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Original Article

Thymoquinone reduced RIPK1-dependent apoptosis caused by valproic acid in rat brain

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Abstract

Aim: Valproic acid (VPA) is a commonly used antiepileptic drug and known to have a neurotoxic effect, but its mechanism is not yet understood. In the present study, we aimed to determine how the VPA causes cell death in the brain and to evaluate the protective effects of thymoquinone (TQ) on VPA-induced brain damage.

Materials and Methods: Male Sprague–Dawley albino rats were divided into three groups: control, VPA (500 mg/kg/day) and VPA + TQ (500 mg/kg/day + 50 mg/kg/day) with seven rats in. At the end of the experiment, rats were sacrificed and brain samples were taken to measure the expression levels of Receptor-interacting serine/threonine-protein kinase-1 (RIPK1) and -3 (RIPK3) genes by quantitative real-time PCR (qRT-PCR), NADPH oxidase-4 (NOX4) and, caspase-3 (CAS-3) expression by immunohistochemistry and the structural changes in the brain tissue by histologically.

Results: RIPK1 gene expression levels were significantly increased in the VPA group compared to the controls ($p<0.05$) and a decrease in VPA + TQ group against the VPA group. Also, NOX-4 and CAS-3 production were increased in the VPA group compared to the control group ($p<0.05$), and there is a markedly decrease in the VPA + TQ group compared to the VPA group.

Conclusion: VPA induced RIPK1-dependent apoptosis, leading to cell deaths in the brain and TQ reduced its effects. Therefore, TQ uptake can be a supportive treatment method for long-term and high-dose VPA users to eliminate undesirable effects.

Keywords: Apoptosis; RIPK1; thymoquinone; valproic acid

INTRODUCTION

Valproic acid (VPA) is a histone deacetylase (HDAC) inhibitor used for treatment of mood disorders and epilepsy. The use of VPA, an anticonvulsant blocking voltage-dependent sodium channels, was supported by clinicians, but later confused due to its side effects and HDAC-dependent and non-dependent damage. In studies conducted with some neurological diseases, VPA has been reported to have protective properties, but it has been observed that VPA-related neurodegeneration is observed in both cultured neuronal cells and experimental animals (1-3). It is stated that VPA is a neuronal protector in some central nervous system injuries (4, 5). In a study, it has been reported that it causes neuronal cell deaths related and not related to HDAC, while in others it causes temporary brain atrophy (6-8). VPA taken during pregnancy increases the incidence of autistic childbirth associated with large brain apoptosis sites (9). VPA has also been shown to cause neuronal cell death that is not dependent on caspase, but the mechanism has not yet been understood (10). Bollino et al. (10) reported that VPA stimulated neuronal cell death by a new calpain-dependent necroptosis pathway with Receptor-interacting serine/threonine-protein kinase 1 (RIP1 or RIPK-1) expression by c-Jun N-terminal protein kinase-1 (JNK1) activation.

Necroptosis is a programmed cell death form and differs from apoptosis mechanistically and morphologically. While the activation of the caspase proteases has a role in apoptosis, RIPK1 and RIPK3 trigger necroptosis (11). The human RIPK gene locates on chromosome 6p25.2 and encodes seven splicing isoforms (RIPK1, RIPK2, RIPK3, RIPK4, RIPK5, RIPK6, and RIPK7) (12,13). RIPK1 and RIPK3 are crucial signaling molecules in necroptosis and are regulated by the caspase pathway and ubiquitination. RIPK1 is the first member of the family and is known to have a function in a variety of cellular pathways associated with cell survival and death. Ubiquitination of RIPK1 leads to cell survival by activating Nuclear Factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), while deubiquitinated form induces the caspase-8 mediated

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apoptosis pathway (14,15). When caspase-8 is inhibited or deficient, RIPK1 assembles with RIPK3 via the C-terminal RIP homotypic interaction motif (RHIM) domain to form the RIP1/RIP3 complex and triggers cells to necroptosis (16) and most important excessive necroptosis may cause many diseases including neurodegeneration (12). Although most studies show that VPA causes pseudo atrophy in the brain, it is also known that VPA causes damage to many tissues and this is caused by various processes such as oxidative stress and inflammation, and apoptosis/necroptosis.

Thymoquinone (TQ) is the most important bioactive ingredient found in black seed (*Nigella sativa*) essential oil and has many health beneficial properties such as antihypertensive, antimicrobial, antidiabetic, anticancer, anti-inflammatory, analgesic, diuretic, and antioxidant activities (17-19). Also, TQ has been reported to display neuroprotective effects (20). Our aim in this study was to examine the potential of VPA to cause brain damage and whether TQ has a protective effect against this damage.

