European Review for Medical and Pharmacological Sciences 2016; 20: 1192-1202

Protective effects of thymoquinone and alpha-tocopherol on the sciatic nerve and femoral muscle due to lower limb ischemia-reperfusion injury

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Abstract. – OBJECTIVE: Thymoquinone (TQ) is an antioxidant and anti-apoptotic substance found in the *Nigella sativa* plant. Alpha-tocopherol (α-TP) is a potent antioxidant. We aimed to determine whether or not TQ and TP have a protective effect against lower limb ischemia-reperfusion (IR) injury of muscle and the sciatic nerve.

MATERIALS AND METHODS: A single dose of TQ 25 mg/kg was given intraperitoneally to the TQ group, a single dose of α-TP 200 mg/kg was given intraperitoneally to the α-TP group. IR was performed for 45 minutes after the drugs' applications.

RESULTS: While serum levels of malondialdehyde (MDA) and interleukin-6 (IL-6) of the IR group were significantly higher than those of the TQ plus α-TP, TQ and α-TP groups ($p<0.001$, $p<0.001$, $p=0.008$, respectively) and IL-6 (all $p<0.001$), the reduced glutathione (GSH) level of the IR group was lower than that of the other three groups. While neuronal nitric oxide synthase activity of nerve tissues of the IR group was significantly lower than that of the TQ plus α-TP group, the muscle tissue caspase-3 activity was higher than that of the TQ plus α-TP group.

CONCLUSIONS: Administration of TQ plus α-TP may strongly protect muscle and nerve tissues against IR injury via their synergistic effects.

Key Words:
Ischemia-reperfusion, Thymoquinone, Alpha-tocopherol, Vitamin E, Nigella sativa, Sciatic nerve, Muscle.

Introduction

Ischemia-reperfusion (IR) remains an important clinical problem. During the ischemic stage, tissue oxygenation is altered due to the impairment of tissue perfusion, leading to the formation of reactive oxygen radicals (ROS) and lipid peroxidation. An intensive increase in the production of cytokines, such as tumor necrosis factor alpha and interleukin (IL)-6, during reperfusion, increases the further formation of ROS, which results in apoptosis and cellular death[1,2]. Peripheral nerves, such as the sciatic nerve, are very sensitive to IR injury, and intensive ROS formation during IR injury is toxic to the sciatic nerve. The elevation of cytokine production, such as in IL-6 and ROS formation, especially during IR of a lower limb, leads to sciatic nerve injury as well as to many other tissues[3,4]. In addition to ROS formation during IR, the antioxidant defensive system becomes impaired. A low level of reduced glutathione (GSH) indicates a poor defensive system[4,5]. The level of the vasodilator substance, nitric oxide (NO), decreases during IR so that the increased vasoconstriction leads to impairment of blood flow of the nerve tissue[6,7]. Thus, the caspase system is activated, which leads to apoptosis and subsequent cellular and tissue death.

Nigella sativa (black cumin), a plant of the Mediterranean and West Asian regions, is widely used in traditional medicine. Thymoquinone (TQ) (2-isopropyl-5-methylbenzo-1, 4-quinone) is an important compound of the volatile oil in the seeds of this plant[8]. Many biological activities of TQ have recently been found to be valuable. It has been reported to have potent anti-inflammato-

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ry, antioxidant and protective effects from carcinogenesis[8-10]. TQ has been reported to exert its anti-inflammatory effects by inhibiting cyclooxygenase and lipoxygenase activities that lead to the reduction of leukotriene synthesis and suppression of proinflammatory cytokine production[9,10]. TQ also prevents reactive oxygen radical (ROS) formation by suppressing polymorph nuclear leukocyte (PMNL) functions[11]. It has been reported to have protective effects on tissues against IR injury in many tissue models such as in models of lower limb IR, abdominal aorta IR and renal, hepatic and muscle tissues[12-17]. Alpha-tocopherol (α-TP) is a substance known to have antioxidant, anti-inflammatory and anti-apoptotic effects[18]. It has been shown to have neuroprotective effects in cerebral IR models[19]. A synergistic effect has also been observed in studies that have used a TQ and α-TP combination[20-23].

In this study, we aimed to investigate whether or not TQ has a protective effect against IR injury of local muscle tissues and the sciatic nerve during IR of lower limb models and whether TQ has a synergistic effect with α-TP during IR injury.

Materials and Methods

Selection of Groups

Thirty S. Dawley male rats aged 6-7 months (180-230 g) were included in the study. Rats were divided into five groups randomly, with six rats per group. The rats were kept in a standard temperature and humidity environment, with a 12-hour light-dark cycle, and were fed standard food and water. The rules of the "Guide for the Care and Use of Laboratory Animals" (NIH, 1985) were followed during the entire experiment. The necessary approvals were obtained from the local Ethics Committee prior to the experiment.

Control group: Neither drug application nor IR were performed on the rats in this group.

IR group: Only IR was applied to this group; no drugs were given.

TQ group: A single dose of thymoquinone (catalogue No. 274666-5G; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) 25 mg/kg was applied intraperitoneally prior to 45 minutes of IR application[24]. A previous study had reported TQ to be adequate if administered 30 minutes prior to IR application[25].

α-TP group: A single dose of alpha-tocopherol acetate (Evigen; Aksu Farma Company, Istanbul, Turkey) at 200 mg/kg was applied intraperitoneally prior to 45 minutes of IR application[26].

TQ plus α-TP group: A single dose of thymoquinone (25 mg/kg) and a single dose of alpha-tocopherol acetate (200 mg/kg) were applied intraperitoneally prior to 45 minutes of IR application.

