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# Thymoquinone Can Improve Neuronal Survival and Promote Neurogenesis in Rat Hippocampal Neurons

*Merve Beker, Tuğçe Dallı, and Birsen Elibol**

**Scope: Thymoquinone (TQ) has been used as a potential therapeutic for diseases such as cancer and diabetes. Herein, we aim to investigate the effect of TQ on behavioral and molecular parameters in healthy rat hippocampus.**
**Methods: TQ (20 mg $kg^{-1}$ $d^{-1}$) is administered intragastrically for 15 days to adult rats. After behavioral tests, the hippocampal tissues are investigated at the histological and molecular levels.**
**Results: In both dentate gyrus and cornu ammonis 1, TQ significantly increases the number of hippocampal neurons. This increase is supported by a significant increase in the doublecortin expression on both gene and protein levels. In addition, TQ significantly decreases the amount of Caspase-3 expression and the cleavage of poly ADP ribose polymerase, indicating a decrease in apoptosis. Further, ERK, GSK-3, JNK, CREB, and iNOS proteins are found to be positively regulated by TQ. However, the gene expression of *synapsin, synaptophysin, NGF, AKT, Bax, NFkB*, and *p53* and the protein expression of BDNF and nNOS are not affected by TQ.**
**Conclusion: These findings suggest that TQ has an enhancing effect on cell survival and neurogenesis in healthy hippocampus, rather inducing apoptosis in damaged neurons. This may proceed via ERK/JNK and CREB signaling pathways as a candidate acting mechanism for TQ.**

## 1. Introduction

Thymoquinone (TQ), an aromatic hydrocarbon is the active compound of *Nigella Sativa*.[1] Previous studies have shown anti-inflammatory, immunomodulatory, and neuroprotective effects of TQ in different models of neurodegeneration and neurotoxicity such as ischemia, depression, temporal lobe epilepsy, diabetes-mediated neuropathy, and toluene-, lead-, or ethanol-induced neurotoxicity.[2–9] Also, TQ has been evaluated as a potential therapeutic agent on cultured neurons.[10–12] Due to its antiproliferative, chemo-preventive, cell-cycle regulatory, and apoptosis-inducing effects, TQ may be a potential nutraceutical for cancer therapy.[9,13–15] It is thought that TQ's recovery effect on the neural tissue occurs via promotion of neurogenesis and nerve-regeneration, in addition to prevention of neuronal degeneration due to its antioxidant activities.[9,16,17]

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TQ has been shown to have a pro-apoptotic and antisurvival activity in many studies.[18–21] Intriguingly, TQ induces DNA damage and apoptosis specifically in cancer cells rather than healthy controls.[19,20] Previously, it was observed that TQ-induced cell death is oxidative stress-mediated and caspase-dependent by an increase in generation of reactive oxygen species (ROS).[21] On the other hand, TQ is a potent antioxidant molecule used as a ROS scavenger for different models of oxidative stress.[13,16,22–25] Superoxide, hydroxyl, and singlet oxygen radicals are found as the molecules that are scavenged by TQ consumption.[2] Furthermore, the increase in brain malondialdehyde (MDA) level in pathological conditions was also inhibited by TQ.[24,26,27] In addition, there is a healing effect of TQ by the increase in the concentration of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in neuronal protection during the conditions that result in oxidative damage to brain such as epileptic seizures.[2,28] The downregulation of the protein levels of cyclooxygenase-2 (COX-2) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) in the brain due to TQ treatment may be accepted as an indication of anti-inflammatory effect of this molecule.[2,4] The effect of TQ on the hippocampus gained more attention on the basis of its enhancing activity on the learning and memory according to studies related amelioration of cognitive decline by preventing oxidative stress in diabetes and restoring oxidative balance and cholinesterase activity in A$\beta$ accumulation to exhibit its neuroprotective effects.[10,29–32]

It was noted that thymoquinone showed its anticarcinogenic effect by decreasing oxidative stress and inhibiting to cell proliferation in vivo cancer models when it was administrated orally at 10 or 20 mg $kg^{-1}$ body weight of rat.[33,34] As a therapeutic agent in neurodegenerative diseases, dosage and administration way of TQ can show varieties among different studies. Similar to cancer studies, oral administration of 10 or 20 mg $kg^{-1}$ of TQ was preferred in the studies related to neuropathological conditions.[5,7] Abukhader et al. investigated the toxic dosage of TQ in different routes of delivery and found out 22.5 mg $kg^{-1}$ in male rats, 15 m /$kg^{-1}$ in female rats can be tolerated when administered intraperitoneally. In case of oral uptake due to elimination in digestive tract, the border of the dosage was determined

 

as 250 mg $kg^{-1}$ in both sexes.[35] Furthermore, the bioavailability of TQ in blood was previously calculated as ≈58% with a lag time of ≈23 min when it was administered by intragastric intubation at a dose of 20 mg $kg^{-1}$.[36] In addition, TQ has been reported to possess potent lipophilicity and, thus, it can cross the blood brain barrier.[15,29,37] In case of exogenous administration under normal conditions, there is limited information about the effect of TQ. Therefore, in this study, we aimed to reveal pure effect of TQ treatment on functional and molecular alterations of healthy hippocampal tissue by intragastric intubation, a more natural way of administration to mimic human use.

## 2. Experimental Section

### 2.1. Animals

Adult (24-week-old) female *Spraque Dawley* rats ($n = 10$) obtained from Bezmialem Vakif University animal laboratory were used in this study. All experimental procedures were approved by the Committee for Animal Research Ethics in Bezmialem Vakif University (2015/229). Animals were maintained under standard conditions with 12-h light/dark cycles, 22 °C, and 60% humidity with ad libitum food and water.

### 2.2. TQ Administration

Rats were randomly divided into two groups; control (C) ($n = 5$) and thymoquinone (TQ) ($n = 5$). TQ (Sigma–Aldrich, Darmstadt, Germany) was dissolved in corn oil as a final concentration of 20 mg $mL^{-1}$ (w/v). All animals were treated with either TQ (20 mg $kg^{-1}$ $d^{-1}$) or corn oil according to their weights by intragastric gavage for 15 successive days.

