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The musculoprotective effects of thymoquinone on ameliorating soleus muscle damage induced by valproic acid in rats

Sıçanlarda valproik asidin neden olduğu soleus kas hasarını iyileştirmede timokinonun koruyucu etkileri

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Abstract

Aim: To determine the potential musculoprotective effects of thymoquinone (TQ) on valproic acid (VPA)-induced muscle damage.

Materials and Methods: Twenty-one male Spraque-Dawley rats were randomly separated into 3 groups (n = 7): Control, VPA, VPA + TQ. Oral VPA (500 mg/kg/day) and TQ (50 mg/kg/day) were given to the rats for a period of 14 days. On the 15th day, soleus muscle tissues were taken for evaluating the expression levels of the Alpha-actinin-3 (ACTN3) and Myosin heavy chain 7 (MYH7) genes and histological analysis.

Results: The VPA + TQ group showed significantly higher ACTN3 and lower MYH7 gene expression, and decreased NADPH oxidase-4 (NOX4) and caspase-3 (CAS-3) levels than the VPA group. Also, histopathological changes were decreased in the VPA + TQ group in comparison with the VPA group.

Conclusion: VPA-induced soleus muscle damage was alleviated due to the antioxidant and antiapoptotic effects of TQ. TQ may be beneficial in treating soleus muscle damage caused by VPA.

Keywords: Thymoquinone; Valproic acid; Apoptosis; Toxicity; Soleus muscle damage.

Öz

Amaç: Çalışmada timokinonun (TQ) valproik asit (VPA) kaynaklı kas hasarı üzerindeki potansiyel koruyucu etkilerinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Yirmi bir erkek Spraque-Dawley sıçan rastgele 3 gruba ayrıldı (n = 7): Kontrol, VPA, VPA + TQ. Sıçanlara 14 gün süreyle oral VPA (500 mg/kg/gün) ve TQ (50 mg/kg/gün) verildi. Onbeşinci günde, Alfa-aktinin-3 (ACTN3) ve Miyozin ağır zincir 7 (MYH7) genlerinin ekspresyon düzeylerinin belirlenmesi ve histolojik analiz için soleus kas dokuları alındı.

Bulgular: VPA + TQ grubunda, VPA grubuna göre önemli ölçüde daha yüksek ACTN3 ve daha düşük MYH7 gen ekspresyonu ile düşük NADPH oksidaz-4 (NOX4) ve kaspaz-3 (CAS-3) seviyeleri gözlendi. Ayrıca VPA + TQ grubunda VPA grubuna göre histopatolojik değişikliklerin azaldığı da görüldü.

Sonuç: TQ'un antioksidan ve antiapoptotik etkileri nedeniyle VPA kaynaklı soleus kas hasarını hafiflettiği ve kas hasarını tedavi etmede faydalı olabileceği sonucuna varılmıştır.

Anahtar Sözcükler: Timokinon; Valproik asit; Apoptoz; Toksisite; Soleus kas hasarı.

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Introduction

For more than a century after its discovery, valproic acid (VPA) has been one of the most effective antiepileptic drugs (AEDs).[1] It is increasingly prescribed for the prophylaxis or the treatment of bipolar and schizoaffective disorders, social phobias, neuropathic pain, and migraine headaches.[2] VPA has a wide range of effects, including pre- and post-synaptic effects, facilitation of GABAergic on glutamatergic transmission, and regulation of ionic currents.[1] Although VPA is widely used, its therapeutic action mechanism is not fully known. However, the multisystemic side effects of VPA include hepatotoxicity, teratogenicity, hyperammonemic encephalopathy, mitochondrial toxicity, neurological toxicity, hypersensitivity syndrome reactions, and metabolic and endocrine adverse events.[3]

VPA, a histone deacetylase (HDAC) inhibitor, affects various critical events related to gene transcription, such as cell cycle control, differentiation, DNA repair, and apoptosis.[4] HDACs are enzymes removing acetyl groups from the amino acid lysine in histone or nonhistone proteins.[5] HDACs are highly important as targets for therapeutic intervention in the treatment of cancer, neurodegenerative disease, diabetes, and muscle degenerative diseases.[6,7] The level of acetylation of proteins in muscles is stabilized by histone acetyltransferases (HATs) and HDACs, and this balance is disrupted in muscle wasting.[8]

VPA causes the generation of reactive oxygen species (ROS), and ROS are byproducts of cellular metabolism produced in mitochondrial oxidation.[9,10] Overproduction of ROS can directly damage DNA, proteins, and lipids, or alter their signaling pathways by affecting transcription factors. Enzymatic and non-enzymatic antioxidant mechanisms are needed to inhibit ROS.[11] On the other hand, thymoquinone (TQ) is a free radical scavenger and a molecule that has various activities both biologically and medically, such as antitumor, anti-inflammatory, and antioxidant activities.[12]