Experimental Procedures

With the exception of the control group, a dose of 50 mg/kg ketamine (Ketalar, Pfizer Limited, Sandwich, Kent, UK) and 10 mg/kg of xylazine (Rompun, Bayer Inc., Toronto, Ontario, Canada) were given intraperitoneally to all the other groups to induce anesthesia prior to the application of IR. A tight tourniquet was formed from a rubber material and connected to the proximal of the left thighs of the rats, without drug application, to induce ischemia in group two and to the other three groups after 45 minutes, with drug application[27,28]. Ischemia was accepted if there were cyanosis, deficits of pulses and absence of microcirculation of the left extremities of the rats. A mini-manual Doppler device (Datascope HNE vascular Doppler, Huntleigh Healthcare Inc., Eatontown, NJ, USA) was used to confirm the presence of ischemic injury when there was suspicious circulation. After 90 minutes of ischemia application, the tourniquet was removed, and the ischemia was terminated; reperfusion was maintained for 90 minutes. After the application of IR, all the rats were put into their cages, kept in the same environment and were fed the same food and water. After one week of IR application, all the groups, including the control group, were taken into the operating room. After maintaining anesthesia by giving 50 mg/kg of ketamine intraperitoneally to all the rats, their abdomens were opened, and blood samples were drawn from the aorta for biochemical analysis. After being sacrificed, the left thigh and tibia regions of the animals were opened, and muscle samples were taken from the distal of the quadriceps and gastrocnemius muscles. Muscle samples taken for biochemical analysis were kept in a phosphate buffer, and muscle samples taken for histopathologic investigation were kept in formaldehyde. The sciatic nerve was dissected, and nervous tissue was then taken from its distal part and kept in formaldehyde for histopathologic instigation. Serum samples taken to measure biochemical parameters were

centrifuged, followed by storage with the muscle samples at -80 C^0 until biochemical analysis was performed.

Tissue Homogenates

The samples were homogenized in PBS at pH 7.4 and centrifuged at 10,000 g for 20 minutes. Supernatants were taken, and aliquots were put into tubes. They were stored at -80°C. The parameters were studied within one month.

Protein Measurement

Tissue homogenate protein levels were measured using the Lowry et al protocol[29]. This method is based on two reactions: 1) the Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu^+, which reacts with the Folin reagent, and 2) the Folin-Ciocalteau reaction.

Serum and Tissue MDA Levels

MDA levels in the serum and muscle tissues of the animals were measured using the Draper and Hadley double heating method[30]. The basic principle of this method is to use a spectrophotometer to measure the color changes after using thiobarbituric acid (TBA) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) with MDA. The measured MDA level was given as μmol/L. Its level was re-calculated, according to the protein level of muscle tissues, and the final results were given as nmol/mg protein.

IL-6 Measurement

The IL-6 level was measured using the enzyme-linked immunosorbent assay (ELISA) method via an IL-6 ELISA rat kit (eBioscience, Vienna, Austria).

Absorbance was measured at a wavelength of 450 ηm using an ELISA reader. The intra-assay and inter-assay coefficient of variation were <5% and <10%, respectively. The sensitivity of the IL-6 assay was 12 pg/ml. The serum level of the IL-6 was given as pg/ml.

The IL-6 levels of muscle tissues were divided into protein levels, and the results for the levels of the muscle tissues were given as pg/ng protein.

Serum and Tissue GSH Measurements

The level of GSH in the serum and muscle tissue samples of the rats were measured using Ellman's method. Serum and tissue samples were homogenized and deproteinized[31]. The basic principle of this method is to use a spectrophotometer to measure the absorbance of the color complexes at 412 nm, which formed after the reaction of Ellman's color reagent (DTNB 40% w/v, in 1% sodium citrate) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) with a supernatant. The serum GSH levels were given as μmol/L, and the GSH levels of the muscles were given as μmol/mg protein.

Histopathologic Method

After being fixed in 10% neutral formaldehyde for 24 hours, muscle and nerve tissues were washed under running water for 8 hours and embedded in liquid paraffin. Tissues were cut into 3 μm thicknesses for immunohistochemical (IHC) staining and into 4 μm thicknesses for hematoxylin-eosin (H&E) staining. These sections were allowed to stay in xylene for 20 minutes before applying an alcohol series (50-100%). The sections were put into an H_2O_2 solution for 10 minutes. After being washed with a phosphate buffered solution (PBS), the sections were heated by 800W for 4-5 minutes in a citrate buffer solution. The sections were then put into a blocker substance for 20 minutes. The primary antibodies were diluted as follows: anti-caspase-3 (ab4051, Abcam Plc, Cambridge, UK) to 1/100 and applied for 75 minutes and anti-nNOS (ab1376, Abcam Plc, Cambridge, UK) to 1/300 and applied for 75 minutes. The slides were rinsed, and a biotinylated secondary antibody (Universal LSAB Kit-K0690, Dako, Denmark) was used for 30 minutes. The sections were then incubated with streptavidin–horseradish peroxidase (HRP) (Universal LSAB Kit-K0690, Dako, Denmark). A diaminobenzidine solution was used as a chromogen, and the sections were counterstained with Mayer's hematoxylin for 3–5 minutes. PBS was used as a negative control. The treated sections were then mounted with Entellan (Code107960, Merck, Darmstadt, Germany) and examined under a light microscope. Employing a blind evaluation protocol performed by a histologist and a pathologist, anti-caspase-3 and anti-nNOS immunoreactivities were divided into four categories according to the percentage of immunopositive reaction areas of the tissues: weak (+), mild (++), moderate (+++) and severe (++++). The blocked tissues were cut into 4μm-thick sections before being stained by H&E. The sections were examined under a light microscope (BX51; Olympus, Tokyo, Japan) and photographed at rele-

Table I. The levels of malondialdehyde, interleukin-6 and reduced glutathione in all groups.

