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THYMOQUINONE AFFECTS THE EXPRESSIONS OF HIPPOCAMPAL miR-26b, miR-124, AND miR-29a microRNAs IN HEALTHY RATS

TİMOKİNON, SAĞLIKLI SIÇANLARDA HİPOKAMPAL miR-26b, miR-124 VE miR-29a MİKRORNA'LARININ EKSPRESYONLARINI ETKİLER

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ABSTRACT

Objective: Thymoquinone (TQ), the main bioactive component of *Nigella sativa*, crosses the blood-brain barrier and exerts neuroprotective and neuromodulatory activities. This study aims to investigate the effect of TQ administration on the expressions of microRNAs (miR) 26b, 124, 29a and 29c in the hippocampus of healthy rats

Methods: TQ (20 mg kg−1 d−1) is administered intragastrically to adult rats for 15 days. MicroRNA levels of related genes were analyzed using real-time polymerase chain reaction.

Results: Administration of TQ significantly downregulated the expression profiles of miR-26b and miR-124 and upregulated miR-29a. No significant change was observed in the expression level of miR-29c.

Conclusion: TQ may have a beneficial effect on healthy brain and/or central nervous system (CNS) function by altering the expression of miR-26b, miR-124, and miR-29a, which are highly expressed in the brain.

Keywords: Thymoquinone, Black seed, Black cumin, microRNA, hippocampus

ÖZ

Amaç: *Nigella sativa*'nın ana biyoaktif bileşeni olan Thymoquinone (TQ), kan beyin bariyerini geçerek nöroprotektif ve nöromodülatör aktiviteler gösterir. Bu çalışmanın amacı, TQ uygulamasının sağlıklı sıçanların hipokampüsünde 26b, 124, 29a ve 29c mikroRNA'larının (miR) ekspresyonları üzerindeki etkisini araştırmaktır.

Yöntem: TQ (20 mg kg-1 d-1) yetişkin sıçanlara 15 gün boyunca intragastrik olarak uygulanmıştır. İlgili genlerin mikroRNA seviyeleri gerçek zamanlı polimeraz zincir reaksiyonu kullanılarak analiz edilmiştir.

Bulgular: TQ uygulaması miR-26b ve miR-124'ün ifade profillerini önemli ölçüde aşağı düzenlerken miR-29a'nın ifadesini yukarı düzenlemiştir. miR-29c'nin ifade düzeyinde anlamlı bir etki gözlenmemiştir.

Sonuç: TQ, beyinde yüksek oranda ifade edilen miR-26b, miR-124 ve miR-29a'nın ifadelerini değiştirerek sağlıklı beyin ve/veya merkezi sinir sistemi fonksiyonlarında faydalı bir role sahip olabilir.

Anahtar Kelimeler: Timokinon, kara tohum, kara kimyon, mikroRNA, hipokampüs.

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Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression post-transcriptionally, thereby providing fine-tuned regulation of hundreds of targets.[1] Accordingly, every biological process is subject to miRNA-dependent regulation. Therefore, deregulation and/or alterations of specific miRNAs could disrupt the maintenance of health and contribute to diseases. It is predicted that approximately 70% of all miRNAs are expressed in the brain, and they play a pivotal role in regulating brain development and function,[2,3] modulating neurodevelopment[4] and neurodegeneration.[5] Abnormal miRNA expression profiles in the hippocampus have been identified as a risk factor in neuropathologies characterized by oxidative stress and apoptosis. [5]

Medicinal herbs are used as an alternative to chemical agents to alleviate health disorders. *Nigella sativa* (NS) has been utilized as a natural source of remedies and health-care in traditional medicine in various cultures for centuries. The seeds of this medicinal plant are often known as “black seed”, “black cumin”, or “black caraway”[6] and are extensively cultivated in the Mediterranean countries, Middle East, Eastern Europe and Western Asia.[7] Black seed and its extracts have been shown in numerous studies to be effective therapeutic agents with hepatoprotective, neuroprotective, cardioprotective, gastroprotective, antioxidant, antihistamine, antidiabetic, anticancer, antihypertensive, anti-inflammatory, antimicrobial, immunemodulatory, analgesic, and spasmolytic properties.[8-10] The broad spectrum of biological activities originated from its bioactive constituents, including thymoquinone (TQ), thymohydroquinone, dithymoquinone, p-cymene, carvacrol, 4-terpineol, t-anethole, sesquiterpene, α-pinene, and thymol.[8] Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) is the major bioactive component (30 – 48%) of the seed's essential oils and is attributed as responsible for the therapeutic properties of *Nigella sativa*.[8,11] TQ has the ability to cross the blood-brain barrier due to its small size and lipophilicity and perform neuromodulatory activities. This makes it an attractive potential substance for targeting the brain in the treatment of neurological disorders.[9]