MATERIALS and METHODS

Animals

A total of 21, 3-4-month-old and 220-290 g male Sprague-Dawley albino rats were purchased and housed in Experimental Research Centre of Fırat Univesity, Elazig/Turkey. They were maintained under a standard 12/12-h light/dark cycle (lights on 6 am and ending at 6 pm) at a constant temperature of 24 °C with 42 ± 5% of relative humidity. The rats were randomly distributed into 3 groups (7 rats/group) and housed in polycarbonate cages with wire lids and given the standard laboratory chow and water throughout the whole experiment. VPA sodium salt (purity >98%) and TQ (purity >98%) were purchased from Sigma-Aldrich.

Experimental design

Group I was the control group and was not treated with anything. Group II (VPA) received oral daily doses of the VPA (500 mg/kg/day) and group III (VPA + TQ) synchronous VPA (500 mg/kg/day) and TQ (50 mg/kg/day) for 14 days (21,22). After treatment with the VPA and TQ for 14 days mentioned above, all the rats were killed ethically. The weight of the rats in the groups was recorded before and after the study. Also after killing ethically, their brains were taken for gene expression and histological and immunohistochemical studies. All of the rats' brains were weighed and recorded. The experiments were performed according to the protocol approved by the Fırat University Animal Experiments Local Ethics Committee on laboratory animals, Elazığ, Turkey (FUDAM 2017/22-252).

RNA extraction

For RNA extraction, an approximate 30 mg brain tissue was taken from the brain and homogenized in a 1.5 ml-zirconiferous Eppendorf tube containing beads with a homogenizer (Bioprep-24, Allsheng). Then the total RNA's were extracted using an AccuZol™ Total RNA Extraction Solution (Bioneer, K-3090) according to the manufacturer's instructions, and quantified by measuring the absorbance at 260/230 nm and 260/280 nm using a NanoDrop spectrophotometer (Denovix DS-11). The purified RNA samples were stored at -80 °C until use.

Quantitative real-time PCR (qRT-PCR) Analysis

qRT-PCR was used to detect the expression level of RIPK1 and RIPK3. First, RNA was reverse transcribed into cDNA [AccuPower® RT PreMix (Bioneer K-2041)] and then qRT-PCR was performed as mentioned before by Tastemir-Korkmaz et al (23). The oligonucleotide sequences were for RIPK1 forward, 5'-AGGTACAGGAGTTTGGTATGGGC-3', and reverse, 5'-GGTGGTGCCAAGGAGATGTATG-3', for RIPK3 forward, 5'-TAGTTTATGAAATGCTGGACCGC-3', and reverse, 5'-GCCAA GGTGTCAGATGATGTCC-3' (24). The $^{2^{\wedge}-\Delta\Delta Ct}$ method was used to calculate the results.

Histological and Immunohistochemical Analysis

All brain tissues were enclosed in 10% neutral formaldehyde for histological and immunohistochemical studies. A routine histochemical procedure was applied to the tissues. Paraffin-wax embedded tissue blocks were produced and 4-5 µm sections were obtained by rotary microtome and stained with hematoxylin-eosin. The preparations obtained were photographed after evaluating with the camera-supported binocular light microscope (ECLIPSE Ni-U, Nikon, Tokyo, Japan). The structural changes examined in the brain tissue sections of the study groups were evaluated according to the scoring made by Refaiy et al (25). Also, tissues embedded in paraffin blocks were cut 4-5 micrometers thick and taken into lysine slides. The slides obtained were stained using caspase-3 (CAS-3) and NADPH oxidase-4 (NOX4) antibodies. Then, a semi-quantitative evaluation was performed under a camera-supported binocular light microscope (ECLIPSE Ni-U, Nikon, Tokyo, Japan).

Statistical Analysis

SPSS 17.0 statistics program was used to evaluate the data obtained from the experiments. For the comparison of body weight and fresh brain/body weight between all groups at the initial and final of the treatment were analyzed with the paired-samples T-test. Values are expressed as mean ± SEM. For the comparison of RIPK1 and RIPK3 expression levels between the groups, one-way analysis of the variance (ANOVA) followed by the LSD post hoc test were used. In histological and immunohistochemical studies, since the measurement values did not show homogeneous distribution, non-parametric tests were studied. Kruskal-Wallis Variance analysis test was used to evaluate the significance of the difference between the groups. Significant variance analysis results were questioned with Mann-Whitney U. The limit of significance was accepted as $p<0.05$.