	IR	α-TP	TQ	TQ plus α-TP	Control
Serum MDA (μmol/l)	0.44±0.07	0.33±0.3[b,E,f]	0.22±0.1[a]	0.21±0.02[a]	0.28±0.06[a]
Serum IL-6 (pg/ml)	79.7±17.9	48.7±16.9[a]	45.1±13.4a	36.5±6.4[a]	39.1±6.5[a]
Serum GSH (μmol/ml)	198.3±59.3	262.6±48.3	289.8±101.9	361.2±75.4[a]	407.6±95.3[a]
Muscle MDA (μmol/μg protein)	12.0±1.7	8.0±2.5[w]	7.6±1.5x	6.3±1.8[a]	5.6±2.8[a]
Muscle IL-6 (pg/ng protein)	203.7±41.5	157.7±30.1[¥,β,#]	121.9±23.0a	104.3±5.5[a]	102.5±31.9[a,¶]
Muscle GSH (μmol/ng protein)	38.7±10.1	62.0±6.1	62.9±16.3≠	77.3±18.4[w]	81.4±35.2[a]

Abbreviations: MDA, malondialdehyde; IL-6, interleukin-6; GSH, reduced glutathione; IR, ischemia-reperfusion; α-TP, alpha-tocopherol; TQ, thymoquinone.
[a]*p<0.001*, [x]*p=0.002*, [w]*p=0.003*, [b]*p=0.008* , [¥]*p=0.011*, [≠]*p=0.046 vs.* IR group. [E]*p=0.013*, [β]*p=0.043 vs.* TQ group. [#]*p=0.004*, [f]*p=0.006 vs.* TQ plus α-TP group. [¶]*p=0.003 vs.* α-TP group.

vant magnifications (X400) with a digital camera (DP72; Olympus, Tokyo, Japan).

Statistical Analysis

All statistical analysis were performed with SPSS (Statistical Package for the Social Sciences, version 18, Chicago, IL, USA). The results were given as mean±SD. The analysis of the biochemical parameters of both the serum and muscle tissue were analyzed using a One-Way ANOVA test with Bonferroni post hoc analysis. A Mann-Whitney U test was used for the analysis of histologic data. A *p* level of *<0.05* was considered to be significant.

Results

Biochemical Parameters

Serum MDA and IL-6 levels of the IR group were significantly higher than those of the other four groups (for MDA: control [*p<0.001*], TQ [*p<0.001*], α-TP [*p=0.008*] and TQ plus α-TP [*p<0.001*]; for IL-6: all *p<0.001*, respectively). The GSH level of the IR group was significantly lower than that of the control and TQ plus α-TP groups (*p<0.001, p<0.001,* respectively). The MDA and IL-6 muscle tissue levels of the IR group were significantly higher than those of the other four groups (For MDA: control [*p<0.001*], TQ [*p=0.002*], α-TP [*p=0.003*] and TQ plus α-TP [*p<0.001*]; for L-6: control [*p<0.001*], TQ [*p<0.001*], α-TP [*p=0.003*] and TQ plus α-TP [*p<0.001*], respectively). The IL-6 level of the α-TP group was observed to be higher than that of the TQ and TQ plus α-TP groups. The GSH level of the IR group was significantly lower than that of the control (*p<0.001*) and TQ plus α-TP groups (*p=0.003*). All biochemical results are given in Table I.

Histopathologic Parameters

The sciatic nerve of the IR group was observed to have mild lymphocytic infiltration in the epineurium, intensive axonal degeneration, intensive dilatations and edema. There was a moderate degeneration observed in the epineurium of the nerve samples of only two animals (20%).

The investigation revealed that, while there was vacuolization (*head of the arrow*) of the axons, vacuolization was increased in edematous regions, tissue integrity was impaired (75%) and a structure similar to partial degeneration was observed (Figure 1A).

Histopathologic investigation of the muscle tissues showed that ischemia had caused severe impairment of the integrity of fibrils, some regions to be stained intensively eosinophilic, cellular boundaries to have disappeared and myolysis structures to be formed. Cellular nuclei were observed to be swollen in the regions of fibrillar degeneration; due to these swollen nuclei, there were scattered vocalizations and deformations. While there was fibrosis of the perimysium around some muscle fibers (35%), the rate of fibrosis of endomysium in the interior regions was observed to be higher (75%) (Figure 2A). While there were dilatations surrounding the epineurium of the α-TP group, they were found to be lower than those of the IR group.

The dilatations within the nerve were observed to be low. However, the regions of axons close to these dilatations had mild degenerations (Figure 1B). The histopathologic investigation of the muscles of this group showed the muscles to be partially protected from ischemia by α-TP. Even though the lymphocytic infiltrations, especially in the regions with dilatations and edema, were not obvious, they were found to be present more often in the regions closest to the skin (Figure 2A).

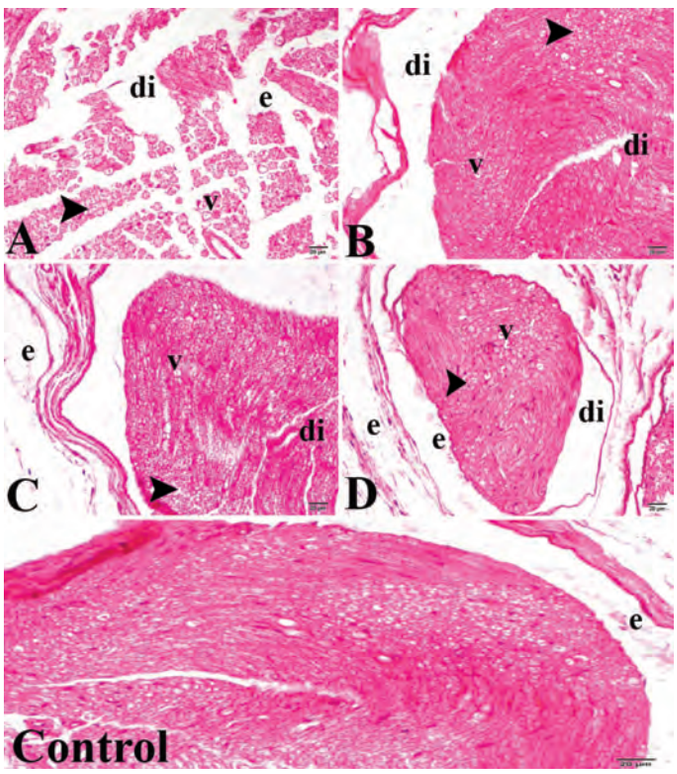

Figure 1. Histopathological examination of the sciatic nerve tissue and its branches, ***A,*** IR group; ***A,*** α-TP group, ***C,*** TQ group, ***D,*** TQ plus α-TP group, di: dilatation, e: edema, v: vacuolization, arrowhead; axon degeneration, H&E stain, (×400).