### 2.3. Behavioral Tests

#### *2.3.1. Morris Water Maze*

Animals were run into Morris water maze (MWM) test at the 16th day of treatment for evaluating the hippocampus-dependent spatial learning and memory. MWM was a circular tank with 210 cm in diameter and 51 cm in height. It was filled to the depth of 45 cm with water maintained at 23 °C (±1) by an automatic heater. A nontoxic paint was used to make the water opaque. Computerized video tracking system (EthovisionXT11, Noldus Information Technology, The Netherlands) was used to track the animal in the pool and to record data. The pool was virtually divided into four quadrants. A movable platform (11 × 11 cm) was located in the center of one of the quadrants. The top of the platform was 2 cm below the surface of the water such that the animal could not see it but could easily climb on it to escape from the water. Experimental room was furnished with several extra-maze cues immobile throughout the entire experimental period. During place learning, rats were given four daily trials, for five consecutive days. Each rat was released into the water facing the pool wall at one of the four starting points, which were used in a pseudorandom order such that each start position was used only once during the daily experimental session. The trial was finished when the animal found the platform or after 60 s. Later, the rat was returned to its cage for a 5-min inter-trial interval. The video-tracking system was automatically recording the swim trajectory, swim velocity, escape latency, and the swim distance to reach the invisible platform. On the completion of place learning, to assess the strength of the acquired place preference, platform has been removed from the pool and a 60 s probe trial was carried out. On the probe trial, the percentage time spent by the animal in the platform quadrant was recorded.[38]

### 2.4. Passive Avoidance Test

One day after MWM probe trial, passive avoidance task was performed. The apparatus consists of two compartments (white-illuminated and black-closed) separated by a guillotine door. Briefly, the test consists of two sessions. In "the acquisition session", each animal was placed in the white-illuminated compartment for 20 s exploration. Then, the guillotine door was automatically opened and the animal was allowed to enter the black-closed compartment. When the animal crossed the dark compartment, a scrambled foot shock (1 mA) was delivered for 2 s and the rat was returned to its home cage. On the following day (24 h later), the "retention session" was performed by placing the rats again to the white-illuminated compartment. After 20 s exploration, the door opened and the step-through latency time to enter the black-closed compartment was measured. In this test session, maximum cut-off time was 150 s.[39]

### 2.5. Histological Analysis

On the completion of behavioral tests, rats were decapitated and total brains were removed for histological analysis and hippocampi were dissected for molecular analysis. For histological staining, brains were frozen on dry ice prior to keeping in –80 °C. Tissue sections were taken from the level of hippocampus with the thickness of 20 $\mu$m by a cryostat. Tissues were stained with Nissl Staining for analyzing viable neurons.[35] Cresyl-violet stained cells were counted from five frames which had area of 70 000 $\mu m^2$ at the cornu ammonis 1 (CA1), CA3, and dentate gyrus (DG).

### 2.6. Molecular Analysis

#### *2.6.1. Enzyme-Linked Immunosorbent Assay (ELISA)*

The dissected hippocampus samples were homogenized with lysis buffer in a mechanical homogenizator. The concentration of Acetylcolinesterase (AChE) was determined according to the instructions for the corresponding ELISA kit (USCN- SEB447Ra, Houston). Briefly, 10 $\mu$L of cell lysate were added to the 96-well plate of kit and allowed to incubation for 2 h at 37 °C. Then, 10 $\mu$L of Detection Reagent A and B was added, and incubated additional 1 h and 30 min, respectively at 37 °C. After

addition of substrate solution and stop solution, the absorbance was recorded at 450 nm for AChE amount (ng $mL^{-1}$).

### 2.7. Quantitative Real-Time PCR Analysis

Right hippocampal tissues were homogenized for RNA extraction. Total RNA was isolated with TRİzol and PureLink RNA mini kit. Briefly, supernatants of homogenized tissue were incubated with 200 $\mu$L of cloroform for 3 min at room temperature. After centrifugation at 12 000 $\times$ *g* for 15 min at 4 C°, transparent part of the supernatant was mixed with equal amounts of 70% ethanol. Then washing steps with special columns were applied and isolated RNA was collected to eppendorf tubes on ice. After determination of the amount of isolated RNA by Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham), cDNA reverse-transcribed and stored in this form. Reaction mixture including SybrGreen (Bioline, Luckenwalde, Germany), DNA polymerase, dinucleotide, and buffer solution was mixed with the template and reactions were performed in appropriate thermal cycle with CFX96 Touch Real-Time PCR (Bio-Rad Laboratories, California).

In our study, we studied genes related neuronal survival and growth (*synaptophysin*, *synapsin*, *doublecortin* (*DCX*), *Nerve Growth Factor* (*NGF*), *protein kinase B* (*AKT*), *Bax*, *Nuclear Factor-κB* (*NFκB*), and *p53*) with housekeeping *β-actin* gene. Primer pairs against these genes were purchased from Sentromer Technology (Istanbul, Turkey). Gene cards were analyzed using the threshold cycle (CT) relative quantification method. CT values were normalized for endogenous reference ($\Delta$CT = CT [GAPDH] − CT [target gene]) and compared with control using the $\Delta\Delta$CT formula ($\Delta\Delta$CT = $\Delta$CT [TQ group] − $\Delta$CT [control]). Data were analyzed using logarithmic transformation of fold induction ratios according to relative quantification (RF) formula ($2^{-\Delta\Delta Ct}$).

