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Sharareh Dariani, Tourandokht Baluchnejadmojarad & Mehrdad Roghani

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Abstract Temporal lobe epilepsy (TLE) is a rather common and difficult-to-treat variant of epilepsy. Nearly one third of people with epilepsy do not respond effectively to currently available anticonvulsants. In this study, we evaluated the protective effect of thymoquinone (TQ), the main constituent of black seed with antioxidant and anti-inflammatory effects, in the intrahippocampal kainate model of TLE in rat. Following kainate injection, seizure activity was observed that was significantly diminished by TQ pretreatment at a dose of 10 mg/kg, p.o. Intrahippocampal kainate also increased malondialdehyde (MDA), nitrite, and nitrate levels and decreased activity of superoxide dismutase and TQ only significantly attenuated MDA. In addition, intrahippocampal kainate caused a significant reduction of neurons in CA1, CA3 and the hilar regions, and TQ significantly attenuated these changes. Timm histochemistry showed a marked mossy fiber sprouting (MFS) in the dentate gyrus of kainate-lesioned rats, and TQ significantly lowered MFS intensity. Meanwhile, a number of reactive astrocytes (astrogliosis) increased significantly in the kainate group, and TQ pretreatment significantly decreased it. These data suggest that TQ pretreatment could attenuate seizure activity and lipid peroxidation, lower hippocampal neuronal loss and MFS, and mitigate astrogliosis in kainate model of TLE.

S. Dariani
Department Physiology, School of Medicine,
Tehran University of Medical Sciences, Tehran, Iran

T. Baluchnejadmojarad (✉)
Department Physiology, School of Medicine,
Iran University of Medical Sciences, Tehran, Iran
e-mail: tmojarad@yahoo.com

M. Roghani
Neurophysiology Research Center, Shahed University, Tehran, Iran

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Introduction

Temporal lobe epilepsy (TLE) is the most frequent kind of epilepsy in adults, characterized by mossy fiber sprouting (MFS) and rewiring in the hippocampal circuits, neuronal loss (Jokeit and Schacher 2004), reactive astrogliosis (Xie et al. 2011), and enhanced oxidative stress (Baluchnejadmojarad and Roghani 2013). Glial fibrillary acidic protein (GFAP) is a biological marker of reactive astrogliosis following neurotoxic insult (Otani et al. 2006; Xu et al. 2011). Intrahippocampal administration of kainate, a potent and neurotoxic analog of glutamate, is widely utilized to develop a reliable model of TLE in rats (Rattka et al. 2013). Since some TLE patients are resistant to current therapies (Freitas et al. 2005), novel treatments are searched for to prevent and inhibit epileptogenic processes (Loscher and Schmidt 2006).

Thymoquinone (TQ) is the major constituent of the black seed volatile oil (Gali-Muhtasib et al. 2006) with anti-inflammatory and antioxidant activity (Khan et al. 2012). TQ has been shown to protect cortical neurons against ethanol-induced apoptosis (Ullah et al. 2012) and preserves cells against cytotoxic agents through oxidative stress offsetting (Mousavi et al. 2010). TQ could also attenuate neurodegeneration in the frontal cortex following toluene exposure (Kanter 2011). Anti-epileptic effect of TQ has been proven in children with refractory seizures (Akhondian et al. 2011) and in pentylenetetrazole-induced seizure model (Hosseinzadeh et al. 2005). TQ may be a good candidate for neuroprotection against kainate-induced TLE. Therefore, we

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decided to investigate its effect in an intrahippocampal kainate model of TLE in rat.

Materials and Methods

All experiments were performed on adult male Wistar rats (270–310 g; n=72) (Pasteur's Institute, Tehran, Iran). They were housed three to four per cage in a temperature-controlled colony room under light/dark cycle with food and water available ad libitum. Procedures involving animals were conducted in conformity with NIH guidelines for the care and use of laboratory animals. In this study, all efforts were made to minimize number of animals and their suffering.