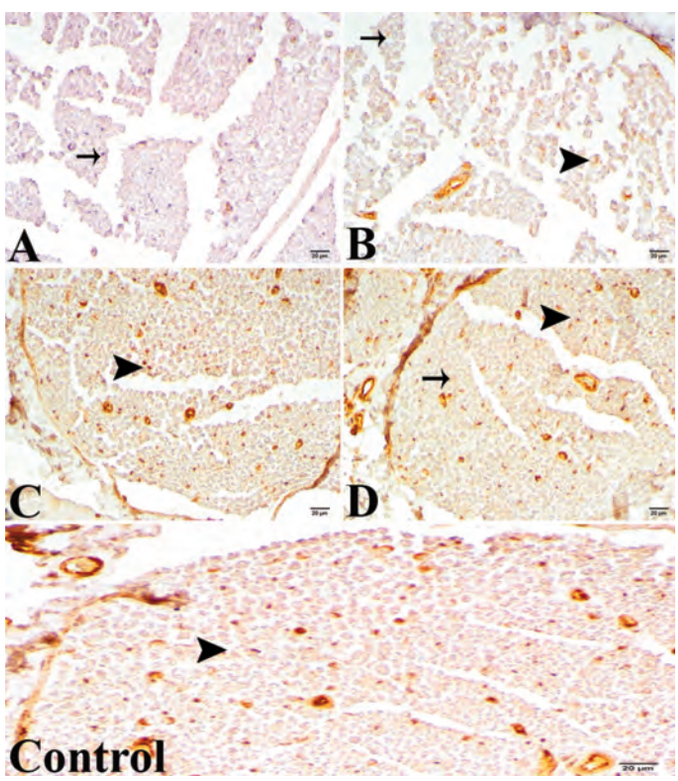

Figure 2. Histopathological examination of the femoral muscle tissue, ***A,*** IR group; ***B,*** α-TP group, ***C,*** TQ group, ***D,*** TQ plus α-TP group, di: dilatation, e: edema, v: vacuolization, arrowhead; axon degeneration, H&E stain, (×400).

Table II. The results of histopathological parameters in all groups.

	Muscle edema	Lymphocytes infiltration in muscle	Vacuolization in muscle	Axonal degeneration	Myelin degeneration	Dilatation of the nerve
IR	3.00±0.57	3.00±0.47	4.00±0.48	4.00±0.52	3.00±0.67	4.00±0.71
α-TP	1.00±0.71^{a}	2.00±0.70^{b}	3.00±0.32^{e}	3.00±0.52^{w}	2.00±0.48^{a}	2.50±0.70^{x}
TQ	1.00±0.70^{a}	2.00±0.71^{b}	1.00±0.71^{a,f}	2.50±0.70^{u}	2.00±0.00^{a}	3.00±0.00^{b}
TQ plus α-TP	1.00±0.44^{a,g}	1.00±0.50^{a,f,k}	1.00±0.73^{a,f}	2.00±0.71$^{a,¥}$	1.00±0.73$^{a,¥,\#}$	2.00±0.33^{a,k}
Control	0.33±0.51^{a,m}	0.50±0.54^{a,R}	0.33±0.51^{a,m}	1.00±0.52$^{a,f,m,\neq,¶}$	0.00±0.50$^{a,f,\neq,¶}$	0.00±0.40$^{a,f,\neq,¶}$

Abbreviations: IR, ischemia-reperfusion; α-TP, alpha-tocopherol; TQ, thymoquinone. $^{a}p<0.001$, $^{u}p=0.002$, $^{w}p=0.005$, $^{e}p=0.008$, $^{x}p=0.014$, $^{b}p=0.024$ *vs.* IR group. $^{f}p<0.001$, $^{¥}p=0.002$, $^{g}p=0.040$ *vs.* α-TP group. $^{¶}p<0.001$, $^{k}p=0.002$, $^{\#}p=0.003$ *vs.* TQ group. $^{\neq}p<0.001$, $^{m}p=0.003$, $^{R}p=0.006$ *vs.* TQ plus α-TP group.

Even though the histopathologic findings of the TQ and α-TP groups were similar, the integrity of nerve tissues of the TQ group was preserved; the degenerations of axon and myelin were lower than those in the IR group (Figure 1C).

The investigation of muscle tissue of the TQ group revealed mild edema and degenerations of myositis. However, the integrity of the tissue was found to be unimpaired.

The investigation of the TQ plus α-TP group showed the nerve tissues to be similar to those of the control group, the dilatations' regions to be similar to the other application groups and the edematous tissues to be increased, starting from the perineural regions (Figure 1D).

Additionally, the lymphocytic infiltration of the muscles was found to be lower, and the regions with edema and dilatations were similar to the other groups (Figure 2D).

All the histopathologic evaluation results are given in Table II.

Immunohistochemistry Stain

The nNOS activity of the sciatic nerve tissues of the IR group was significantly lower than that of the control, TQ and TQ plus α-TP groups