RESULTS

Effects of VPA and TQ on body and fresh brain weight gain/loss

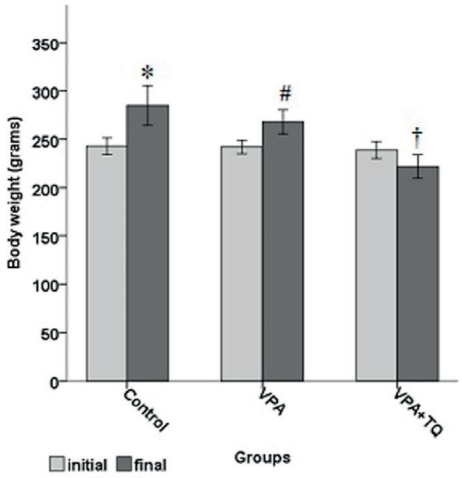
The body weight measurements showed that the body weight of the animals at the initial and final of the treatment were increased and this increase was statistically

significant in control and VPA groups (p=0.001 and p=0.004, respectively). On the contrary, a decrease was found in the body weight of the VPA + TQ group statistically (p=0.027) (Table 1, Figure 1). Additionally, the ratio of fresh brain weight/body weight differed between the VPA + TQ group and the others statistically (p≤0.05) (Table 2).

Table 1. Body weight (g) of animals during treatment

Design of Treatment	Control	VPA	VPA + TQ
Initial of Study	242.83 ± 2.98	242.00 ± 2.56	238.85 ± 3.17
Final of Study	284.83 ± 6.97	267.85 ± 4.77	222.00 ± 4.60
Statistical comparison (Initial of study vs final of study)			(p)
Control			0.001
VPA			0.004
VPA + TQ			0.027

Changes in the body weight of experimental rats. Values are expressed as mean ± SEM. The groups were compared with the paired-samples T-test at the beginning and end of the treatment. p<0.05. Abbreviations: VPA: valproic acid; TQ: thymoquinone; VPA: 500 mg/kg VPA; VPA + TQ: 500 mg/kg VPA + 50 mg/kg TQ



Values are expressed as mean ± SEM. The groups were compared with the paired-samples T-test at the beginning and end of the treatment. *, #, † In each column, different superscript letters mean significant differences at p<0.05. Abbreviations: VPA: valproic acid; TQ: thymoquinone; VPA: 500 mg/kg VPA; VPA + TQ: 500 mg/kg VPA + 50 mg/kg TQ

Figure 1. Changes in the body weight of experimental rats

Table 2. Comparison of fresh brain weight (g) and fresh brain weight/body weight ratio of the study population

	STUDY GROUPS		
	Control	VPA	VPA + TQ
Fresh brain weight (g)	1.8633 ± 0.0384	1.8386 ± 0.0216	1.8100 ± 0.0114
Fresh brain weight/body weight ratio	0.0065 ± 0.0001[c]	0.0068 ± 0.0001[c]	0.0078 ± 0.0003[a,b]

Each group represents the mean ± SEM for experimental rats. [a]: Significant from control; [b]: Significant from VPA; [c]: Significant from VPA+TQ. p<0.05. Abbreviations: VPA: valproic acid; TQ: thymoquinone; VPA: 500 mg/kg VPA; VPA+TQ: 500 mg/kg VPA+50 mg/kg TQ

RIPK1 and RIPK3 gene expression levels in rat brain

Table 3 shows the effects of the VPA and TQ treatments on the RIPK1 and RIPK3 gene expressions in all the study groups. The RIPK1 expression increased in the VPA group when compared with the control and this increase was found statistically significant (p=0.000). However, RIPK1 expression appears to decrease in the group given TQ with VPA (p=0.009), and this decrease was found significant when compared with the VPA group (Table 3). The RIPK3 expression increased in the VPA and VPA + TQ groups but didn't find significant from control (p>0.05) (Table 3).

