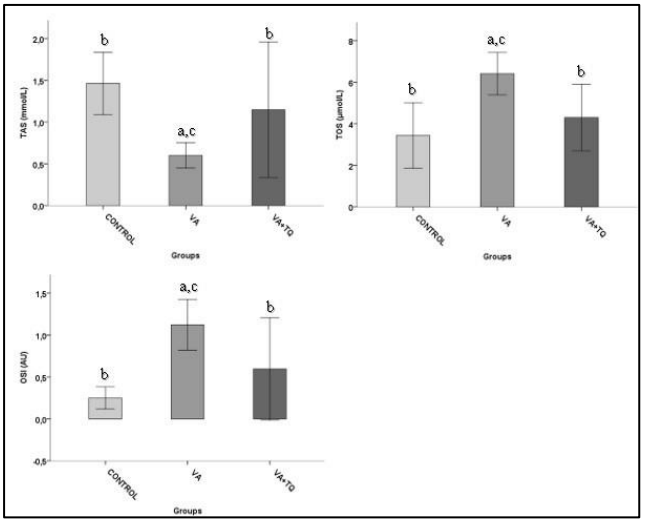

Fig. 1 Effects of VA, TQ, and their coadministration on the serum level of TAS, total antioxidant status; TOS, total oxidant status; OSI, Oxidative stress index in rats after two weeks. Values are expressed as mean ± SEM of seven animals. ANOVA followed by the LSD post hoc test were used. a $p<0.05$ versus control; b $p<0.05$ versus VA treated rats; c $p<0.05$ versus VA + TQ treated rats. Abbreviations: VA: valproic acid; TQ: thymoquinone; VA: 500 mg/kg VA; VA + TQ: 500 mg/kg VA + 50 mg/kg TQ. AU: Arbitrary Units.

3.2. COX1 and COX2 Gene Expression Levels

Table 2 shows the expression levels of COX-1 and COX-2 genes in control, VA, and VA + TQ groups. Statistically, COX-1 expression levels were decreased in the VA group, while COX-2 expression levels were increased ($p < 0.05$). In the VA + TQ group, COX-1 expression levels increased, while COX-2 expression levels decreased ($p < 0.05$).

3.3. Histopathological and Immunohistochemical Findings

Table 3 shows the histopathological findings in the present study. No other findings than normal histological structures were found in the histological examination of the kidney tissue sections of the control group (Table 3, Figure 2: a-b). Significant structural changes including dilatation and

degeneration in proximal-distal tubules, vascular congestion, hemorrhagic areas, glomerular degeneration, tubular dilatation in the medulla, and mononuclear cell infiltrations were observed in the VA group compared with the control group ($p < 0.05$) (Table 3, Figure 2: c-c1-c2-c3). An improvement in histopathological findings was observed in the VA + TQ group compared to the VA group ($p < 0.05$) (Table 3, Figure 2: d-e).

Table 2 Effects of VA and TQ on the expression of COX-1 and COX-2 genes in rat kidneys

Groups	Expression value (Ct value; Mean ± SEM)	
	COX-1	COX-2
Control	35.10 ± 1.05 [b]	32.70 ± 0.77 [b]
VA	30.57 ± 1.22 [a,c]	39.07 ± 1.42 [a,c]
VA + TQ	34.98 ± 1.64 [b]	34.57 ± 1.51 [b]

Each group represents the mean ± SEM of seven rats. a: Significant from control; b: Significant from VA; c: Significant from VA + TQ; $p < 0.05$

Table 3 Histopathological changes of renal samples in the experiment groups

Parameters/Score	Experimental groups		
	Control	VA	VA + TQ
Tubular dilatations in the medulla	–	+++ [a]	++ [b]
Proximal - distal tubule dilatation	–	+++ [a]	++ [b]
Proximal - distal tubule degeneration	–	+++ [a]	++ [b]
Glomerular degeneration	–	+++ [a]	++ [b]
Vascular congestion	–	+++ [a]	++ [b]
Interstitial mononuclear cell infiltration	–	+++ [a]	++ [b]
Hemorrhagic area	–	+++ [a]	++ [b]

Scoring system is as described in the methods section. n = 7. Abbreviations: VA, Valproic acid; TQ, thymoquinone. a: valproic acid increased renal damage, $p < 0.05$ vs. control. b: thymoquinone reduced renal damage, $p < 0.05$ vs. VA.