Skeletal muscle fiber size, with the ability to significantly alter the amount of force, is influenced by a wide variety of physiological inputs such as nutrient levels, activity levels, cytokines, levels of various growth hormones, and other secreted factors.[13] Skeletal muscle which has a heterogeneous phenotype depending on its anatomical location and function in each organism is characterized by the ratio of fast and slow twitch fibers in a muscle.[14] Fibers of skeletal muscle vary in their metabolic, electrical, and contractile characteristics. There are four main types of fibers in limb muscles and the mammalian trunk, which are called I, IIA, IIX, and IIB, from the slowest to the fastest.[15] While Type I fibers are slow twitch fibers, and Type IIA IIX and IIB fibers are fast twitch fibers.[16] In plantaris and soleus, ACTN3 (Alpha-actinin-3) and MYH7 (Myosin heavy chain 7) are fast and slow specific genes, respectively. Slow-twitch skeletal muscles can withstand high energy expenditure, glucose uptake, stress, and fatigue.[17] Fast-twitch skeletal muscles represent explosiveness, strength, and high capacity for phosphate separation and lactate formation, but are more strenuous.[18] Many studies have shown that there are changes in skeletal muscle phenotypes and properties regarding many stimuli, for example, unloading, exercise, and gene mutation and that the stimuli do not entirely induce the transition from fast twitch to slow twitch in muscle fiber phenotype, and this occurs due to the epigenetic variations of the muscle types.[19]

Alpha (α)-actinins, located in the Z line of skeletal muscle and forming actin-actin cross-links, have two isoforms; ACTN2 and ACTN3.[20] ACTN3, expressed in type II muscle fibers, is a gene that encodes the alpha-actinin-3 protein.[21] ACTN3 is a special gene that affects muscle strength and fiber type distribution and changes muscle function by interacting with the signal protein calcineurin during growth.[22] ACTN3 expression is limited to fast glycolytic muscle fibers, and its deficiency reduces glycogen phosphorylase activity, resulting in a shift of energy use towards more oxidative pathways.[23] A muscle rich in slow-twitch fibers is characterized by higher blood flow, maximum oxygen uptake, and lower peripheral resistance during exercise and rest.[24] The MYH7 gene is expressed in

slow-twitch type I skeletal and cardiac muscle.[25] MYH7 is expressed by slow fibers encoding myosin heavy chain (MyHC-1), which are rich in mitochondria and have oxidative metabolism and fatigue resistance.[26] Studies have reported that VPA inhibits by interacting with N-acetyl glutamate synthase expressed in mitochondria.[27] Myosins are responsible for establishing the work-energy balance which is important for lifelong homeostasis and heart function. Morphological and metabolic abnormalities in myosins promote adverse clinical outcomes. In this case, it is thought that treatment with natural antioxidants such as TQ may limit adverse clinical outcomes, especially in patients with hypertrophic cardiomyopathy, by improving re-stabilization, contraction, and metabolic phenotypes.

TQ which is a fundamental component of the essential oil of *Nigella sativa* seeds has numerous pharmacological effects including anti-microbial, anti-tumor, anti-inflammatory, immunomodulatory, anti-histaminic, and antioxidant effects.[28]

Studies have shown that TQ has a therapeutic effect on preventing and treating diseases such as overgrowth and migration of smooth muscle cells, pulmonary arterial hypertension, and restenosis.[29] TQ has protective effects against muscle tissue damage caused by lower extremity ischemia-reperfusion due to oxidant damage mechanisms, radical scavenging, and antioxidant effects.[30] In this study, we aimed to examine the potential protective effect of TQ against muscle damage induced by VPA.

Materials and Methods

Chemicals

VPA (purity > 98 %) (Cas No: 99-6-1), TQ (purity > 98 %, St. Louis, MO) (Cas No: 490-91-5) and the other chemicals were bought from Sigma Aldrich.

Animal experiments

Twenty-one adult male Sprague-Dawley rats that weighed approximately 200-300 g were included. The rats were maintained on a 12:12-hour light-dark cycle at a controlled temperature of 21 °C and were allowed access to food and water freely. Approval was obtained from the Animal Experiments Local Ethics Committee by the Guidelines for the Care and Use of Laboratory Animals (Protocol No. #2021/10).

Experimental design

During treatment, the rats were separated into 3 groups of 7 animals each randomly as follows: control group (saline solution), VPA group (500 mg/kg), VPA + TQ group (500 mg/kg VPA and 50 mg/kg TQ). VPA (500 mg/kg) and TQ (50 mg/kg) doses were given by using gavage once a day for 14 days. In VPA + TQ group, TQ was given to the rats 30 min prior to the VPA treatment. The administered VPA and TQ doses were taken from the previous studies.[31,32,33]

At the end of the 14th day, the rats were euthanized with an intraperitoneal injection of ketamine and xylazine under anesthesia. Soleus muscle tissues were rapidly removed and washed with ice-cold saline and split into two parts; one fixed in 10% neutral formalin for histopathological assessment and the other preserved at -80 °C until the analysis of gene expression.