Table 3. Comparison of RIPK1 and RIPK3 gene expression levels between the groups

	STUDY GROUPS		
	Control	VPA	VPA + TQ
RIPK1	14.522 ± 0.943	20.023 ± 0.926[a,b]	16.178 ± 0.693
RIPK3	14.698 ± 0.728	17.266 ± 1.261	17.130 ± 1.037

Each group represents the mean ± SEM for experimental rats. [a] Significant from control, [b] Significant from VPA + TQ. Abbreviations: VPA: valproic acid; TQ: thymoquinone; VPA: 500 mg/kg VPA; VPA + TQ: 500 mg/kg VPA + 50 mg/kg TQ

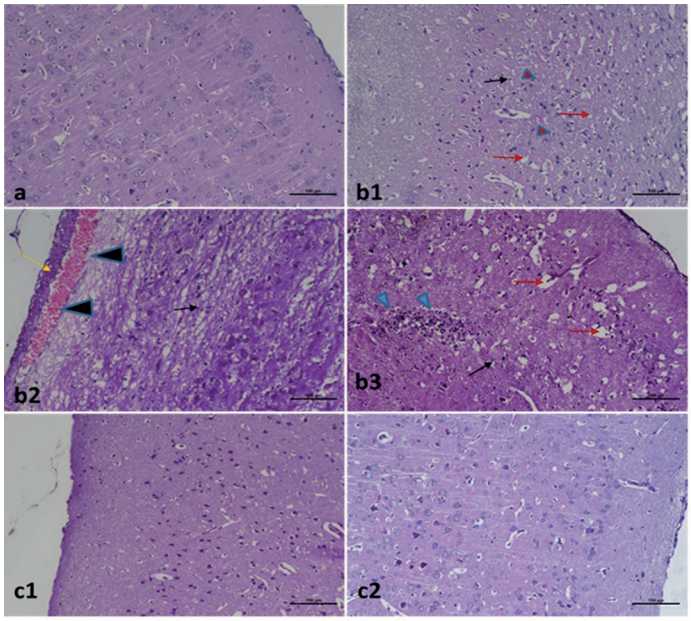
Histological results

The structural changes examined in the brain tissue sections of the control and experimental groups were evaluated according to the scoring made by Refaiy et al. (25) (Table 4). In the histological examination of the brain tissue sections of the control group, no findings other than normal histological structures belonging to this organ were found (Figure 2a). In the VPA group, significant histopathological changes were seen when compared to the control group (p≤0.05). These changes were determined as neuron degeneration, mononuclear cell infiltration, molecular layer degradation, decrease in the number of neurons, hemorrhagic areas, neuropil vacuolization, and piconotic nuclei in neurons (Figure 2: b1-b2-b3). In the VPA + TQ group, a significant improvement was observed in histopathological findings compared to the group with VPA (p≤0.05) (Figure 2: c1-c2).

Immunohistochemical analyses

In immunohistochemical staining, a significant difference was found between the control group and the experimental groups (VPA and VPA + TQ groups) in brain tissue sections (p<0.05). In samples marked with CAS-3 and NOX4, more positive markings were observed in the sections of VPA

and VPA + TQ groups compared to the control group brain tissue. Among the experimental groups; more positive markings were observed in the VPA group compared to VPA + TQ (Figure 3, Figure 4). Mean immunostaining scores of experimental groups were given in Table 5.



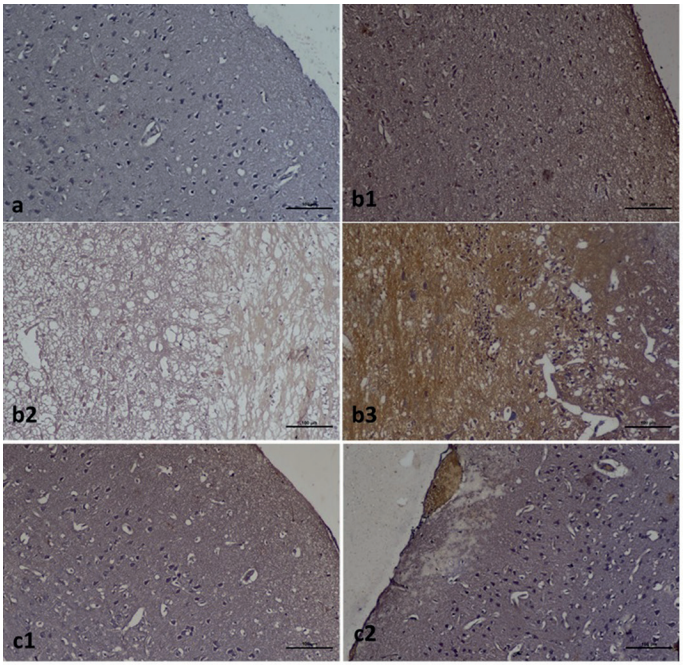
(a) Normal histological appearance was observed in the brain tissue sections of the control group (Group I). (b1-b2-b3) brain tissue sections of the VPA group (Group II): Red arrow; neuropil vacuolization, black arrowhead; vascular congestion, black arrow; picnotic nuclei, blue arrowhead in neurons, mononuclear cell infiltration, yellow arrow; molecular layer degradation, red arrowhead; shows neuron degeneration. (c1-c2) brain tissue sections of VPA + TQ group (Group III); Compared to Group II, histopathological findings decreased (H – E, a-b1- b2-b3-c1-c2; x20)