### 2.8. Western Blot Analysis

Left hippocampus was dissected, complemented with RIPA lysis buffer, homogenized, and centrifuged at 4 °C for 15 min. The collected supernatants were used for SDS-PAGE. Equal amounts of protein which were determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham) by Multiskan GO Microplate Spectrophotometer were diluted in sample buffer, boiled, and loaded onto 4–20% Bis-Tris polyacrylamide gels and transferred into polyvinyl diflouride membranes. Membranes were incubated in blocking solution (5% milk powder in 0.1% Tween 20/0.1 M Tris-buffered saline), and immersed with primary antibodies; phosphorylated/total AKT, phosphorylated/total ERK, phosphorylated/total c-Jun N-terminal Kinase (JNK), phosphorylated/total Glycogen Synthase Kinase-3 $\alpha/\beta$ (GSK3$\alpha/\beta$), Bcl-2, Poly ADP Ribose Polymerase (PARP) (Cell Signaling, Danvers, USA), DCX (Thermo Fisher Scientific, Paisley, England), caspase 9, caspase 3, phosphorylated/total cAMP Response Element Binding Protein (CREB) (Millipore, Darmstadt, Germany), Bax (Novus, Abingdon, UK), Brain Derived Neurotrophic Factor (BDNF), inducible Nitric Oxide Synthase (iNOS) (Santa Cruz, Dallas, USA), and neuronal Nitric Oxide Synthase (nNOS) (Abcam, Cambridge, USA) each diluted in 5% milk powder in 0.1% Tween 20/0.1 M Tris-buffered saline. Membranes were rinsed and incubated in peroxidase-coupled secondary antibodies which are diluted in same solvent as the primary antibodies. Signal detection was made with luminol substrate (Advansta, San Francisco, USA) under CCD camera with Fusion $FX_7$ system (Vilber Lourmat). Protein loading was controlled with a monoclonal mouse antibody against $\beta$-actin and $\beta$-tubulin (Thermo Fisher Scientific, Paisley, England). Immunoreactive protein bands were quantified densitometrically using ImageJ analysis system (NIH; Washington, USA).

### 2.9. Statistical Analysis

Group comparisons were carried out using Student's *t*-test. A repeated-measures analysis of variance was conducted on the MWM training data. Statistical significance was acceptable to a level of $p \leq 0.05$. Data analysis was performed using the Statistical Package for Social Sciences (SPSS) version 18 software.

## 3. Results

### 3.1. Evaluation of Animal Behavior after TQ Administration

During training in the MWM, in both groups, a decrease in the latency to reach the hidden platform was observed (**Figure 1**A). This decrease was also observed for both groups from the first day to the fifth day of the training in the swim distance (Figure 1B). Two-way repeated measure analysis of variance (treatment X day) yielded significant day effect (for latency $F_{(4:24)} = 4.713$ $p = 0.006$ and for distance $F_{(4:24)} = 6.165$ $p = 0.001$) with an insignificant treatment effect ($p > 0.05$). Independent Student's *t*-test performed for each training day indicated that there was no significant difference in both escape latency and swim distance between the groups ($p > 0.05$). On the probe trial, TQ-treated rats spent more time in the platform quadrant, however, it did not reach accepted level of significance (Figure 1C). The retention test of the passive avoidance, which was conducted 24 h after training, revealed insignificant difference in the step-through latency to the dark compartment between control and TQ-treated groups (Figure 1D).

### 3.2. Histological and Biochemical Measurements in Hippocampal Tissues after TQ Administration

Nissl staining performed for analyzing viable neurons is illustrated in **Figure 2**. Neurons counted in both CA1 and DG regions of hippocampus were significantly higher in TQ-treated animals than corn oil received control animals ($p = 0.026$ for CA1, $p = 0.015$ for DG). However, there was no significant between-group difference in the pyramidal neurons of CA3 region of hippocampus ($p = 0.219$). The hippocampal content of AChE in TQ group was higher compared to the control group, however, it did not reach accepted level of significance ($p = 0.135$) (**Figure 3**).

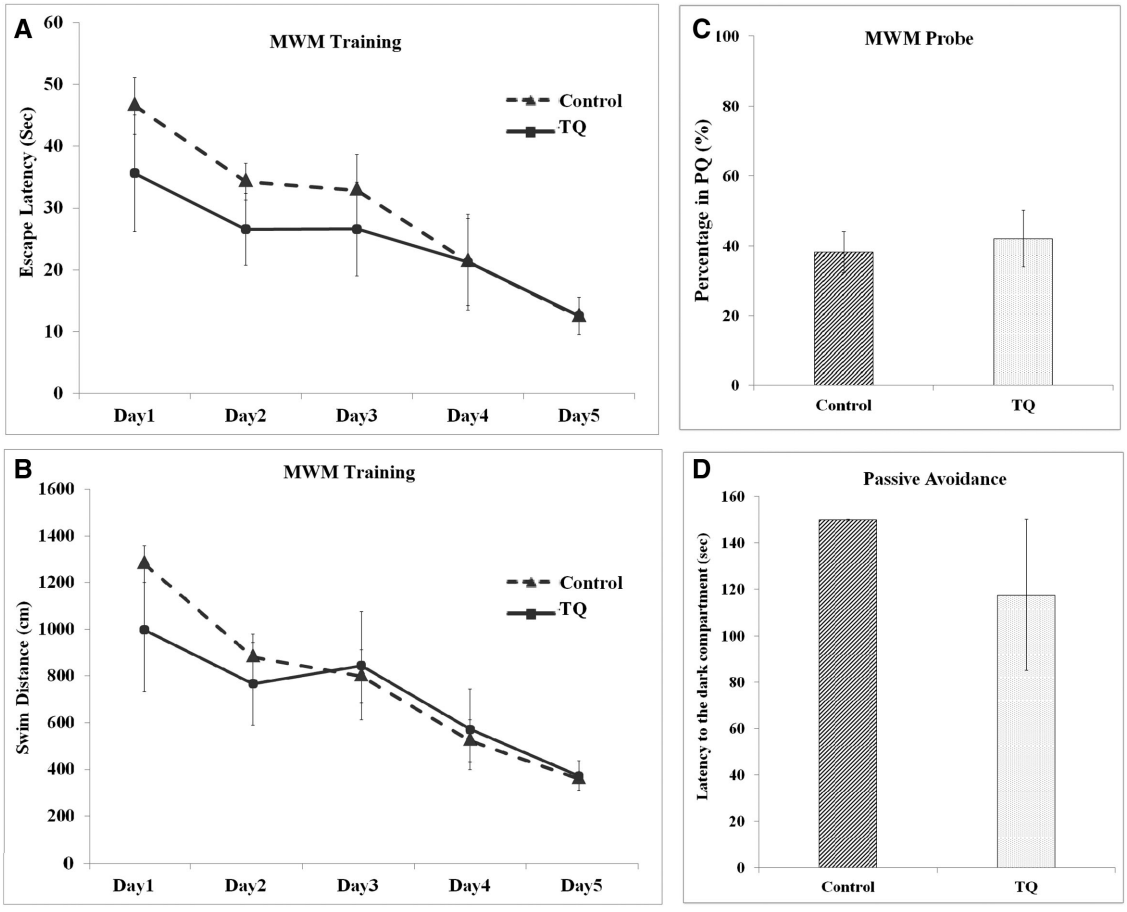


**Figure 1.** Mean swim latency (A) and mean swim distance (B) calculated for the first 5 days of MWM training and mean percent time spent in the platform quadrant on the probe trial of MWM (C). D) The time to the entrance to the dark side of Passive Avoidance. Error bars denote SEM.