Real-time PCR (RT-PCR) analysis

Frozen muscle tissue (30 mg) was homogenized for 1 min in 500 µl of Tissue Lysis Buffer (Bioprep-24, Allsheng). Total RNA was extracted using the ExiPrepTM Tissue Total RNA isolation kit (Bioneer, K-3325) according to the manufacturer's instructions. The quality and the concentration of RNA were measured at an absorbance of 230-260 nm and 260/280 nm through a NanoDrop spectrophotometer (Denovix DS-11). Reverse transcription was transcribed into cDNA with the high-capacity AccuPower® RT PreMix (Bioneer, K-2041) as per the instructions of the manufacturer. SYBR green-based RT-PCR was performed using SYBR green master mix (Hibrigen MG-SYBR-01-400) to measure the mRNA expression levels of ACTN3 and MYH7 genes through the ExiCyclerTM96 Real-Time Quantitative PCR system (Bioneer). Thermal cycling conditions were as follows: 5 minutes at 95 °C, followed by 45 cycles of 15 seconds at 95 °C and 25 seconds at 60 °C. Primer sequences were used

for ACTN3 and MYH7 (Bioneer, S-1001) as given in Table 1. $2^{-\Delta\Delta Ct}$ method was used for the calculation of the expression levels of the genes. For normalization of the genes, GAPDH was used.

Table 1. Nucleotid sequences of primers.

Name	Sequence (5'-3')
ACTN3	**Forward** 5′-GGAATGGGATGATGGAACCTG-3′
	Reverse 5′-TGCTCTGAGGGACAGTGGAATC-3′
MYH7	**Forward** 5'-GCGGACATTGCCGAGTCCCAG-3'
	Reverse 5'-GCTCCAGGTCTCAGGGCTTCACA-3'
GAPDH	**Forward** 5'-CAACTCCCTCAAGATTGTCAGCAA-3'
	Reverse 5'-GGCATGGACTGTGGTCATGA-3'

Histochemical analysis

Muscle tissues collected from animals were fixed in a 10% neutral formaldehyde solution. Following the fixation, the tissue samples were washed. The washing process was respectively as follows: They were dehydrated by passing through a series of alcohol, cleared with xylol, and embedded in paraffin. From the obtained paraffin blocks, sections of 4-5 μm were taken. Muscle tissues of each group were stained with Hematoxylin-Eosin to make the histopathological evaluation. Histopathological findings were assessed under the topics as follows: Disorganization of muscle fibers (loss of striations, increase in the connective tissue between muscle fibers), findings related to necrosis in muscle fibers (irregular eosinophilia in fibers, loss of interfiber connections), findings related to inflammation (mononuclear cell infiltration). For the evaluation of the findings, a modified semi-quantitative scoring system was used [(-): no sign, (+): mild sign (++): moderate sign (+++): severe sign].[34] Imaging and photographs of the samples were obtained through a photomicroscope.

Immunohistochemical analysis

Caspase-3 (CAS-3) and NADPH oxidase-4 (NOX4) receptor activity were determined by immunohistochemical methods in sections taken from muscles. Sections of 4-5 μm which were obtained from paraffin blocks were taken on a lysine slide. Sections were deparaffinized by using xylol and heat. Afterward, sections were run through an alcohol series and washed with PBS. They were incubated with 3% hydrogen peroxide, a non-immune blocking solution, primary antibody, secondary antibody, and streptavidin peroxidase, respectively. DAB marking and nuclei staining with hematoxylin were performed. The immunoreactivity of histological preparations was evaluated according to the degree of staining with a modified semi-quantitative scale [(-): No staining, (+): Weak staining, (++): Moderate staining, (+++): Intense staining].[34] Sections taken were visualized with an imaging-assisted light microscope (ECLIPSE Ni-U, Nikon, Tokyo, Japan).

Statistical analysis

IBM SPSS Statistics V22.0 was used for the statistical analysis. The obtained data are presented as mean ± SD. The level of normality was assessed through the Shapiro-Wilk test. Significant differences were examined with one-way ANOVA after LSD for the data obtained in the inter- and intra-group comparisons of parametric values in genetic parameters. The Mann-Whitney U test was applied to calculate the statistical significance of the non-parametric values and histopathological scores. $p \leq 0.05$ values were considered statistically significant.

Results

Effect of VPA and TQ on expressions of ACTN3 and MYH7 genes

Figure 1 demonstrates the effects of TQ treatment against the VPA administration on the mRNA expression of ACTN3 and MYH7 genes level in each study and control group. ACTN3 gene expression was decreased significantly in the VPA group in comparison to the control group, while MYH7 gene expression was increased significantly ($p \leq 0.05$). When the VPA + TQ group was compared with the VPA group ($p \leq 0.01$) (Figure 1), ACTN3 gene expression was significantly increased, while MYH7 gene expression was significantly decreased.