Figure 2. Results of the histopathological analysis

Table 4. Evaluation of structural changes detected in rat brain tissue

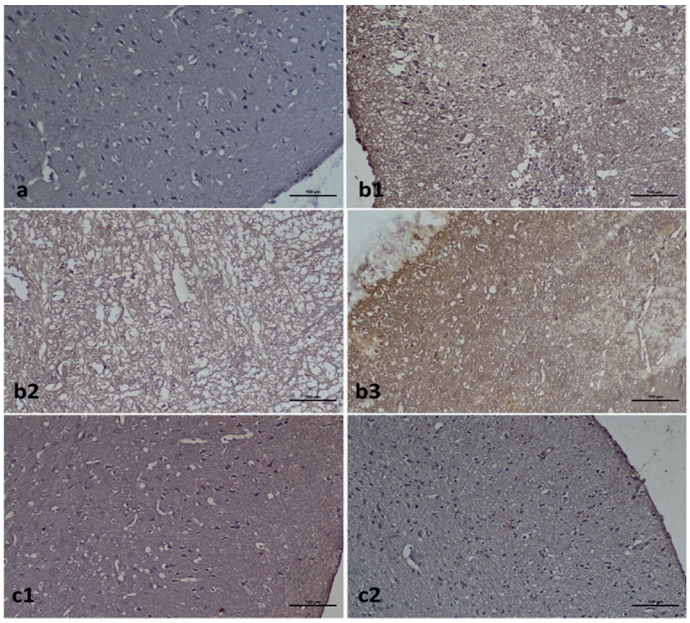
Parameters/scores	Control	VPA	VPA + TQ
Neuronal degeneration	–	+++[a]	++[b]
Molecular layer degradation	–	+++[a]	+[b]
Hemorrhagic areas	–	+++[a]	++[b]
Mononuclear cell infiltration	–	+++[a]	++[b]
Vacuolization of neuropropyl	–	+++[a]	++[b]
Picnotic nucleus in neurons	–	+++[a]	++[b]
Decrease in the number of neurons	–	+++[a]	+[b]

Each group represents the mean ± SEM for seven rats. Histological (structural) evaluation of the experimental parameters was scored. -, no damage; +, mild damage; ++, moderate damage; +++, severe damage. [a]VPA increased brain tissue oxidative damage vs. control, [b]TQ reduced brain tissue oxidative damage vs. VPA, Abbreviations: VPA: valproic acid; TQ: thymoquinone



(a) Control group (group I) brain tissue sections; CAS-3 was negatively stained and brown areas were not observed. (b1-b2-b3) Brain tissue sections of the VPA group (group II); a large number of brown areas were positively stained with CAS-3. (c1-c2) Brain tissue sections of VPA + TQ group (group III); a smaller amount of positive stained brown areas was observed with CAS-3 compared to VPA group (Immune staining, a-b1-b2-b3-c1-c2; x20)

Figure 3. CAS-3 marked brain tissue section belonging to the control group and experimental groups



(a) Brain tissue sections of the control group; brown areas that were negatively stained with NOX4 were not observed. (b1-b2-b3) Brain tissue sections of the VPA group; a fairly large amount of brown areas was observed, positively stained with NOX4. (c1-c2) Brain tissue sections to the VPA + TQ group; less positive stained brown areas were observed with NOX4 when compared with VPA group (Immunostaining, a-b1-b2-b3-c1-c2; x20)

Figure 4. Brain tissue section marked with NOX4 belonging to the control group and experimental groups

Table 5. Immunoreactivity scores for CAS-3 and NOX-4

Target stained immunohistochemically	Experimental groups		
	Control	VPA	VPA + TQ
CAS-3	+	+++[a]	+ / ++[b]
NOX-4	+	+++[a]	+ / ++[b]

Each group represents the mean ± SEM for seven rats. Immunoreactivity was scored using a semi-quantitative scale: -, no staining; +, weak staining; ++, moderate staining; +++, intense staining. [a]VPA increased brain tissue oxidative damage vs. control, [b]TQ reduced brain tissue oxidative damage vs. VPA. Abbreviations: VPA: valproic acid; TQ: thymoquinone

DISCUSSION

VPA is commonly used in epilepsy and mood disorders (19,26) and although considered a safe medication in the elderly, it causes side effects in various organs including the brain (10,19,27).