### 3.3. The Effect of TQ Administration on Gene Expression

Real-time PCR analysis made in order to evaluate the effect of TQ on the expression of several genes such as *synapsin, synaptophysin, DCX, NGF, AKT, Bax, NFκB*, and *p53* which are involved in cell survival, cellular growth, synaptic plasticity, neurogenesis, and apoptosis processes demonstrated intriguing results (**Figure 4**). The most notable difference was observed in *DCX* and *synapsin* with 7.59- and 2.92-fold increases in TQ exposed animals in respect to control animals. In addition, TQ administration upregulates *synaptophysin, AKT*, and *NFκB* while it downregulates *NGF, Bax*, and *p53* (Figure 4). Solely, the upregulation of *DCX* expression by TQ administration was significant ($p = 0.052$).

### 3.4. The Effect of TQ Administration on Protein Expression

The upregulation of neuronal migration marker, *DCX*, gene expression was also promoted by the upregulation of DCX protein expression by TQ administration ($p \leq 0.001$) (**Figure 5**A). On the other hand, when compared to control group, TQ treatment seems not to influence noticeably the protein levels of BDNF which is a neurotrophin influencing the brain functions (Figure 5B).

**Figure 6** shows the effects of TQ on the cell survival and apoptosis. According to the Student's *t*-test, the ratio of Bcl-2 which is a member of anti-apoptotic Bcl-2 family to Bax which is a member of pro-apoptotic Bcl-2 family significantly increased in TQ-treated animals ($p = 0.026$) (Figure 6A). In this study, we used the protein expression of PARP, caspase 9, and caspase 3 to determine the contribution of TQ on apoptotic pathway. First, we investigated the protein level of PARP which acts as a "molecular sensor" to identify DNA breaks. Herein, we found that TQ treatment significantly decreased the cleavage of PARP which is a marker for induction of apoptosis ($p = 0.015$) (Figure 6B). The levels of caspase 9 and caspase 3, the two members of intrinsic apoptosis pathways, were also significantly affected upon TQ exposure. Expression level of cleaved caspase-9 significantly increased ($p = 0.001$) whereas the level of cleaved caspase-3 significantly decreased in TQ-treated animals when compared to control group ($p = 0.047$) (Figure 6C, D).

Next, we investigated the major elements of AKT pathway including the proteins related with cell survival which were significantly altered with TQ therapy (**Figure 7**). The expression ratio of phosphorylated (Ser473) AKT to total AKT was significantly

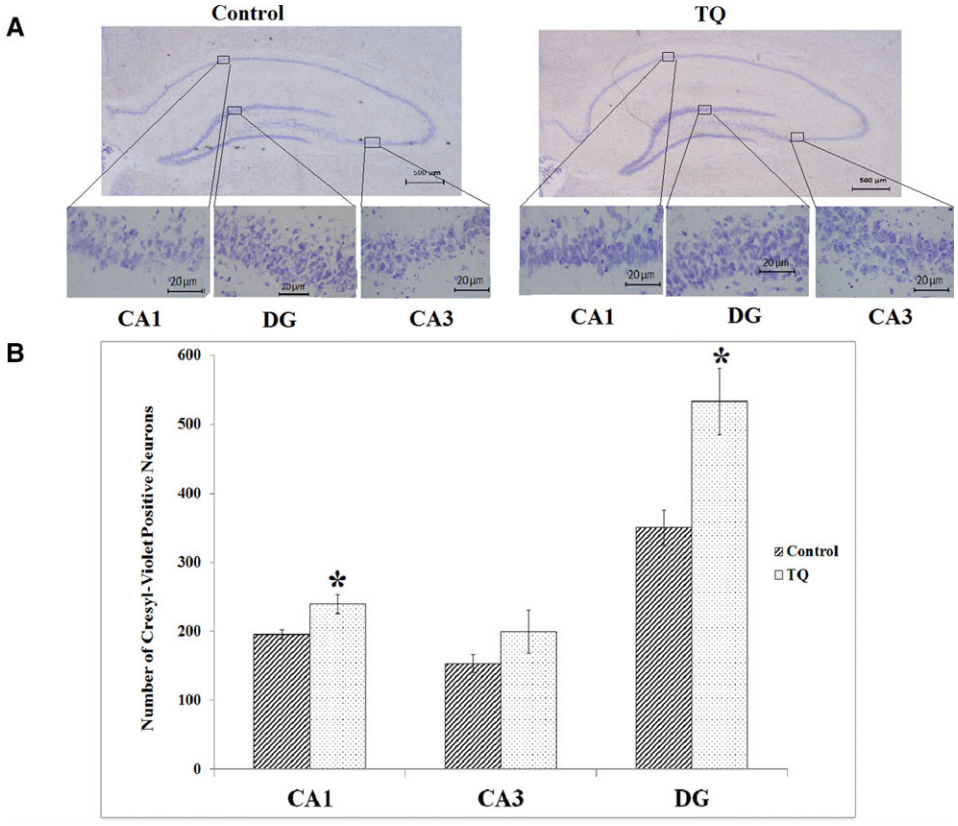


**Figure 2.** The representative images for Nissl staining (A), and the number of cresyl violet positive viable neurons (B) of CA1, CA3, and DG region of hippocampus in control and TQ-treated animals. The degree of significance is denoted as * for $p \leq 0.05$. Error bars indicate SEM.