Experimental Procedure

Rats were divided into sham operated (Sham), TQ-treated sham-operated (Sham+TQ10), kainate, and TQ-treated kainate (Kainate+TQ10) groups. For intrahippocampal injections, rats were anesthetized with chloral hydrate (350 mg/kg; i.p.), placed into the stereotaxic frame (Stoelting Co., USA) with the incisor bar set at 3.3 mm below the interaural line. The dorsal surface of the skull was exposed and a burr hole was drilled using the following coordinates according to the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson 1986) with the bregma point as the reference: anteroposterior, 4.1 mm; lateral, 4.1 mm, and ventral to the dura, 4 mm. Freshly prepared kainate (kainic acid; Sigma-Aldrich, USA) solution (5 μL of normal saline containing 4 μg of kainate) was injected into the right side of the hippocampus at a rate of 1 μL/min using a Hamilton microsyringe. The syringe was slowly withdrawn and the rat scalp was sutured. The sham group received an equivalent volume of normal saline at the same stereotaxic coordinate. The Sham+TQ group received TQ (Sigma-Aldrich, USA) p.o. at a dose of 10 mg/kg/day starting 1 week before surgery and the last treatment was 1 h pre-surgery. TQ was dissolved in propylene glycol. The dose of TQ was chosen according to previous reports (Gilhotra and Dhingra 2011; Hosseinzadeh et al. 2007). The kainate+TQ group received TQ with the same protocol and then received intrahippocampal injection of kainate solution.

Behavioral Assessment of Seizure

All animals were assessed for kainate-induced seizures during the first 24 h post-surgery. At 6th week post-surgery, all animals were re-evaluated for behavioral progression of seizures 4 h/day for 5 consecutive days to record the spontaneous seizures and scored according to Racine's classification: 0, no reaction; 1, stereotypic mounting, eye blinking, and/or mild facial clonus; 2, head nodding and/or multiple facial clonus; 3, myoclonic jerks in the forelimbs; 4, clonic convulsions in the forelimbs with rearing; and 5, generalized clonic convulsions and loss of balance (Racine et al. 1972).

Assessment of Oxidative Stress Markers

Measurement of Hippocampal MDA Concentration

The rats (n=6 for each group) were anesthetized with diethyl ether and decapitated. The hippocampi were isolated and blotted dry, and then weighed and prepared as a 10 % tissue homogenate in ice-cold 0.9 % saline solution. After centrifugation (1,000×g, 4 °C, 10 min), the supernatant was aliquoted and stored at −70 °C until assayed. The concentration of malondialdehyde (MDA), used as a marker of lipid peroxidation, was calculated by measuring thiobarbituric acid reactive substances in the supernatant as described previously (Baluchnejadmojarad and Roghani 2011).

Determination of Hippocampal Nitrite Concentration

Supernatant nitrite (NO_2^-) content was assayed by the Griess method as described before (Baluchnejadmojarad and Roghani 2011).

Assay of Hippocampal SOD Activity

Superoxide dismutase (SOD) activity was measured as previously reported (Baluchnejadmojarad and Roghani 2011).

Protein Assay

The protein content of the supernatant was measured by the Bradford method, using bovine serum albumin (Sigma Chemical, USA) as the standard (Bradford 1976).

Nissl and Timm Staining

Animals (n=6 for each group) were deeply anesthetized with ketamine (150 mg/kg) and perfused through the ascending aorta with 50 mL of heparinized normal saline followed by 100 mL of sulfide solution (1.2 % Na_2S and 1.0 % NaH_2PO_4) and then with 50–100 mL of 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Following perfusion, the brains were removed from the skull, hippocampal blocks were prepared and immersed in 30 % sucrose in phosphate buffer at 4 °C for 2–3 days. Then, sections were cut at a thickness of 30 μm on a freezing microtome (Leica, Germany). Every

second section was Nissl-stained with 0.1 % cresyl violet (Sigma) and alternate sections were used for Timm staining. In Nissl-stained sections, neuronal loss was quantified in CA1, CA3, and hilar regions of the hippocampus in at least three sections at a level range between −3.6 and −4.3 mm from the bregma using an image capturing and analysis system (Bel Engineering, Italy). The process was repeated at least two times for each section and its average was taken as the final value. Counting was done blind to the treatments received.

Mossy fibers from granule cells in the dentate gyrus undergo reorganization of their terminal projections in epilepsy (Baluchnejadmojarad and Roghani 2013). Timm staining is an accepted method for the visualization of zinc-containing neuronal elements. To visualize MFS in the inner molecular layer of the dentate gyrus (DG), we used a modified procedure to label the zinc-containing axons of the granule cells (Karoly et al. 2011). The slices were immersed for 5 min in 100 % alcohol, 5 min in 70 % alcohol, and 10 min in distilled water. The slices were then developed in the dark under continuous agitation for 60 min in Timm working solution with the following composition: 60 mL of 50 % gum Arabic, 10 mL of 2 M sodium citrate buffer (pH 3.7), 30 mL of 5.6 % hydroquinone, and 0.5 mL of 17 % silver nitrate solution. The staining process was terminated with 2 % sodium acetate and the unreacted silver ions were removed with 5 % sodium thiosulphate. Assessment of MFS (as Timm index) was obtained from the absolute value of the area of Timm granules divided by the length of DG (Baluchnejadmojarad and Roghani 2013). The Timm index for each animal was the mean of four sections. All procedures and analyses were done blind to the treatments.