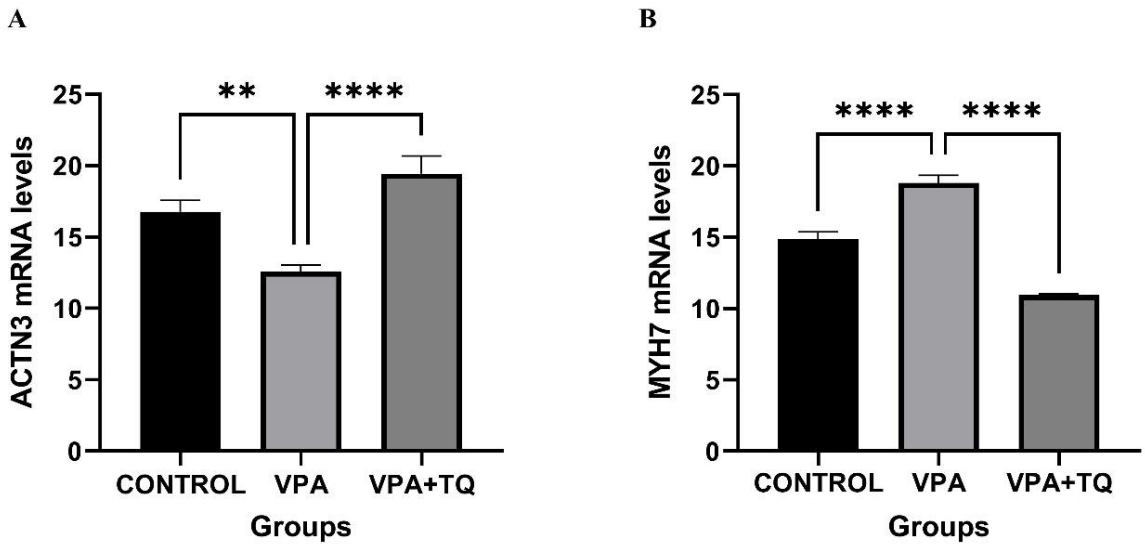

Figure 1. Effects of VPA and TQ on the expression of ACTN3 and MYH7 genes in rat muscle tissue. Each group represents the mean ± SD for seven rats. Abbreviations: VPA, valproic acid; TQ, thymoquinone; ACTN3, Alpha-actinin-3; MYH7, Myosin heavy chain 7. **** $p \leq 0.0001$ ** $p \leq 0.01$

Histochemical Findings

In the control group, normal muscle tissue histology was detected in both transverse and longitudinal sections (Figure 2 A,B). In muscle fibers in VPA groups; disorganization of muscle fibers, irregular eosinophilia in some muscle fibers and loss of interfiber connections were observed (Figure 2 C,D). While a decrease in these findings was observed in the VPA + TQ group, nuclear clusters were observed in certain areas within the muscle tissue, especially in longitudinal sections, which can be evaluated in favor of mononuclear cell infiltration (Figure 2 E, F). Obtained findings are displayed in Table 2.

Table 2. Histopathological scoring of muscle sections of experimental groups

Groups	Disorganization of muscle fibers	Findings associated with necrosis in muscle fibers	Inflammation-related findings
1 (CONTROL)	-	-	-
2 (VPA)	+++	+++	+++
3 (VPA + TQ)	-	+	++
The significance rate as a result of the comparison of the groups			
1 - 2	$p = 0.001$	$p = 0.001$	$p = 0.001$
1 - 3	$p = 0.002$	$p = 0.002$	$p = 0.002$
2 - 3	$p = 0.003$	$p = 0.002$	$p = 0.006$

$p \leq 0.05$ were considered statistically significant. The relationships between groups are assessed by Mann-Whitney U test.
(-), negative score: No structural changes
(+), 1 positive score: Light structural changes
(++), 2 positive score: Middle structural changes
(+++), 3 positive score: Serious structural changes

Immunohistochemical Findings

The findings of the evaluation are as in Table 3. In the control group, CAS-3 immunoreactivity in muscle tissue was not observed (Figure 3 A,B). Intense labeling with CAS-3 was detected in the VPA groups (Figure 3 C,D). A weaker labeling rate was detected in the VPA + TQ groups (Figure 3 E,F). In studies with NOX4, no labeling was detected in the control groups (Figure 4 A,B). While VPA groups were intensely marked with NOX4 (Figure 4 C,D), no obvious labeling was observed in VPA + TQ groups (Figure 4 E,F).