TQ has been shown to provide neuroprotective effects in several degenerative diseases of the central nervous system, including cerebral ischemia,[11] epilepsy,[12] Parkinson's disease[13] and Alzheimer's disease.[14] It has been reported that TQ enhances antioxidant capacity and inhibits neuroinflammation.[13] Besides, it improves the memory and cognitive function,[15] sleep quality and ameliorates stress, anxiety[16] and depression.[17] The hippocampus is the region in which learning, memory, emotional regulation and pain perception occurs.[18] Therefore, in the present study, we aimed to investigate the effects of TQ on the expression of brain abundant microRNAs, including miR-26b, miR-29a, miR-29c and miR-124 related to brain development and function, neurodevelopment and neurodegeneration,[3,4] in the hippocampus of a healthy rat brain.

Methods

Animals

In the present study, 24-week-old female Spraque Dawley rats obtained from Bezmialem Vakif University were used. Animals were housed under standard laboratory conditions (12h light/dark cycles, 22 °C, and 60% humidity) with ad libitum food and water. All experimental procedures were performed according to the ethical approval obtained from the Committee for Animal Research Ethics at Bezmialem Vakif University (2015/229).

Thymoquinone (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 days.

Quantitative real-time PCR analysis

The homogenization for total RNA extraction was performed to the right hippocampal tissues of rats. Using the miRNA isolation kit (Thermo Fisher Scientific, Inc.), miRNAs were isolated from total RNA, which is obtained by TRIzol and the PureLink RNA mini kit. Firstly, at room temperature, supernatants of tissue homogenates were incubated with 200 µL of chloroform for 3 min. After centrifugation at 12 000 × g for 15 min at 4°C, 70% ethanol was added to the transparent part of the supernatant in a 1:1 ratio. After washing steps with special columns, 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 by miRNA specific cDNA synthesis kit. A reaction mixture including SybrGreen (Bioline, Luckenwalde, Germany), DNA polymerase, dinucleotide, and buffer solution was mixed with the template and reactions were performed in an appropriate thermal cycle with CFX96 Touch Real-Time PCR (Bio-Rad Laboratories, California). Primer against miR-26b, miR-29a, miR-29c, and miR-124 with housekeeping U6 genes (Table 1)[19] 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 (ΔCT = CT [U6] − CT [miRNA gene]) and compared with control using the ΔΔCT formula (ΔΔCT = ΔCT [TQ group] − ΔCT [control]). Data were analyzed using logarithmic transformation of fold induction ratios according to the relative quantification (RF) formula (2−ΔΔCt).

Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Unpaired t-test with Welch’s correction were performed to calculate the statistical significance between groups using GraphPad Prism 8.01 (GraphPad Software Inc., La Jolla, CA, USA). The statistically signi-

ficant difference was considered as *p < 0.05, ** p < 0.01, *** p < 0.001.

Table 1. Information about the primer sets

Gene	Forward sequence	Reverse sequence
miR-26b	TTCAAGTAATTCAGGATAGGT	-
miR-29a	TAGCACCATCTGAAATCGGTTA	-
miR-29c	TAGCACCATTTGAAATCGGTTA	-
miR-124	TAAGGCACGCGCTGAATGCC	-
U6	CGCAAAUUCGUGAAGCGUUC	-

Results

As shown in Figure 1 and Table 2, RT-PCR analysis revealed that TQ administration led to alterations in the expression of miR-26b, miR-124 and miR-29a in the healthy rat brain. No significant change was determined in the expression of miR-29c. The expression of miR-26b ($p > 0.01$) and miR-124 ($p > 0.05$) were significantly down-regulated (0.38-fold and 0.52-fold, respectively) in the TQ-treated rat group compared to the control group. However, the expression of miR-29a ($p > 0.05$) was markedly up-regulated (1.36-fold) in the TQ-treated rat group relative to the control group.