GFAP Immunohistochemistry

The animals (n=5 for each group) were anesthetized with ketamine, perfused with normal saline followed by 4 % paraformaldehyde, the brains were removed, the hippocampal blocks were prepared, and sections were cut as mentioned before for Nissl staining. Sections were washed with phosphate buffer saline (PBS). After permeabilization with 0.4 % Triton X-100/PBS for 15 min, nonspecific staining was blocked by incubation with 10 % normal goat serum in PBS for 1 h at room temperature. Then, sections were incubated with rabbit polyclonal anti-GFAP primary antibody (Abcam, USA) at a dilution of 1/500 in a moist atmosphere at room temperature overnight. Thereafter, slides were washed in PBS and incubated for 2 h with goat anti-rabbit antibody conjugated with HRP (Abcam, USA) at a dilution of 1/500 in PBS. Following several rinses in PBS, slides were incubated with 3,3-diaminobenzidine (Sigma-Aldrich, Germany) and 0.01 % (v/v) H_2O_2 in PBS for 5–10 min in the darkness to visualize GFAP-immunoreactive astrocytes. Slides were then washed, mildly counterstained with 0.1 % cresyl violet, dehydrated in a graded series of alcohol, cleared in xylene, coverslipped with Entellan, and microscopically analyzed. The stratum lucidum area of the hippocampus in at least four sections at a level range between −3.6 and −4.3 mm from the bregma was evaluated for astrogliosis. Evaluation and counting was done blind to the treatments.

Statistical Analysis

Data were expressed as means±SEM. To compare the experimental groups, non-behavioral data were analyzed using one-way ANOVA followed by Tukey's post hoc test. Seizure-related behavioral data were analyzed using the non-parametric Kruskal–Wallis test. Percentage of rats with spontaneous seizure was examined by χ^2 test. In all analyses, the null hypothesis was rejected at a level of 0.05.

Results

All rats except for two rats from the kainate group and one rat from kainate+TQ10 tolerated the experimental procedures until the end of the study.

Behavioral Assessment of Seizure Activity

Sham and Sham+TQ10 groups showed no signs of seizure activity during the first 24 h post-surgery and/or after 5-weeks. In contrast, all rats (100 %) in the kainate group exhibited high scores of seizures within the first 24 h post-surgery and 76.4 % of them had seizure activity on the 6th week. Meanwhile, rats injected with KA and pretreated with TQ showed only lower seizure activity as compared to the kainate group within the first 24 h (56.2 %) and only 43.8 % of them had seizure activity on the 6th week and this difference versus kainate group was significant for both time periods (p<0.01) (Table 1).

Table 1 Numbers and rates of animals with seizures during the first 24 h post-surgery and on the 6th week in experimental groups

	Within 24 h post-surgery	Rate (%)	On the 6th week post-surgery	Rate (%)
Sham	0/18	0	0/18	0
Sham+TQ10	0/18	0	0/18	0
Kainate	17/17	100	13/17	76.4
Kainate+TQ10	9/16	56.2*	7/16	43.8*

*p<0.01 (versus Kainate)

Oxidative Stress Markers

Rats receiving TQ10 in the sham group did not show a significant change in the hippocampal level of MDA and nitrite and nitrate and SOD activity as compared to the vehicle-pretreated sham group. In contrast, rats in kainate group showed a significant elevation of MDA ($p<0.05$) and nitrite and nitrate content ($p<0.05$) and reduction of SOD activity ($p<0.05$) and pretreatment of kainate group with TQ10 only significantly attenuated the elevated MDA ($p<0.05$). However, nitrite and nitrate level was lower and SOD activity was higher in the Kainate+TQ10 versus kainate group at a nonsignificant level (Fig. 1).