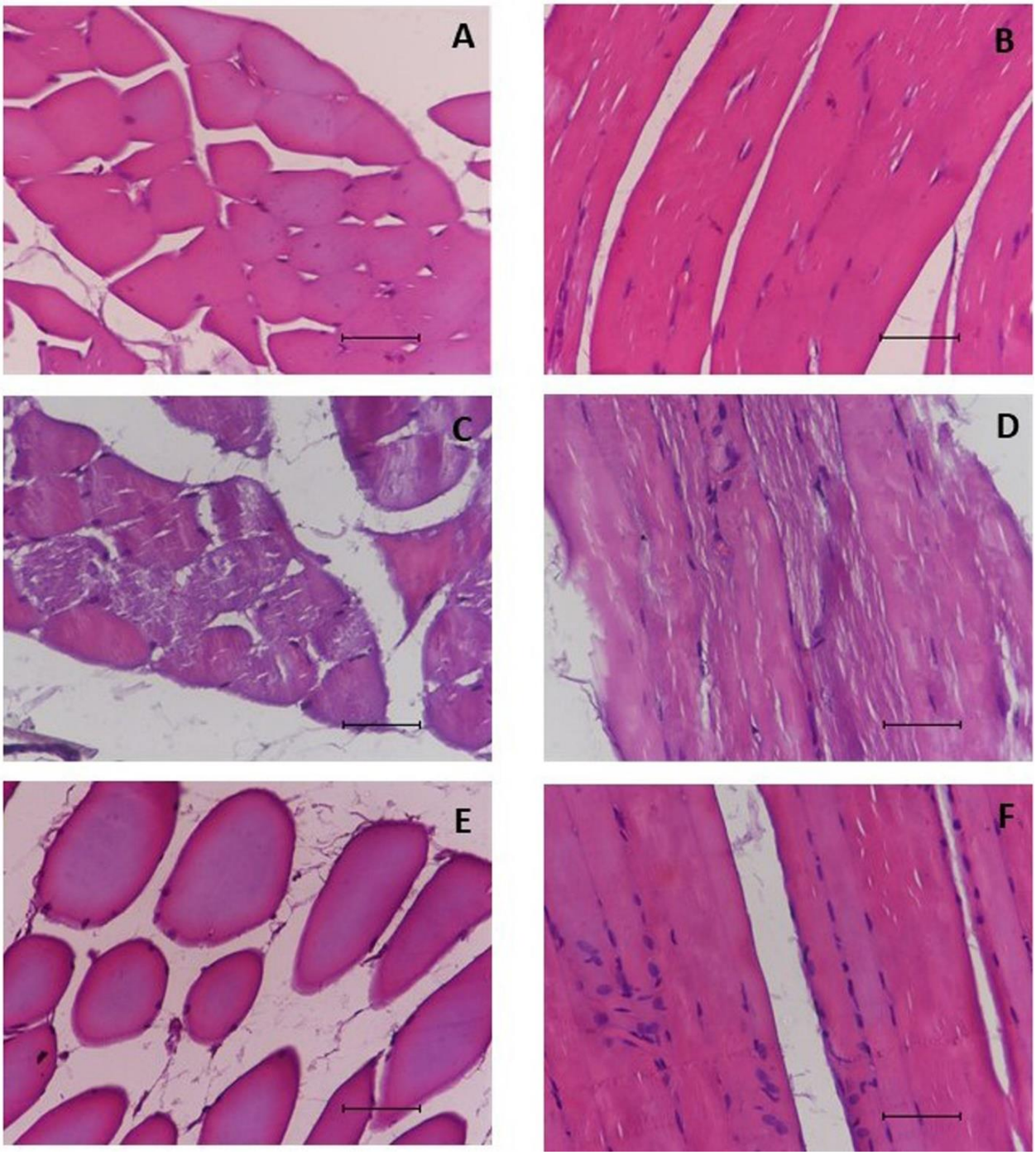

Figure 2. Rat muscle tissue section. **A)** Control group transverse section, **B)** Control group longitudinal section, **C)** VPA group transverse section, **D)** VPA group longitudinal section, **E)** VPA + TQ group transverse section, **F)** VPA + TQ group longitudinal section. H&E, scale bar 50 µm, x400. Abbreviations: VPA, valproic acid; TQ, thymoquinone

Table 3. Immunoreactivity scores for CAS-3 and NOX4

Groups	**CAS-3**	**NOX4**
1 (CONTROL)	**-**	**-**
2 (VPA)	**+++**	**+++**
3 (VPA + TQ)	**+**	**-**
The significance rate as a result of the comparison of the groups		
1 - 2	*p = 0.001*	*p = 0.001*
1 - 3	*p = 0.002*	*p = 0.102*
2 - 3	*p = 0.003*	*p = 0.003*

p≤0.05 were considered statistically significant. The relationships between are assessed by Mann-Whitney U test.
(-), negative score: No staining
(+), 1 positive score: Light staining
(++), 2 positive score: Middle staining
(+++), 3 positive score: Serious staining