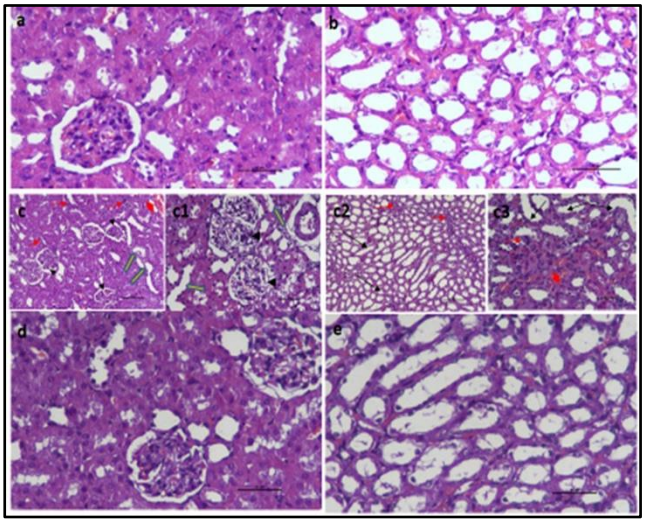

Fig. 2 Kidney tissue sections of the control and experimental groups. a (cortex) and b (medulla); normal histological appearance is observed in the kidney tissue sections of the control group. (a; cortex; Bowman's capsule, distal, proximal tubules b; medulla; collecting tubules are observed normally (H–E, x20). c - c2 (cortex), c1 - c3 (medulla); kidney tissue sections belonging to the group given VA: red arrows; mononuclear cell infiltrates, yellow filled arrows; prochymal – distal tubule dilatations, black arrowheads; glomerular degenerations, red arrowhead; hemorrhagic area, black arrows; tubular dilatations in the medulla (c- c2; H–E, x20, c1-c3; H–E, x40). d (cortex) and e (medulla); Renal tissue sections of the group given VA + TQ: compared to VA group, a significant improvement is observed in the cortex and medulla (H–E, x20).

The immunochemical findings are shown in Table 4 and Figures 3 - 4. In the immunohistochemical staining of kidney tissue sections, a significant difference was found between the control group and the VA and VA + TQ groups ($p < 0.05$). In the kidney samples, CAS-3 immunoreactivity was observed intensely in the VA group but not in the control group. Interestingly, it was poorly observed in VA + TQ group (Table 4, Figure 3). At the same time, NOX-4 immunoreactivity was also observed similar to CAS-3 immunoreactivity results (Table 4, Figure 4).

Table 4 Immunreactivity grades of CAS–3 and NOX–4 in renal tissues of experimental groups.

Target stained immunohistochemically	Experimental groups		
	Control	VA	VA + TQ
CAS–3	–	+++ [a]	+ [b]
NOX–4	–	+++ [a]	+ [b]

(n = 7 for each group). Scoring system is described in the methods section shows the apoptotic renal ratio. Abbreviations: VA, valproic acid; TQ, thymoquinone. a: valproic acid increased renal damage, $p < 0.05$ vs. control. b: thymoquinone reduced renal damage, $p < 0.05$ vs. VA.