Histochemistry of the Hippocampus in Nissl Staining

The number of neurons per unit area in the CA1, CA3, and hilar regions was counted and compared among groups (Fig. 2). Our results showed that TQ10 pretreatment of the sham group did not produce any significant change in this regard. In contrast, intrahippocampal injection of kainate led to a marked and significant degeneration and reduction of neurons in CA1 ($p<0.05$), CA3 ($p<0.005$), and hilar ($p<0.01$) regions of the hippocampus versus sham group. In this regard, the neurodegeneration in the hippocampus was typified by an apparent cell loss in the dentate hilus and considerable thinning of cell layers in the CA1 and CA3 regions and the dentate gyrus of the kainate group exhibited granule cell dispersion and displacement and it was two to threefold broader as compared to the contralateral side in the upper border. In addition, TQ pretreatment of the kainate group rats significantly attenuated these changes in CA1 ($p<0.05$), CA3 ($p<0.01$), and hilar ($p<0.01$) regions versus kainate group, indicating the neuroprotective potential of TQ against kainate neurotoxicity.

Timm Histochemistry

Our observation concerning zinc histochemistry in the hippocampus was in agreement with the literature (Baluchnejadmojarad and Roghani 2013). In this study, KA lesion-induced aberrant MFS was shown by Timm method at 6th week post-lesion that selectively labeled synaptic terminals of mossy fibers due to their high zinc content (Baluchnejadmojarad and Roghani 2013). In the sham groups, little MFS was observed in the DG molecular layer. On the contrary, in the kainate group, Timm staining showed a robust MFS that extended into the dentate supragranular layer and in the TQ10-pretreated group, supragranular MFS was less intense and more dispersed, though it was still denser than the sham group. We further compared the average width and Timm staining density (as indicated by Timm index) between kainate and TQ10-pretreated kainate groups and found out that this index is significantly higher versus the sham group ($p<0.001$) and

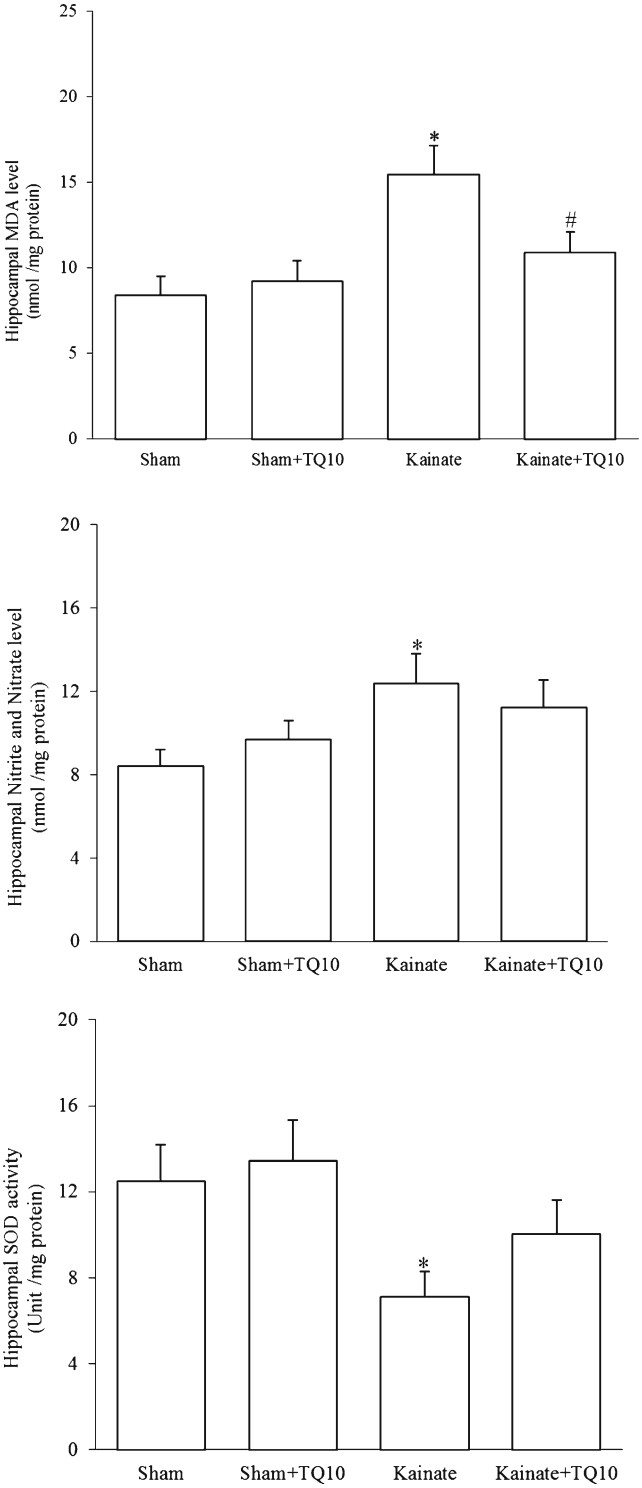

Fig. 1 Malondialdehyde (*MDA*) concentration, nitrite and nitrate content, and superoxide dismutase (*SOD*) activity in hippocampal homogenate. $^{*}P<0.05$ (versus Sham); $^{\#}P<0.05$ (versus Kainate)