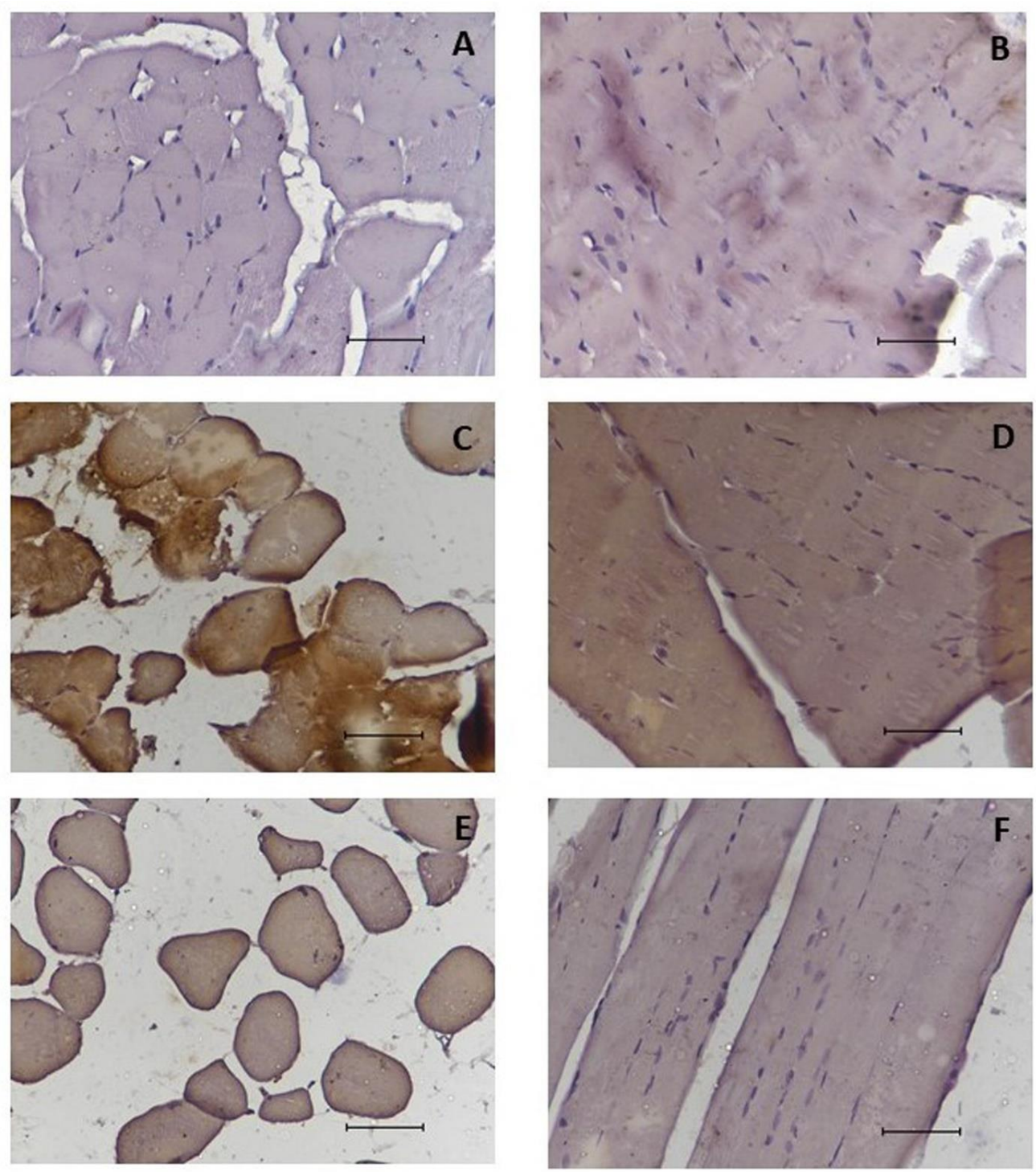

Figure 3. CAS-3 immunostaining. **A)** Control group transverse section, **B)** Control group longitudinal section, **C)** VPA group transverse section, **D)** VPA group longitudinal section, **E)** VPA + TQ group transverse section, **F)** VPA + TQ group longitudinal section. Scale bar 50 µm, x400. Abbreviations: VPA, valproic acid; TQ, thymoquinone; CAS-3, Caspase 3.

Discussion

VPA is an antiepileptic drug that is commonly used and highly effective in adults and children.[35] Although VPA is generally well tolerated, it has been reported to have significant side effects that may occur during treatment. Hyperammonemic encephalopathy, increased liver enzymes, leukopenia, thrombocytopenia, and pancreatitis are among the side effects of VPA.[36,37] Studies have shown that the mechanism of hyperammonemia in epileptic seizures includes muscle contractions as well as ammonia production through acidosis.[38]