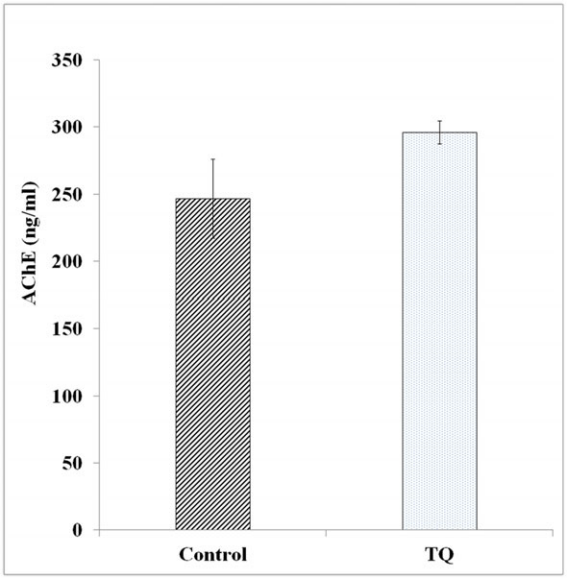


**Figure 3.** The amount of acetylcholinesterase (AChE) in control and TQ-treated animals. Error bars indicate SEM.

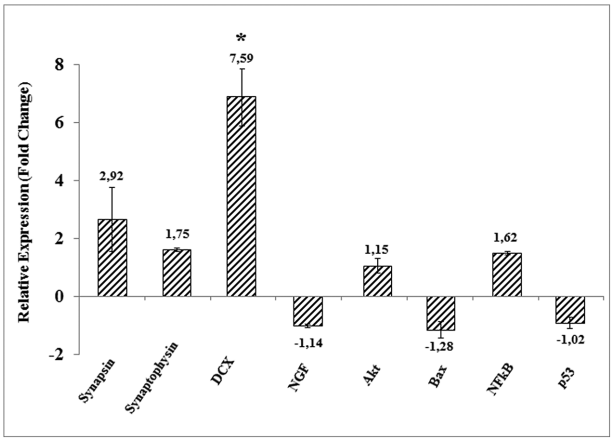


**Figure 4.** Real-time PCR analysis for *synapsin*, *synaptophsin*, *DCX*, *NGF*, *AKT*, *Bax*, *NFκB*, and *p53* genes. The values put onto each graph represents the relative fold change calculated by calibrating the $\Delta C_t$ data of TQ group according to control group. Positive values define increases in fold change while negative ones define decreases. The degree of significance is denoted as * for $p \leq 0.05$. Error bars indicate SEM.

downregulated in the TQ-treated animals ($p = 0.003$) (Figure 7A). In contrast, activation of GSK3$\alpha/\beta$, a downstream molecule of AKT pathway, by phosphorylation in Ser219 residue was significantly increased by TQ administration ($p \leq 0.001$) (Figure 7B). This significant upregulation by TQ was also observed for activation of both subunits of the mitogen activated kinase ERK protein (ERK1/2, phosphorylated in Thr202/Tyr204 residues) ($p \leq 0.001$) (Figure 7C). We further examined the stress activated kinase

 

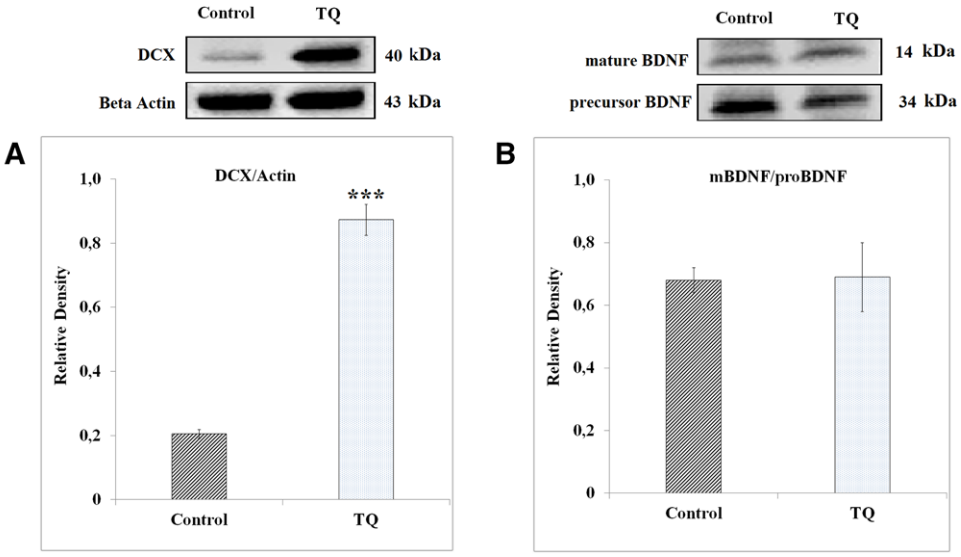


**Figure 5.** Representative pictures and relative amounts of DCX to beta actin (A) and mature BDNF to precursor BDNF (B) analyzed by western blot for both control and TQ-treated animals. The degree of significance is denoted as *** for $p \leq 0.001$. Error bars indicate SEM.

JNK1/2 which is one of the key molecules responsible from the fate of the cell. TQ treatment significantly increased the phosphorylation of JNK2 (Tyr185) ($p = 0.001$) while it did not influence the phosphorylation of JNK1 (Thr183) (Figure 7D). In addition, the phosphorylation ratio of CREB (Ser133), one of the activated transcription factor by these kinases, was higher in the TQ-treated animals ($p = 0.026$) (Figure 7E).

Lastly, to consider the anti-inflammatory effects of TQ, we investigated the expression of the NOS enzymes. In the hippocampus of TQ treated animals, iNOS expression was higher than control animals ($p = 0.002$), while the levels of nNOS were quite similar in TQ-treated and control groups (**Figure 8**A, B).

## 4. Discussion

Most of therapeutic molecules such as resveratrol and polyphenols behave differently in diseased-state and in normal/healthy tissue.[40,41] In the present study, we found same phenomena for TQ on healthy hippocampal tissue, the memory operation center of brain.