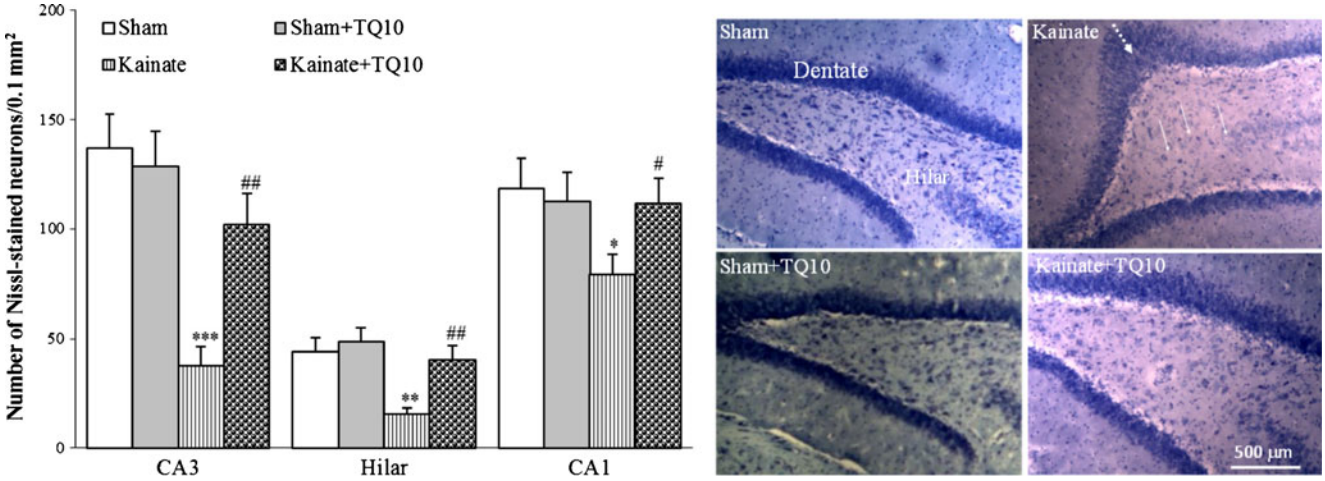

Fig. 2 Number of Nissl-stained neurons in different areas of the hippocampus (*left panel*) and a photomicrograph of coronal sections through the dentate region (*right panel*). Severe reduction in the number of neurons in the hilar region and neuronal dispersion in the upper blade (*white arrow*) were observed in the Kainate group, but no such marked changes were noticed in the TQ10-treated kainate group. *$p<0.05$; **$p<0.01$; ***$p<0.005$ (versus Sham); $^{\#}p<0.05$ (versus Kainate)

TQ10 pretreatment could significantly reduce it in dentate gyrus ($p<0.05$) (Fig. 3).

GFAP Immunohistochemistry

GFAP is a specific marker of both normal and reactive astrocytes, which can be distinguished by the differences in their morphology, i.e., in the normal brain, only a few astrocytes express GFAP with thin and long processes, in contrast, reactive astrocytes are larger with elongated processes. Following KA administration, the number and size of astrocytes are increased, as well as their length and the thickness of their processes (Xie et al. 2011). Quantification of the number of reactive astrocytes in the stratum lucidum area revealed a marked astrogliosis in the kainate group, and TQ10 pretreatment significantly decreased it versus the kainate group ($p<0.01$). In addition, no activated astrocytes were observed in the hippocampus of the sham and Sham+TQ10 groups and the observed astrocytes had a normal morphology (Fig. 4).