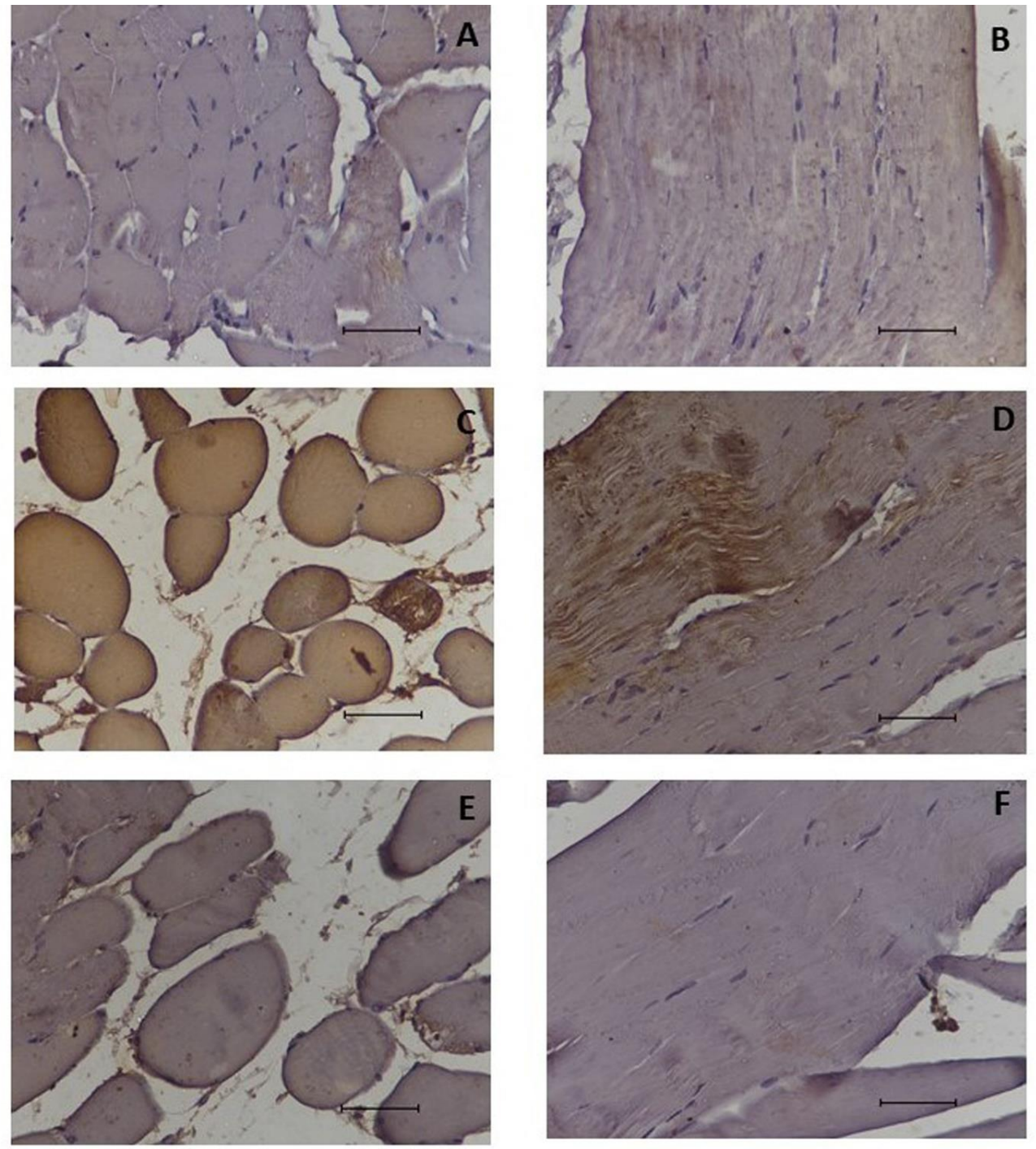

Figure 4. NOX4 immunostaining. **A)** Control group transverse section, **B)** Control group longitudinal section, **C)** VPA group transverse section, **D)** VPA group longitudinal section, **E)** VPA + TQ group transverse section, **F)** VPA + TQ group longitudinal section. Scale bar 50 μm, x400. Abbreviations: VPA, valproic acid; TQ, thymoquinone; NOX4, NADPH Oxidase-4.

In this study, ACTN3 and MYH7 mRNA were measured by qRT-PCR to determine whether VPA administration changes the expression of genes involved in fiber type and energy metabolism (glycolysis and mitochondrial respiration) in skeletal muscle. According to the findings, decreased muscle mass and fiber diameter, inflammation, necrosis, and irregularity in muscle fibers were observed with decreased ACTN3 and increased MHY7 expression and increased oxidative enzyme activity with VPA application.[39] Studies have shown that ACTN3 deficiency alters skeletal muscle metabolism by increasing fatty acid oxidation and glycogen storage.[40] Studies have reported that the absence of ACTN3 causes higher rates of muscle breakdown in long-term running.[41]

Some studies have shown that with the increase of MYH7 expression in the muscles of mice, atrophy, protein synthesis disorder, and transition from glycolytic to oxidative fibers were detected.[42] Specific genes in slow twitch muscle are moderately activated, while histone modification regulates the genes in both fast and slow twitch skeletal muscles.[19] In a study, it was found that while free carnitine levels decreased in serum, red blood cells, and muscles of rats given VPA for a period of 28 days, carnitine levels and carnitine to free carnitine ratio increased.[30]