TQ has been shown to recover cognitive functions in distinct models of neurodegeneration.[4,8,24] There are also studies demonstrating the positive action of *Nigella sativa* seeds and/or its constituents on the spatial memory and fear-conditioned memory.[29,42] In contrary, despite the fact that our control animals seem to learn later than TQ treated ones, there was no functional improvement eventually due to TQ consumption as compared to control subjects. Also, we did not observe any significant difference in the memory retention both in spatial memory and fear-conditioned memory which dependent to hippocampal circuits. It is well known that the neurotransmitter acetylcholine plays a role in learning and memory. When the disruption of acetylcholine by AChE enzyme decreased, an increase in acetylcholine, and thus, an increase in memory performance was obtained using AChE inhibitors such as *Nigella sativa*.[43] In the current study, TQ had no effect on the AChE amount and also on the BDNF protein expression which has a modulatory action on learning and memory,[44] parallel to our behavioral outcomes. Therefore, we can say that the enhancement of learning and memory performance by TQ seen in pathological conditions was not observed in a healthy tissue at this dosage and time of exposure.

In contrast to lack of effect functionally, we observed TQ-related significant alterations in both cellular and molecular level. First, at cellular level, we observed an increase in the amount of neuronal viability both in pyramidal neurons and granular cells of hippocampus. In pathological conditions, TQ also shows its protective effects by increasing neuron numbers. For example, in a study on cultured hippocampal and cortical neurons which are suffered with amyloid beta 1–42 peptide, TQ treatment increased the survival rate of neurons.[10]

In order to see the mechanisms background of this enhancing activity of TQ on hippocampal cell numbers, we made investigations for expression of some major genes and proteins for cell survival, neurogenesis, signal transduction, synaptic transmission, and plasticity. Interestingly, by TQ treatment, we observed a dramatic increase in the *DCX* gene expression followed by an upregulation in the DCX protein a microtubule associated protein which is a critical factor of effective neuronal migration to the destination areas, and thus a marker for defining migrating newborn neurons.[45] In one of our previous studies, a significant decrease in the granular cells was observed with a significant decrease in the DCX expression.[46] Therefore, we can say that the increase in the cell number in hippocampus can be interpreted as TQ-triggered increase in the neurogenesis of hippocampus. Correspondingly, there is a study that *Nigella sativa* promotes neurite outgrowth that is crucial for neuro-regeneration.[47] Although, it does not reach the desired value of significance, the increase in the synapsin mRNA levels (nearly threefold) demonstrated the enhancing effect of TQ on the synaptic plasticity of hippocampus because of its role in synaptic transmission for carrying out

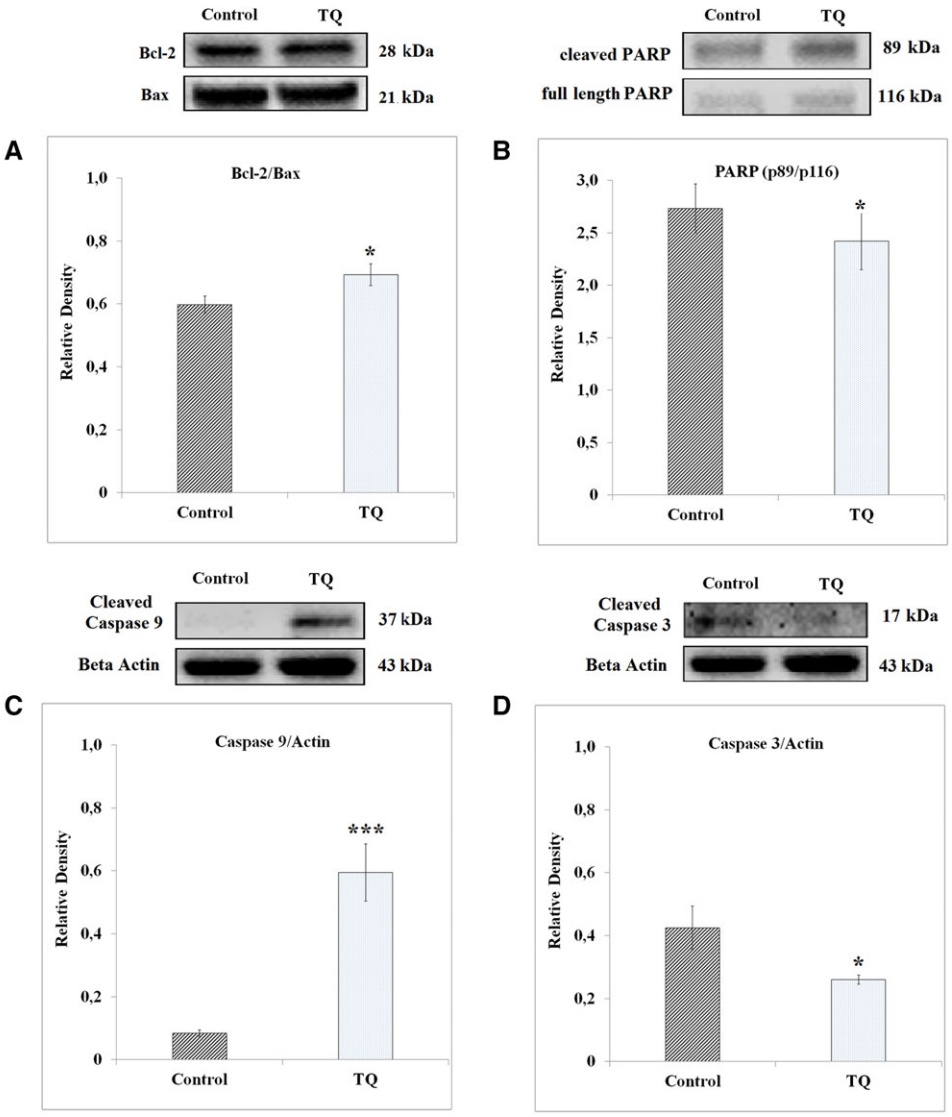


**Figure 6.** Representative pictures and relative amounts of Bcl-2 to Bax (A), cleaved PARP to full length PARP (B), caspase 9 to beta actin (C), and caspase 3 to beta actin (D) analyzed by western blot. The degree of significance is denoted as * for $p < 0.05$ and *** for $p \leq 0.001$. Error bars indicate SEM.

learning.[48] However, we observed that this increase in the neurogenesis by TQ was independent from BDNF.