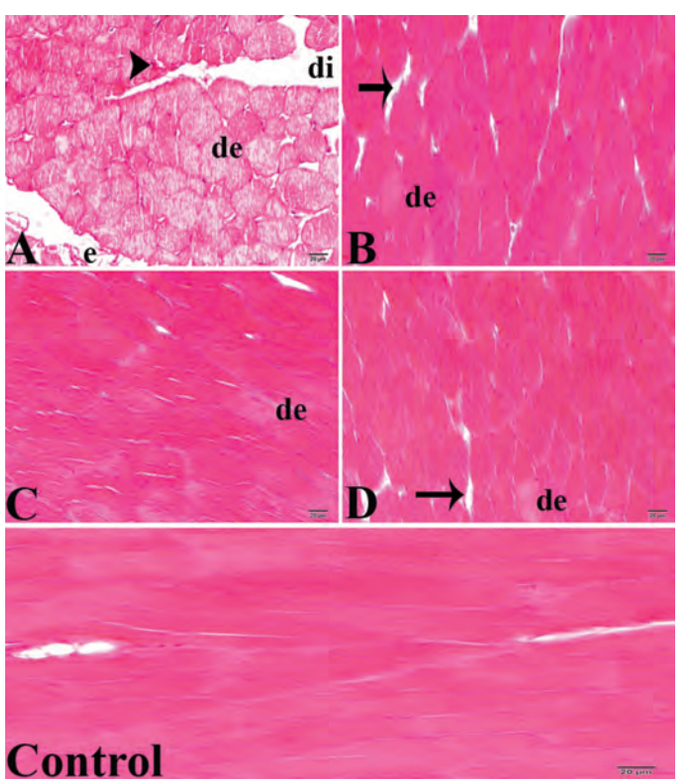

Figure 3. Histopathological examination of the sciatic nerve tissue and its branches by immunohistochemical methods, A: IR group; B; α-TP group, C: TQ group, D: TQ plus α-TP group, arrowhead; dense immunoreactivity, arrow; mild immunoreactivity, Anti-nNOS stain, (×400).

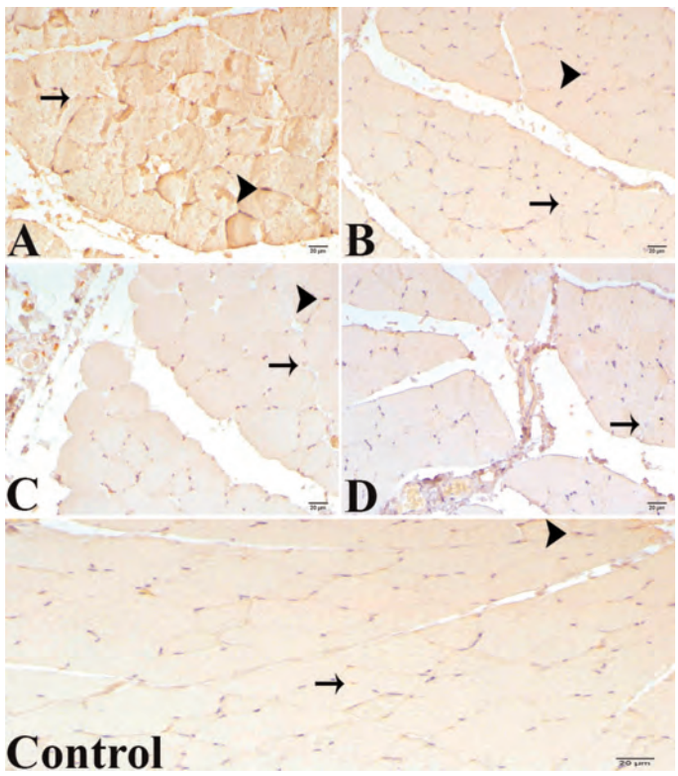

Figure 4. Histopathological examination of the femoral muscle tissue by immunohistochemical methods, ***A,*** IR group; ***B,*** α-TP group, ***C,*** TQ group, D: TQ plus α-TP group, arrowhead; dense immunoreactivity, arrow; mild immunoreactivity, Anti-Caspase-3 stain, (×400).

(Control [$p<0.001$], TQ [$p<0.001$], α-TP [$p=0.007$], TQ plus α-TP [$p<0.001$], respectively). The nNOS activity of the TQ plus α-TP group was significantly higher than that of both the α-TP and TQ groups (Figure 3). Caspase-3 activity of the muscle tissues of the IR group was significantly higher than in the other four groups (Control [$p<0.001$], TQ [$p=0.001$], α-TP [$p=0.002$], TQ plus α-TP [$p<0.001$], respectively). Caspase-3 of the TQ plus α-TP group was significantly lower than that of the α-TP and TQ groups (Figure 4). All immunohistochemical results are given in Table III.

Table III. The results of immunohistochemistry stain in muscle and sciatic nerve.

	Anti-nNOS antibody in sciatic nerve	Anti-Caspase-3 antibody in muscle
IR	1.00±0.67	3.00±0.67
α-TP	2.00±0.67^{a}	2.00±0.57^{b}
TQ	2.50±0.53^{E,g}	2.00±0.67^{f}
TQ plus α-TP	3.00±0.50^{E,k,u}	1.00±0.33$^{E,m,\#}$
Control	4.00±0.50^{E,R,x,w}	0.00±0.40$^{E,R,x,¥}$

Abbreviations: IR, ischemia-reperfusion; α-TP, alpha-tocopherol; TQ, thymoquinone. $^{E}p<0.001$, $^{f}p=0.001$ $^{a}p=0.007$, $^{b}p=0.002$ vs. IR group. $^{R}p<0.001$, $^{m}p=0.001$, $^{k}p=0.006$, $^{g}p=0.013$ vs. α-TP group. $^{x}p<0.001$, $^{\#}p=0.003$, $^{u}p=0.030$ vs. TQ group. $^{w}p=0.002$, $^{¥}p=0.016$ vs. TQ plus α-TP group.

Discussion

In this study, we found that TQ protected the sciatic nerve and muscle tissues against IR injury more prominently in the TQ plus α-TP applied group. This is the first study to show that TQ and TQ plus α-TP protect the sciatic nerve and local muscle tissues against IR injury. A previous study had shown that TQ prevents muscle tissue damage during IR of a lower limb by decreasing lipid peroxidation in local muscle tissues and increasing antioxidant capacity[16]. However, that study was very different from ours. We evaluated muscle and nerve tissues immunohistochemically and found TQ to prevent IR injury by decreasing caspase-3 activity in muscle tissues and increasing nNOS in nerve tissues.