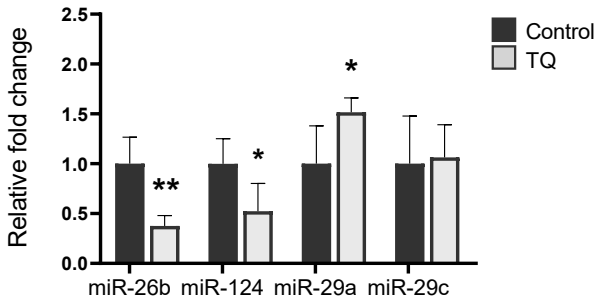

Figure 1. MicroRNA expression with Real-time RT-PCR. The microRNA expression levels of miR-26b, miR-124, miR-29a and miR-29c in the TQ administrated healthy rat hippocampus relative to control group (n = 5 in each group). Error bars represent SEM (standard error of mean). Statistically significance difference; *p < 0.05 and **p < 0.01.

Discussion

The present study investigated the effect of TQ supplementation on the expression of miR26a, miR-124, miR-29a and miR-29c in the healthy rat brain by RT-PCR. The results of the present study showed that TQ treatment reduced the mRNA expression of miR-26b and miR-124 while increasing the expression of miR-29a in comparison to the control group (Table 1, and Fig. 1). The expressions of miR-29c did not exhibit a notable difference.

It is well known that mRNAs are highly expressed in the brain and play a pivotal role in regulating brain development and function.[2,3] miR-26 has been identified as a functional miRNA that plays a role in a number of bio-

Table 2. Expression level of MicroRNAs (miRNAs) analysed by qRT-PCR in control and hippocampus.

miRNA	Control	TQ-treated Hippocampus
miR26b	0,04247 ± 0,01129	0,01598 ± 0,00442** ↓
MiR124	0,05880 ± 0,01478	0,03084 ± 0,01633* ↓
MiR29a	0,26712 ± 0,10160	0,36314 ± 0,09917* ↑
miR29c	0,17419 ± 0,08326	0,18525 ± 0,05720 ↑

The results are shown as mean ± SEM. The degree of significance is denoted as *p < 0.05 and **p < 0.01.

logical processes, including neural cell specification,[4] cell proliferation, apoptosis and the development of normal tissues and tumors.[20] It has been reported that loss of miR-26b results in protective effects on oxidative stress damage by regulating neuronal apoptosis.[21] Dill et al.[22] showed that it regulates neuronal differentiation. Caputo et al.,[23] found that miR-26b negatively regulates brain-derived neurotrophic factor (BDNF) expression at the post-transcriptional level that is involved in neuronal development and plasticity. Reduced BDNF expression is thought to be a risk factor for major depressive disorder (MDD). A previous study showed that miR-26b expression increased in MDD patients.[23] Additionally, decreased levels of BDNF, in the hippocampus are strongly associated with cognitive impairments in animals with Alzheimer disease.[24] In addition, miR-26b and miR-207 were found to be consistently dysregulated in obstructive sleep apnea patients following intermittent hypoxia, a characteristic pathophysiological change of obstructive sleep apnea, which can alter the expressions of apoptosis/anti-apoptosis proteins in the hippocampus coexisting with mnemasthenia.[25] It has also been identified as related to Alzheimer's disease since increased expression of miR-26b activates cell cycle entry, tau-phosphorylation, and apoptosis in postmitotic neurons.[22] It has recently been demonstrated that healing effect on AD patients through down-regulated miR-29c and miR-26b expressions in the Aβ1–42-induced rat hippocampus by increasing cell viability and decreasing apoptosis rate.[19] Considering all these studies, it can be concluded that decreased miR-26b expression may provide neuroprotective effects and reduce the risk of CNS diseases in healthy individuals.

miR-124, a brain-enriched mRNA, regulates neuroinflammation,[26] neural development and differentiation.[27] It is extensively expressed in neurons[4], suggesting its key function in the CNS. The expression level of miR-124 is undetectable or extremely low in neural progenitors, while its expression increases gradually in differentiating and mature neurons.[4,28] Highly expressed miR-124 can induce neuron-specific differentiation and govern the dendritic plasticity of neural stem cells.[29,30] miR-124 has been reported to be up-regulated in chronic stress and acts as a regulator of structural plasticity and behavioral responses to chronic stress.[31] Bahi et al.[32] also found that stress in the rat hippocampus causes increased expression of miR16 and miR-124, linked to the development of depressive-like symptoms. Pan-Vazquez, et al.[33] demons-