In literature, there is a considerable amount of knowledge about the apoptotic effect of TQ, especially on cancer cells.[9,15,19,21,38] Nevertheless, we observed that TQ increases the survival factors with respect to death signals which was deduced from an increase in the ratio of anti-apoptotic protein Bcl-2 to pro-apoptotic protein Bax giving an idea about the decision of the living or dying the cell. In addition to the apoptosis-inducing effect of TQ by downregulation of Bcl-2 and upregulation of Bax, the selective inhibitory activity of TQ on cancer cells is associated with upregulation of activated caspases (caspase 3, 7, 8, and 9).[19–21,49–53] In the current study, the decrease in both caspase-3, effector caspase which ends up the programmed cell death process, and PARP, one of the targets of caspase-3, supported the apoptosis-inhibiting effect of TQ in healthy tissue. However, we observed a TQ-dependent increase in the expression of caspase-9, the initiator caspase which cleaves and activates other elements of the pathway. The decrease in the apoptosis in contrast to the increase in caspase 9 can be explained by dimerization of caspase-9 because of bringing molecules closer together due to the higher expression levels and unbalance between the expression levels of caspase-9 and its modulators, as stated in the study of Druskovic et al.[51] Moreover, it has been shown that the serine/threonine protein kinase B (AKT) downregulates the caspase-9 activity by phosphorylating its Ser196 residue.[52] In our study, we also observed a decrease in the AKT expression by TQ treatment suggesting in the activation of caspase 9 due to increase in its cleavage. The protective effect of TQ against to apoptosis was also observed in neurodegenerative conditions, such as neurotoxicity induced by chemicals such as A$\beta$, ethanol, toluene, and lead and PTZ-induced seizures, via increasing Bcl-2 and decreasing

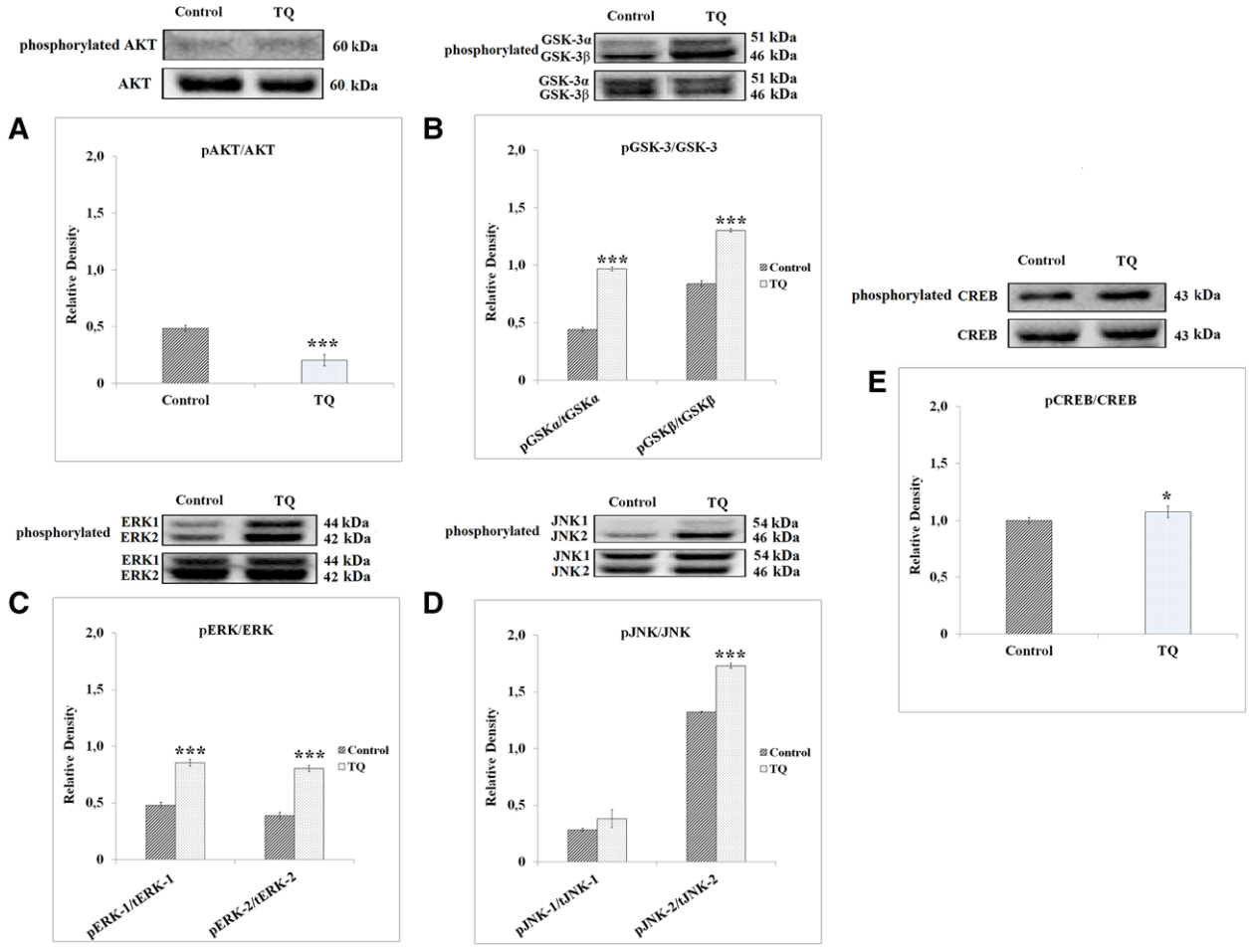


**Figure 7.** Representative pictures and relative amounts of phosphorylated AKT to total AKT (A), phosphorylated GSK-3 to total GSK-3 for both $\alpha$ and $\beta$ subunits (B), phosphorylated ERK1/2 to total ERK1/2 (C), phosphorylated JNK1/2 to total JNK1/2 (D), and phosphorylated CREB to total CREB (E) analyzed by western blot for both control and TQ-treated animals. The degree of significance is denoted as * for $p \leq 0.05$, *** for $p \leq 0.001$. Error bars indicate SEM.