Peripheral nerve damage, especially sciatic nerve injury, is still an important problem for clinicians. Lipids, the major component of myelin fibers, allow nerves to become easily in-

jured due to impaired oxygenation of the tissues during the ischemic stage of IR, which then leads to excess ROS formation and lipid peroxidation[32]. Nucleotide oxidation and protein carbonylation, due to excessive ROS formation, are responsible for nerve injury. The production of proinflammatory cytokines increases during the reperfusion stage, leading to further increases in ROS formation[33]. Elevated ROS and cytokine levels directly activate the caspase enzyme pathway, which induces apoptosis and leads to cellular death[34]. TQ, which is one of the active compounds of *Nigella sativa*, has been reported to be an antioxidant, immune regulator and to have an antineoplastic effect. TQ is also known to decrease ROS formation, to have a protective effect in the antioxidant defense system and to prevent lipid peroxidation[8-10]. Experimental IR models have shown TQ to have renal, ovarian and testicular tissue protective and neuroprotective effects by significantly decreasing ROS lipid peroxidation and increasing the antioxidant enzyme system such as in glutathione peroxidase[12,13,16,35,36]. Moreover, in a study of systemic IR injury performed by clamping the abdominal aorta, TQ was reported to have a protective effect on many tissues by increasing the total antioxidant capacity and significantly decreasing ROS formation[14]. Glutathione is an important reductive and antioxidant agent of the organism; it maintains oxidative-reduction balance in the cell and thus prevents the cell from endogenous and exogenous harmful substances[4,5]. Elevated superoxide and hydrogen radicals increase ROS formation by lowering the GSH level. Normally, GSH is oxidized to glutathione by glutathione reductase. Thus, a decrease in GSH indirectly indicates low glutathione peroxidase activity[37]. TQ has been reported to have a tissue injury lowering effect by preventing a decrease in the level of GSH and decreasing lipid peroxidation and oxidative stress during renal and gastric IR injury[15,38]. In our study, while serum and muscle tissue levels of MDA – a good marker for lipid peroxidation – of the IR group were higher than in the other groups, the GSH levels of the IR group were lower than in the other groups. ROS formation was found to be suppressed, and the antioxidant system was found to be preserved in the TQ plus α-TP group. This effect in both the TQ and α-TP groups was found to be lower than in the TQ plus α-TP group.

IL-6, a major cytokine with a pleiotropic effect, maintains cytokine balance. Excessive production of IL-6 stimulates the production of other proinflammatory cytokines[39]. Excessive cytokine production increases ROS formation and simultaneously stimulates a direct caspase pathway, which leads to apoptosis and cellular death[40]. TQ is known to prevent immune damage by decreasing the levels of many proinflammatory cytokines such as IL-6[41,42]. In this study, the IL-6 levels in serum and muscle tissues of the IR group were significantly higher than what was found in the other groups. While the IL-6 level of the TQ group was lower than that of the α-TP group, its level of TQ plus α-TP was similar to that of the control group. The decrease in the nNOS level of nervous tissue during IR leads to low production of NO[43]. NO is essential for nerve regeneration and cell survival[44]. Low NO activity of vasa nervorum in nerve tissues leads to nutritional supplement impairment via vasoconstriction, which is why nNOS has potent neuroprotective activity[45]. The production of NO may increase during IR injury, and excessive release of NO leads to neural injury by the formation of peroxynitrite radicals[46]. Both TQ and α-TP regulate NOS activity during IR and prevent IR injury by regulating both the increase and decrease in the NOS level[38,47,48]. In this study, while lipid peroxidation and ROS of the sciatic nerve of IR were significantly increased, nNOS activities of the TQ and α-TP groups were significantly higher than those of the control group. The nNOS activity of the TQ plus α-TP group was higher than that of the TQ and α-TP groups. The nNOS activity of the TQ and plus α-TP groups was significantly higher than that of the IR group. However, histopathologic investigation of the sciatic nerve tissue showed that it had prevented any IR injury of nerve tissues of the TQ and plus α-TP groups to a greater extent than that of the IR group. Both TQ and α-TP may increase nNOS activity and NO formation, which may protect the organism against increased ROS. Thus, they may show a neuroprotective effect by maintaining the NO-related vasodilatation in the vasa nervorum of the sciatic nerve. The application of both TQ and α-TP together may lead to a more powerful neuroprotective effect due to the synergistic effect of nNOS. TQ and α-TP were reported to decrease caspase-3 activity and apoptosis[9,49]. The hepatic IR model has shown TQ to decrease hepatic tissue injury by decreasing ROS formation and the activities of caspase 3, 8 and 9[50]. In our study, we found caspase-3 activity and its histologic damage of muscle tissues to be sig-

nificantly high in the IR group. Caspase-3 activity and histopathologic injury were significantly low in the TQ and α-TP groups. The anti-apoptotic effect of the TQ plus α-TP group was found to be higher.