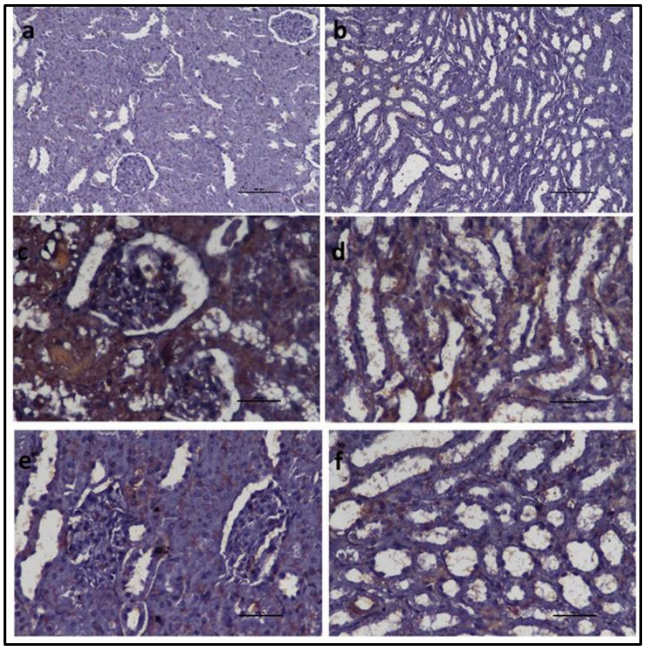

Fig. 3 Kidney tissue section of the control and experimental groups immunostaining with CAS-3. a (cortex) and b (medulla); Control group kidney tissue sections were stained negative with CAS-3, brown areas are not observed (immunostaining, x20). c (cortex) and d (medulla) VA group kidney tissue sections stained positively with CAS-3, with no large amounts of brown areas observed (immune staining, x40). VA + TQ group kidney tissue sections e (cortex) and f (medulla) show less positive staining brown areas with CAS-3 compared to VA group (immune staining, x20).

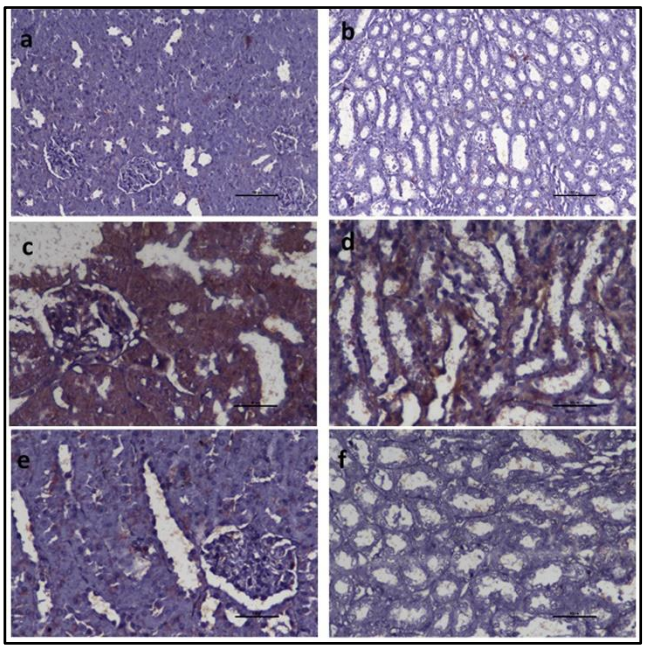

Fig. 4 Kidney tissue section of the control and experimental groups immunostaining with NOX-4. Control kidney tissue sections a (cortex) and b (medulla) stained negatively with NOX-4 and no brown areas were observed (immune staining, x20). c (cortex) and d (medulla) VA group kidney tissue sections stained positively with NOX-4, with no large amounts of brown areas observed (immune staining, x40). VA + TQ group kidney tissue sections e (cortex) and f (medulla) show less positive staining brown areas with NOX-4 compared to VA group (immune staining, x20).

4. Discussion

VA is a well-tolerated, effective antiepileptic drug that is widely used as a mood stabilizer (Ornoy et al. 2020). Previous studies have reported markers of tubular and renal glomerular injury as a result of chronic use of some antiepileptic drugs such as VA (Hamed 2017). In previous studies, it was observed that kidney and liver functions were impaired, thrombocytopenia and coagulopathy developed after 3 months of VA treatment (Yaman et al. 2013). A previous study has reported that exposure to various drugs for therapeutic or diagnostic purposes causes damage and clinical manifestations in renal tubules, interstitium, glomerulus, and renal microvasculature (Malyszko et al. 2017).

In this study, BUN and Cr levels increased as a result of kidney function loss. Makris and Spanou (2016) have reported that acute kidney injury is characterized by increased blood levels of BUN and Cr, as well as oliguria and electrolyte disturbances. It was found that BUN and Cr levels were significantly higher in VA-treated rats than in the control group, indicating damage in the function and structure of the kidneys. TQ used in this study is to reduce the nephrotoxic effect of VA. It was found that VA + TQ treatment significantly reduced BUN and Cr levels. TQ is a well-known ROS scavenger and antioxidant (Karimi et al. 2019). The findings of this study demonstrate the protective effects of TQ against VA-induced nephrotoxicity in rats. Studies have reported that TQ reduces serum BUN and Cr levels and protects kidney function (Badary et al. 2000; Jalili et al. 2017).