One of the side effects of VPA is weight gain. In the present study, both the control and VPA groups gained weight and the VPA + TQ group lost during the study. Among the side effects of VPA, weight gain is frequently reported, although the real incidence and size of this problem is unknown (28,29). Pseudo atrophy in the brain caused by VPA was reported by some researchers (30-32). Therefore, we compared the ratio of fresh brain/body weight between the groups. It was seen that in the VPA and VPA + TQ group, the fresh brain weight decreased when compared with the control group. However, the comparison of the ratio of fresh brain/body weight among groups showed no statistical differences, but an increase found in the VPA + TQ group compared to the other groups. This increase was probably due to the weight loss of the rats in this group.

It is known that VPA has been recognized as neurotoxic since the 1970s, but the underlying mechanisms are not well understood. A study has demonstrated that in neonatal rats treated with VPA for anticonvulsant action, common apoptotic neurodegeneration in some regions of the brain including the frontal cortex, thalamus, hippocampus, parietal cortex, etc. was found (33). Although some reports showed that VPA exacerbates the death of cerebellar granule neurons, reduces the proliferation of hippocampal neurons, and induces apoptosis in various types of neuronal cells (10,34), some reported it to reduce neuronal damage and find neuroprotective (35,36). Also, Bollino et al (10) showed that VPA causes neuronal cell death by a caspase-independent mechanism called calpain-dependent necroptosis. RIPK1 is a mediator of apoptosis, necroptosis, and inflammation, and involved in some signaling pathways such as interferon, interleukin (IL)1α, tumor necrosis factor (TNF), and Toll-like receptor (TLR). Several studies found that active RIPK1 complexed with RIPK3 can induce production of inflammatory cytokines or mixed lineage kinase domain-like pseudokinase (MLKL)-dependent necroptosis, or Fas-associated protein with death domain (FADD) resulting with activated caspase-8 and finally induce apoptosis following DNA damage or TLR signaling. A defect in the activities of RIPK1 has been associated with some diseases such as cancer, ischemic injuries, chronic and acute inflammatory diseases, autoimmune diseases, axonal degeneration, and neutrophilic dermatosis (37). In our study, it was observed that while the RIPK1 level increased significantly in the VPA group, the RIPK3 level did not. Increased RIPK1 level indicates that VPA leads the cells to apoptosis and causes brain cells' death.

It also confirms in histological data that VPA caused neuronal damage and apoptosis induced increasing CAS-3 immunoreactivity, which is an indicator of apoptosis. While no CAS-3 immunoreactivity was detected in the control group, it was observed to be less in the VPA + TQ group than in the VPA group, and it was also observed that TQ reduced damage and decreased apoptosis.

Previous studies showed that the major cause of oxidative stress is an accumulation of reactive oxygen species (ROS). NADPH oxidase (NOX), the major enzyme responsible for ROS generation, has seven family members (NOX1-NOX5, dual oxidase 1 (DUOX1), and DUOX2) demonstrated in various tissues of mammals. Especially, NOX2 and NOX4 have been found responsible for ROS production in brain tissue (38). Also, animal and human post-mortem studies have determined increased NOX2 and NOX4 levels in the injured brain showing the importance of these two NOXs in the pathogenesis of traumatic brain injury (TBI) (39,40). We found that the level of NOX4 in the VPA group increased significantly compared to the control and VPA + TQ groups. This result showed that VPA increased the NOX4 level and subsequently ROS level, and finally caused oxidative stress in the brain tissue. We verified that TQ decreased oxidative stress caused by VPA by inhibiting the level of NOX4 and prevent VPA induced brain damage.

CONCLUSION

VPA leads to apoptosis in brain tissue when used in higher doses and a long time, and mediated by increased RIPK1 expression and oxidative stress and consequently cause brain damage. TQ could be a candidate compound to reduce the damage of VPA in organs. However, much more studies are needed for TQ to be administered with VPA.

Competing Interests: The authors declare that they have no competing interest.

Financial Disclosure: There are no financial supports.

Ethical Approval: This study was approved by the Firat University Animal Experiments Local Ethics Committee on laboratory animals, Elazig, Turkey (FUDAM 2017/22-252).

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