Conclusions

TQ and α-TP are substances with potent anti-inflammatory, antioxidant and anti-apoptotic effects. Both TQ and α-TP may have similar protective effects in muscle and nerve tissues against IR injury during IR of a lower limb. The application of TQ and α-TP in combination may further protect muscle and nerve tissues against IR injury by their synergistic effect.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Cure E, Cumhur Cure M, Tumkaya L, Kalkan Y, Aydin I, Kirbas A, Yilmaz A, Yuce S, Yücel AF. Adalimumab ameliorates abdominal aorta cross clamping which induced liver injury in rats. Biomed Res Int 2014; 2014: 907915.
2) Cure MC, Cure E, Tumkaya L, Kalkan Y, Aydin I, Kirbas A, Efe H, Sehitoglu I, Yuce S. Topiramate has protective effect on renal injury. Bratisl Lek Listy 2015; 116: 259-263.
3) Iida H, Schmeichel AM, Wang Y, Schmelzer JD, Low PA. Orchestration of the inflammatory response in ischemia-reperfusion injury. J Peripher Nerv Syst 2007; 12: 131-138.
4) Ozyigit F, Kucuk A, Akcer S, Tosun M, Kocak EF, Kocak C, Kocak A, Metineren H, Genc O. Different dose-dependent effects of ebselen in sciatic nerve ischemia-reperfusion injury in rats. Bosn J Basic Med Sci 2015; 15: 36-43.
5) Valek L, Kanngiesser M, Häussler A, Agarwal N, Lillig CH, Tegeder I. Redoxins in peripheral neurons after sciatic nerve injury. Free Radic Biol Med 2015; 89: 581-592.
6) Lin H, Hou C, Chen D. Altered expression of inducible nitric oxide synthase after sciatic nerve injury in rat. Cell Biochem Biophys 2011; 61: 261-265.
7) Toda N, Okamura T. The pharmacology of nitric oxide in the peripheral nervous system of blood vessels. Pharmacol Rev 2003; 55: 271-324.
8) Boudiaf K, Hurtado-Nedelec M, Belambri SA, Marie JC, Derradji Y, Benboubetra M, El-Benna J, Dang PM. Thymoquinone strongly inhibits fMLF-induced neutrophil functions and exhibits anti-inflammatory properties in vivo. Biochem Pharmacol 2016 epub ahead of print doi: 10.1016/j.bcp.2016.01.006.
9) Sener U, Uygur R, Aktas C, Uygur E, Erboga M, Balkas G, Caglar V, Kumral B, Gurel A, Erdogan H. Protective effects of thymoquinone against apoptosis and oxidative stress by arsenic in rat kidney. Ren Fail 2016; 38: 117-123.
10) Vaillancourt F, Silva P, Shi Q, Fahmi H, Fernandes JC, Benderdour M. Elucidation of molecular mechanisms underlying the protective effects of thymoquinone against rheumatoid arthritis. J Cell Biochem 2011; 112: 107-117.
11) Mollazadeh H, Hosseinzadeh H. The protective effect of Nigella sativa against liver injury: a review. Iran J Basic Med Sci 2014; 17: 958-966.
12) Silachev DN, Plotnikov EY, Zorova LD, Pevzner IB, Sumbatyan NV, Korshunova GA, Gulyaev MV, Pirogov YA, Skulachev VP, Zorov DB. Neuroprotective effects of mitochondria-targeted plastoquinone and thymoquinone in a rat model of brain ischemia/reperfusion injury. Molecules 2015; 20: 14487-14503.
13) Mete Ural Ü, Bayo lu Tekin Y, ehito lu , Kalkan Y, Cumhur Cüre M. Biochemical, histopathological and immunohistochemical evaluation of the protective and therapeutic effects of thymoquinone against ischemia and ischemia/reperfusion injury in the rat ovary. Gynecol Obstet Invest 2016; 81: 47-53.
14) Aydin MS, Kocarslan A, Kocarslan S, Kucuk A, Eser I, Sezen H, Buyukfirat E, Hazar A. Thymoquinone protects end organs from abdominal aorta ischemia/reperfusion injury in a rat model. Rev Bras Cir Cardiovasc 2015; 30: 77-83.
15) Farag MM, Ahmed GO, Shehata RR, Kazem AH. Thymoquinone improves the kidney and liver changes induced by chronic cyclosporine a treatment and acute renal ischaemia/reperfusion in rats. J Pharm Pharmacol 2015; 67: 731-739.
16) Hosseinzadeh H, Taiari S, Nassiri-Asl M. Effect of thymoquinone, a constituent of Nigella sativa L., on ischemia-reperfusion in rat skeletal muscle. Naunyn Schmiedebergs Arch Pharmacol 2012; 385: 503-508.
17) Abd El-Ghany RM, Sharaf NM, Kassem LA, Mahran LG, Heikal OA. Thymoquinone triggers anti-apoptotic signaling targeting death ligand and apoptotic regulators in a model of hepatic ischemia reperfusion injury. Drug Discov Ther 2009; 3: 296-306.
18) Sharma AK, Ponery AS, Lawrence PA, Ahmed I, Bastaki SM, Dhanasekaran S, Sheen RS, Adeghate E. Effect of alpha-tocopherol supplementation on the ultrastructural abnormalities of peripheral nerves

in experimental diabetes. J Peripher Nerv Syst 2001; 6: 33-39.

19) Varshosaz J, Taymouri S, Pardakhty A, Asadi-Shekaari M, Babaee A. Niosomes of ascorbic acid and α-tocopherol in the cerebral ischemia-reperfusion model in male rats. Biomed Res Int 2014; 2014: 816103.

20) Al-Johar D, Shinwari N, Arif J, Al-Sanea N, Jabbar AA, El-Sayed R, Mashhour A, Billedo G, El-Doush I, Al-Saleh I. Role of Nigella sativa and a number of its antioxidant constituents towards azoxymethane-induced genotoxic effects and colon cancer in rats. Phytother Res 2008; 22: 1311-1323.

21) Sultan MT, Butt MS, Karim R, Iqbal SZ, Ahmad S, Zia-Ul-Haq M, Aliberti L, Ahmad AN, De Feo V. Effect of Nigella sativa fixed and essential oils on antioxidant status, hepatic enzymes, and immunity in streptozotocin induced diabetes mellitus. BMC Complement Altern Med 2014; 14: 193.

22) Soleimani H, Ranjbar A, Baeeri M, Mohammadirad A, Khorasani R, Yasa N, Abdollahi M. Rat Plasma Oxidation Status After Nigella Sativa L. Botanical Treatment in CCL(4)-Treated Rats. Toxicol Mech Methods 2008; 18: 725-731.

23) Gündüz H, Dede S, Agaoglu ZT, Atasoy N, Mert N. Serum trace elements status of rabbits supplemented with Nigella sativa, vitamins C and E, and selenium against damage by N-methyl-N'-nitro-N-nitrosoguanidine. Biol Trace Elem Res 2002; 89: 65-73.

24) Randhawa MA, Alghamdi MS, Maulik SK. The effect of thymoquinone, an active component of Nigella sativa, on isoproterenol induced myocardial injury. Pak J Pharm Sci 2013; 26: 1215-1219.