In this study, TQ inhibited elevated TOS and OSI levels and increased TAS, ameliorated impaired kidney function, and reshaped histopathological changes in the kidney. According to the study, TOS and OSI levels increased and TAS levels decreased in VA-treated rats (Takeuchi et al. 2005). On the other hand, the results showed that TQ protects against VA-induced kidney injury by suppressing oxidative stress and apoptosis, as reported in previous studies (Tekbas et al. 2018).

It has been reported that COX-2 expression is associated with inflammation and cancer proliferation, while COX-1 plays a cleaning role in the homeostatic process (de Leval et al. 2000). In this study, COX-1 expression levels decreased in the VA group, while COX-2 expression levels increased. COX-1 inhibition has positive effects on seizure suppression, while COX-1 inhibition has been reported to cause gastrointestinal toxicity (Wallace et al. 2000; Barbalho et al. 2016). COX-2 is increased in PG production, clinical inflammation, and some types of human cancer, particularly colon cancer (Crofford 1997). In the study, while COX-1 expression levels increased in the TQ group, COX-2 expression levels decreased. COX-1 inhibition can cause side effects such as kidney and liver failure (Dhir 2019). COX-2-induced inflammation contributes to epileptogenesis and neuronal damage that develops after brain injuries (Polascheck et al. 2010). Anti-inflammatory

treatments such as selective COX-2 inhibitors can be used as anti-epileptogenesis in brain injuries such as cerebral ischemia, head injury, or status epilepticus (SE) (Polascheck et al. 2010). In the study, it was reported that the inhibition of COX-1 upregulated the expression of COX-2.

When the histological results were evaluated, normal kidney histology was observed in the control group. In the VA group, important histopathological changes such as tubular dilatations, proximal-distal tubule dilatation, proximal-distal tubule degeneration, glomerular degeneration, vascular occlusion, interstitial mononuclear cell infiltration, and hemorrhagic area were observed in the medulla. Histopathological changes were very few in the group treated with VA + TQ. According to the histological results, it can be said that TQ has a corrective effect on kidney damage.

CAS-3 is a protease with a well-known role in apoptosis (Shalini et al. 2015). CAS-3 is known to have a role in cell growth and differentiation, cytokine maturation, and apoptosis (Yan et al. 2013). Generation of ROS and upregulation of CAS-3 lead to apoptosis (Rana 2008). CAS-3 immunoreactivity, a marker of apoptosis, was not seen in the control group, whereas immunostaining of CAS-3 was strong in the VA group. CAS-3 immunoreactivity was higher in the VA group than in the VA + TQ group. From this point of view, it can be said that TQ reduces VA-induced apoptosis. TQ was found to prevent programmed cell death by inhibiting CAS-3.

NOX-4, the major isoform of NADPH in the kidney, produces H_2O_2 and contributes to redox processes by activating multiple signaling pathways in kidney diseases such as acute kidney injury, diabetic nephropathy, and hypertensive nephropathy (Yang et al. 2018). Upregulation of NOX-4, an important source of ROS, causes kidney damage. In the present study, NOX-4 immunoreactivity was not seen in the control group, but it was seen at high intensity in the VA group. The immunoreactivity was weaker in the VA + TQ group compared to the VA group. TQ appears to prevent VA-induced nephrotoxicity with its antioxidant properties. Previous studies have shown that TQ protects by showing anti-inflammatory and antioxidant properties in various kidney diseases such as renal ischemia-reperfusion, diabetic nephropathy, and nephrotoxicity caused by inflammation and oxidative stress (Shaterzadeh-Yazdi et al. 2018; Erdemli and Yigitcan 2020; Azirak et al. 2022).

5. Conclusion

This study demonstrated the beneficial effects of TQ against VA-induced nephrotoxicity by reducing oxidative stress due to its antioxidant, and anti-inflammatory effects. However, more clinical studies are needed to confirm these results.

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Authors' contributions:

Study design (SA, MKÖ), Biochemical analysis (SB), Genetic analysis (SA, DTK), Histological and immunohistochemical analysis (MÖ), Analysis and interpretation of the data (SA, DTK, SB, MÖ, MKÖ), The drafting of the paper (SA, DTK), Final approval of the version to be published (SA, DTK, SB, MÖ, MKÖ)

Conflict of interest disclosure:

There is no conflict of interest.

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