VPA treatment increased the expression levels of high transcription genes, especially MYH7, by increasing H3 acetylation.[19] MYH7 (encoding the β-myosin heavy chain) is expressed during fetal stages and shows almost no expression after the maturation period.[43] Therefore, the reexpression of MYH7 is considered an important indicator of the pathological process of cardiomyocyte hypertrophy.[44] Chronic inflammation and oxidative stress cause cardiac hypertrophy due to obesity.[24]

Hereditary myosin myopathies result from mutations occurring in the skeletal muscle myosin heavy chain (MyHC) genes.[45] It has been reported that mutation in the MYH7 gene causes severe muscle weakness and skeletal deformity.[46] Loss of muscle activity in fast-twitch fibers causes muscle fiber atrophy.[19]

When the VPA + TQ group was compared with the VPA group ($p \leq 0.01$) (Figure 1), ACTN3 gene expression was significantly increased, while MYH7 gene expression was significantly decreased. Supporting the findings of this study, previous studies reported that mRNA expression of MYH7 was significantly reduced by carvacrol treatment in mice with T1DM.[47] In clinical studies, TQ, the main component of *N. sativa*, has been found to have antimicrobial, antioxidant, antidiabetic, anti-inflammatory, antitumor, therapeutic effects on metabolic syndrome, urinary, cardiovascular, neuronal, respiratory, gastrointestinal, and reproductive disorders.[48,49] Studies have shown that TQ inhibits vascular smooth muscle cell proliferation, ROS formation and induces apoptosis.[29]

As stated in the histological results of the study, VPA treatment caused muscle damage. According to the findings obtained, the findings related to an irregularity in muscle fibers and necrosis in muscle fibers increased. In the TQ groups, on the other hand, findings related to muscle fiber irregularity and muscle fiber necrosis were observed to be significantly reduced in comparison with the VPA group. TQ use was found to reduce the pathological changes within the VPA + TQ group. From the histopathological findings, it can be suggested that TQ protects the histological structure of the muscle against the damage induced by VPA. Studies have reported that intramuscular VPA administration in dogs causes a very high risk of inflammation and muscle damage, with mild myonecrosis at low concentrations and diffuse myonecrosis at higher concentrations, and increased tissue destruction after repeated doses.[50] Denervation-related effects may result from changes in the transcriptional program due to increased histone acetylation and muscle inactivation. It was observed that H3 acetylation increased significantly with the administration of VPA, an HDAC inhibitör.[19] Previously conducted studies have shown that HDAC inhibitors, including VPA, increase the differentiation of myoblasts through the regulation of myogenic regulatory factors (MRF).[51]

VPA-induced oxidative stress stimulates several signaling pathways, such as the activation of CAS-3 and it leads to apoptosis.[52,53] Excessive ROS generation inside the cell causes oxidative stress and damage occurs in macromolecules such as DNA, RNA, and proteins.[54-56] Treatment with VPA increases CAS-3 activity in muscle tissues and therefore induces apoptosis.[57] Increased immunoreactivity of CAS-3 in the group treated with VPA points to the apoptosis of muscle tissues. Moreover, the CAS-3 level in the muscle tissues of the VPA + TQ treated group was significantly lower than the muscle tissues of the VPA group. The findings show the ability of TQ to prevent and treat muscle damage induced by VPA, thanks to its antioxidant feature.

NOX4, a significant marker of apoptosis and oxidative stress in muscle tissue, plays a

role in the dysfunction of muscles.[58] Inhibition of NOX4 may be a potential substitution to treat VPA-induced muscle apoptosis. In this study, we found that NOX4 levels increased significantly in the VPA group in comparison with the VPA + TQ group. This suggests that ROS induced by VPA causes oxidative stress in muscle tissues. It was confirmed that TQ reduces oxidative stress induced by VPA through the inhibition of NOX4 level, in line with a previously conducted study.[59] Besides, therapy with TQ reduced ROS generation and reduced muscle apoptosis induced by VPA. It has been revealed in earlier studies that TQ led to the tension of tracheal smooth muscle decreasing.[60]

Conclusion

In the study, it was found that TQ blocked adverse effects on VPA-induced gene expressions, reduced apoptosis of muscle cells, suppressed oxidative stress, prevented histological changes, and protected rat muscle tissues from VPA-induced damage as a result. Consequently, TQ is considered a potential aid in the prevention and treatment of muscle damage when administered with VPA. According to the results, it can be suggested that TQ may be a promising candidate for treating muscle damage. However, further studies measured the protein levels of associated genes with ELISA and western blotting methods are needed to explore the clinical applications of TQ.

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Ethics Committee Approval

Approval was obtained from Animal Experiments Local Ethics by the Guidelines for the Care and Use of Laboratory Animals (Protocol No. #2021/10).

Authors' Contributions

All of the authors contributed at every stage of the study.

Conflict of Interests

No potential conflicts of interest were reported.

Financial Disclosure

This study hasn't received any financial support.

Statements

These research results have not been presented anywhere previously.

Peer-review

Externally peer-reviewed

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