25) Tas U, Ayan M, Sogut E, Kuloglu T, Uysal M, Tanriverdi HI, Senel U, Ozyurt B, Sarsilmaz M. Protective effects of thymoquinone and melatonin on intestinal ischemia-reperfusion injury. Saudi J Gastroenterol 2015; 21: 284-289.

26) Hsu CC, Wang JJ. L-ascorbic acid and alpha-tocopherol attenuates liver ischemia-reperfusion induced of cardiac function impairment. Transplant Proc 2012; 44: 933-936.

27) Sharma JP, Salhotra R. Tourniquets in orthopedic surgery. Indian J Orthop 2012; 46: 377-383.

28) Duehrkop C, Rieben R. Refinement of tourniquet-induced peripheral ischemia/reperfusion injury in rats: comparison of 2 h vs 24 h reperfusion. Lab Anim 2014; 48: 143-154.

29) Lowry, OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.

30) Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 1990; 186: 421-431.

31) Owens CW, Belcher RY. A colorimetric micromethod for the determination of glutathione. Biochem J 1965; 94: 705-711.

32) Dong S, Cao Y, Li H, Tian J, Yi C, Sang W. Impact of ischemic preconditioning on ischemia-reperfusion injury of the rat sciatic nerve. Int J Clin Exp Med 2015; 8: 16245-16251.

33) Turkoglu E, Serbes G, Dolgun H, Oztuna S, Bagdatoglu OT, Yilmaz N, Bagdatoglu C, Sekerci Z. Effects of α-MSH on ischemia/reperfusion injury in the rat sciatic nerve. Surg Neurol Int 2012; 3: 74.

34) Wang Y, Schmeichel AM, Iida H, Schmelzer JD, Low PA. Ischemia-reperfusion injury causes oxidative stress and apoptosis of Schwann cell in acute and chronic experimental diabetic neuropathy. Antioxid Redox Signal 2005; 7: 1513-1520.

35) Erboga M, Kanter M, Aktas C, Sener U, Fidanol Erboga Z, Bozdemir Donmez Y, Gurel A. Thymoquinone ameliorates cadmium-induced nephrotoxicity, apoptosis, and oxidative stress in rats is based on its anti-apoptotic and anti-oxidant properties. Biol Trace Elem Res 2016; 170: 165-172.

36) Awad AS, Kamel R, Sherief MA. Effect of thymoquinone on hepatorenal dysfunction and alteration of CYP3A1 and spermidine/spermine N-1-acetyl-transferase gene expression induced by renal ischaemia-reperfusion in rats. J Pharm Pharmacol 2011; 63: 1037-1042.

37) Picklo MJ, Long EK, Vomhof-DeKrey EE. Glutathionyl systems and metabolic dysfunction in obesity. Nutr Rev 2015; 73: 858-868.

38) Magdy MA, Hanan el-A, Nabila el-M. Thymoquinone: Novel gastroprotective mechanisms. Eur J Pharmacol 2012; 697: 126-131.

39) Simpson RJ, Hammacher A, Smith DK, Matthews JM, Ward LD. Interleukin-6: structure-function relationships. Protein Sci 1997; 6: 929-955.

40) Sehitoglu I, Tumkaya L, Bedir R, Kalkan Y, Cure MC, Yucel AF, Zorba OU, Yuce S, Cure E. Zoledronic acid aggravates kidney damage during ischemia reperfusion injury in rat. J Environ Pathol Toxicol Oncol 2015; 34: 53-61.

41) Samarghandian S, Azimi-Nezhad M, Mehrad-Majd H, Mirhafez SR. Thymoquinone ameliorates acute renal failure in gentamicin-treated adult male rats. Pharmacology 2015; 96: 112-7.

42) Alkharfy KM, Ahmad A, Khan RM, Al-Shagha WM. Pharmacokinetic plasma behaviors of intravenous and oral bioavailability of thymoquinone in a rabbit model. Eur J Drug Metab Pharmacokinet 2015; 40: 319-323.

43) Qi WN, Yan ZQ, Whang PG, Zhou Q, Chen LE, Seaber AV, Stamler JS, Urbaniak JR. Gene and protein expressions of nitric oxide synthases in ischemia-reperfused peripheral nerve of the rat. Am J Physiol Cell Physiol 2001; 281: 849-856.

44) Keilhoff G, Fansa H, Wolf G. Nitric oxide synthase, an essential factor in peripheral nerve regeneration. Cell Mol Biol (Noisy-le-grand) 2003; 49: 885-897.

45) Ii M, Nishimura H, Kusano KF, Qin G, Yoon YS, Wecker A, Asahara T, Losordo DW. Neuronal nitric oxide synthase mediates statin-induced restoration of vasa nervorum and reversal of diabetic neuropathy. Circulation 2005; 112: 93-102.

46) Stavniichuk R, Shevalye H, Lupachyk S, Obrosov A, Groves JT, Obrosova IG, Yorek MA. Peroxynitrite and protein nitration in the pathogenesis of diabetic peripheral neuropathy. Diabetes Metab Res Rev 2014; 30: 669-678.

47) Aycan Ö, Tokgöz O, Tüfek A, Alabalik U, Evliyao lu O, Turgut H, Çelik F, Güzel A. The use of thymoquinone in nephrotoxicity related to acetaminophen. Int J Surg 2015; 13: 33-37.

48) Rifaioglu MM, Nacar A, Yuksel R, Yonden Z, Karcioglu M, Zorba OU, Davarci I, Sefil NK. Antioxidative and anti-inflammatory effect of thymoquinone in an acute Pseudomonas prostatitis rat model. Urol Int 2013; 91: 474-481.

49) Fouad AA, Jresat I. Thymoquinone therapy abrogates toxic effect of cadmium on rat testes. Andrologia 2015; 47: 417-426.

50) Abd El-Ghany RM, Sharaf NM, Kassem LA, Mahran LG, Heikal OA. Thymoquinone triggers anti-apoptotic signaling targeting death ligand and apoptotic regulators in a model of hepatic ischemia reperfusion injury. Drug Discov Ther 2009; 3: 296-306.