trated that exercise has a positive effect on stress resilience via increased expression of the glucocorticoid receptor (Nr3c1) and decreased expression of miR-124 in the hippocampus. Moreover, it was shown that expression levels of miR-124 up-regulated in the peripheral blood of MDD patients affect alterations in brain miRNA expression directly. Its expression levels were also found to be significantly correlated with the connectivity of both intra- and inter-networks in the brain.[34] Additionally, miR-124 has been shown to function as a regulator to alleviate cell death correlated with the expression of BACE1/β-secretase in Alzheimer's disease (AD).[35] Another study reported repressed tyrosine-protein phosphatase non-receptor type 1 (PTPN1) by up-regulation of miR-124 in the temporal cortex and hippocampus in AD patients, which indicates region-specific abnormalities linked to synaptic, plasticity and memory dysfunction.[36] Furthermore, miR-124 regulates translation of GluA2 primarily in the somatic cytoplasm of neurons rather than in dendrite, which could cause functional alterations in synaptic strength and connectivity.[37] Khan et al.[38] suggest that TQ may be directly linked with the miR-124 expression levels, which are involved in neuronal development and differentiation and this fact decreases ERK phosphorylation and ameliorates cognitive impairments in the rat hippocampus. Therefore, we may suggest that TQ-induced down-regulated miR-124 expression may improve memory, synaptic strength and plasticity. As mentioned before, the expression level of miR-124 has been found to be increased in chronic stress, AD and major depressive disorder; thereby, reduced miR-124 expression can also protect individuals from the development of neurodegenerative diseases.

The miR-29 family, consisting of miR-29a, miR-29b and miR-29c, has been implicated in neuronal proliferation, differentiation, plasticity, and survival.[39] miR-29a is abundant in the brain, and particularly in hippocampal neurons[40] and regulates dendritic spine morphology,[41] neural development and morphology.[40] In addition, it is important in fine-tuning motor function.[42] miR-29a, has been shown to be up-regulated during normal aging in the CNS.[43] Dysregulation of the miR-29 family is associated with many neurodegenerative disorders including AD, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis and Parkinson's disease.[39] Ma et al.[44] found that overexpression of miR-29a regulates neurite outgrowth and development of neuronal stem cells by targeting extracellular matrix-related genes. Thus, increaseed miR-29a expression levels due to TQ treatment may affect neural development and morphology.

TQ, a major bioactive compound of *Nigella sativa* seed's oil, has been extensively studied for its biological activities and therapeutic potential and shown to possess neuroprotective, antioxidant and anti-inflammatory properties. It has been shown that TQ exerted a strong neuroprotective effect on Aß-induced neurotoxicity and aggregation by increasing cell viability and decreasing apoptosis rate via inhibition of ROS formation, and mitochondrial membrane depolarization in the hippocampal and cortical neurons.[45] The diminished TQ effect on Aß-induced inhibition of synaptic vesicle recycling was also reported in the same study. Bin Sayeed et al.[15] demonstrated that *Nigella sativa* (500 mg capsule twice daily for 9 weeks) improved age-related cognitive decline, memory and attention in healthy humans. The authors also reported that daily consumption of one NS capsule (500 mg for 4 weeks) as a nutritional supplement stabilized mood, decreased anxiety, and improved memory in healthy adolescent males.[46]

Overall, these studies and our results showed that TQ administration alters the expression of miR-26b, miR-124 and miR-29a, which play a role in maintaining healthy brain function. All these three miRNAs are abundant in the brain, indicating the importance of their activity in the CNS and their activities affect neuronal development, differentiation, plasticity, survival and synaptic function. Therefore, TQ may be suggested to be used as a natural supplement for the maintenance and improvement of brain health.

Conclusion

The results of the present study indicate that the TQ administration can affect brain-enriched microRNA expressions, which have functions in neural development and differentiation, neuroinflammation, memory, synaptic strength and plasticity. Regular expressions of these brain enriched miRNAs are critical for the maintenance of healthy CNS. Administration of TQ may help to improve memory, cognitive abilities and prevent the development and progression of neurodegenerative diseases. However, further research is required to identify the full potential of these natural agents for CNS in health and diseases.

Compliance with Ethical Standards

The experimental protocol of this study was approved by the Committee for Animal Research Ethics at Bezmialem Vakif University (2015/229).

Conflict of interest

The authors declare no conflict of interest.

Authorship Contributions

B.E. designed research and performed data analysis; T.D. performed research together with M.B., who also aided data analysis; and A.D contributed to data analysis and wrote the paper. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

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Financial Disclosure

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