Bax and caspase 3 levels in hippocampus of rats.[7,8,11,17,53] In our study, the correlation between alterations in the expressional levels of caspase 3 and PARP provides us a strong conclusion about the anti-apoptotic effect of TQ because PARP gives an information about the activity of caspase-3 due to the fact that cleavage of PARP by caspase-3 binds DNA irreversibly, and prevents arrival of repair enzymes to DNA to start apoptosis.[54]

Especially in cancer cells, TQ also showed a hindering effect on proliferation due to over-phosphorylation of some protein kinases such as AKT, ERK, and JNK.[55,56] Herein, we observed that an increase in the phosphorylation of all studied kinases (GSK-3, ERK1/2, and JNK-2) and factors (CREB) except activation of AKT suggesting the activation of a cell survival cascade parallel to the increase in the surviving neurons in hippocampal regions by TQ administration. GSK-3 may be a sensor determining the neuronal cell fate in the brain and GSK-3$\beta$, which is active in resting cells, is inactivated by phosphorylation.[57] In literature, it was stated that dephosphorylation of GSK-3 by some chemicals cause ROS production and activation of Bax as well as caspase-3 in developing mouse brain to induce apoptosis and inhibits the cell proliferation by inhibiting the cyclin D1.[58,59] While activation of ERK1/2 contributes to the increased proliferation in cells, activation of JNK1/2 controls apoptosis.[60] As seen in our study, a parallel activation of the ERK1/2 may oppose the apoptosis-inducing JNK activation both in vitro and in vivo.[61] In addition, the phosphorylation level of CREB, a target of MAPK, plays an important role in memory and neuronal survival, increased by TQ treatment, as stated before.[53]

Lastly, we considered the consequences of TQ treatment on the expression of NOS enzymes to determine the action of TQ on the inflammation. Here, we showed an increase in iNOS levels depending on TQ treatment in contrast to the previous experiments performed under pathophysiological conditions such as lipopolysaccharide induction, ischemia, and diabetes.[5,27,62] In a pathological condition, the high levels of NO is produced from iNOS by activated macrophages, which represent a major cytotoxic principle of those cells.[63] The inhibitory effects of TQ as a decrease in the iNOS expression of macrophages were confirmed for protection of neuroinflammation which occurred due to some pathophysiological conditions.[24,64] Moreover, there are some studies showing the increased activity of iNOS as a protective mechanism[65–67] due to the anti-apoptotic properties

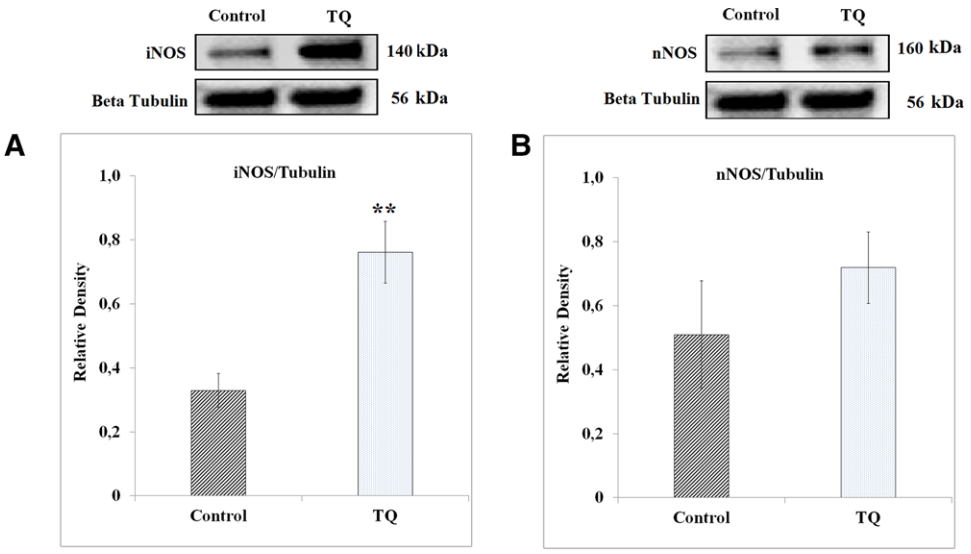


**Figure 8.** Representative pictures and relative amounts of iNOS (A) and nNOS (B) proteins. Normalization for proteins was made with tubulin. The degree of significance is denoted as ** $p \leq 0.01$. Error bars indicate SEM.

of NO derived from iNOS.[68] In molecular level, the MAPK pathway most likely contributes to iNOS gene expression, as parallel to our results.[69] In addition, it was noted that the inhibition of NO synthesis creates impaired memory and learning tasks in different mouse models.[70,71] Therefore, we can propose that the anti-apoptotic action of TQ may produce a homeostatic mechanism through upregulation of iNOS expression by activation of MAPK pathway in the healthy hippocampus to enhance cognitive function.

In conclusion, we observed that TQ administration in physiological conditions causes a wide range of molecular alterations in proteins related neurogenesis, cell survival, and apoptosis. In a healthy brain tissue, TQ consumption increased cell survival and decreased apoptosis in molecular level in contrast to the effects of TQ on cancer tissue. In cellular level, we observed an increase in the number of hippocampal cells due to TQ-related increase in the neurogenesis. However, we did not observe any functional enhancement in the learning and memory due to TQ consumption in contrast to the considerable alterations both in gene and protein levels. Indeed, these data confirm that the addition of TQ in our diets shapes the hippocampus in a positive manner for a healthy brain.

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B.E. designed research and contributed to data analysis; T.D. performed research together with M.B. who also aided data analysis; B.E. and M.B. wrote the paper.

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## Conflict of Interest

The authors have declared no conflict of interest.

## Keywords

apoptosis, cell survival, hippocampus, neurogenesis, thymoquinone

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