Discussion

Temporal lobe epilepsy is regarded as a chronic and resistant-to-treat neurological disorder hallmarked with recurrent

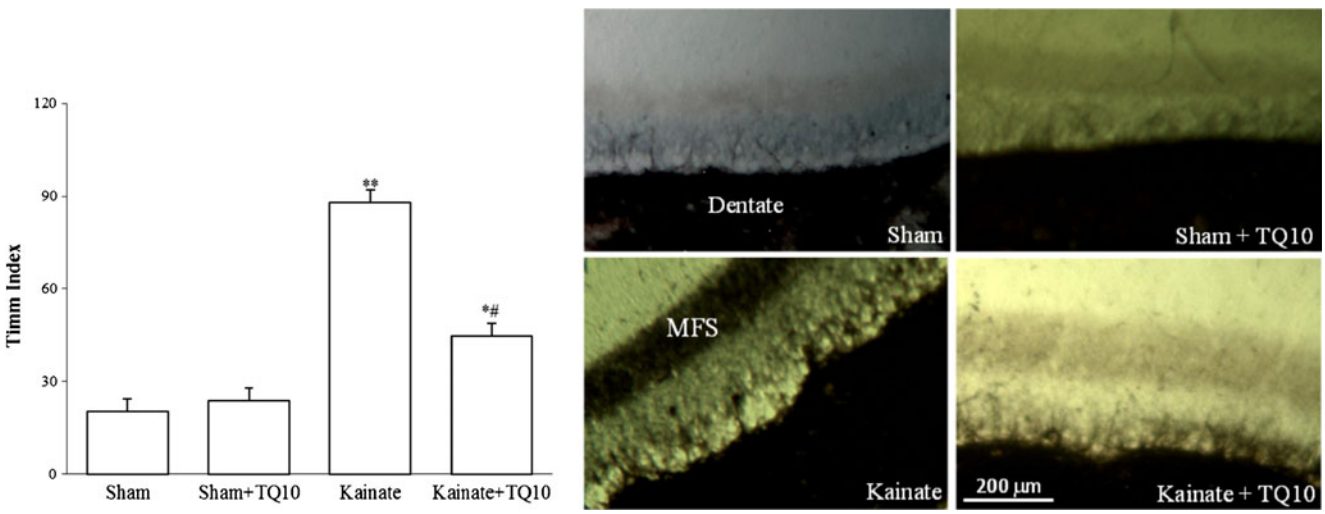

Fig. 3 Timm index as an indicator of mossy fiber sprouting (*MFS*) (*left panel*) and a photomicrograph through the hippocampus (dentate region) (*right panel*). *$p<0.05$; **$p<0.001$ (versus Sham); $^{\#}p<0.01$ (versus Kainate)

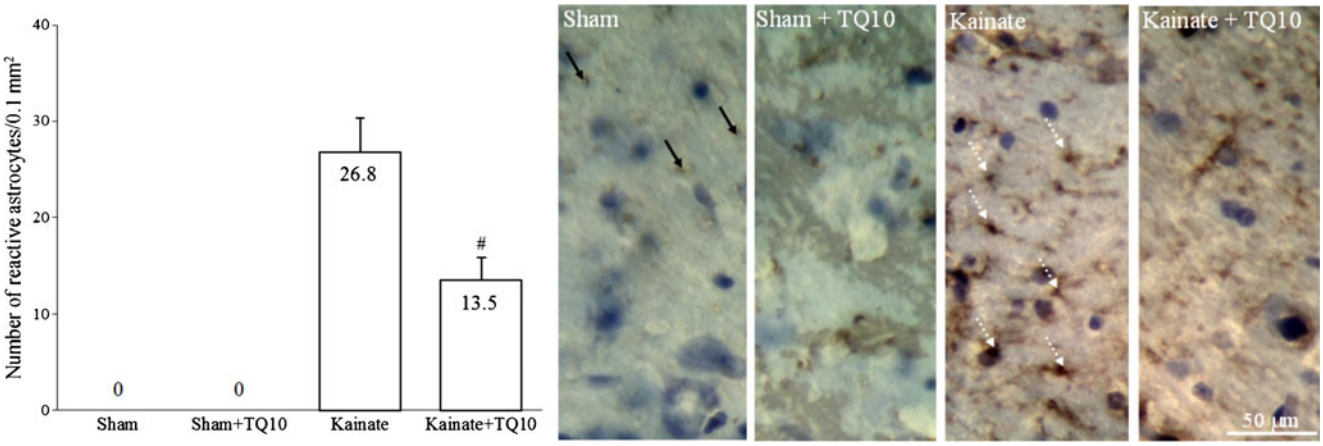

Fig. 4 Effect of kainate or kainate plus thymoquinone (*TQ*) pretreatment on GFAP immunostaining. Photomicrographs show GFAP immunopositive cells in stratum lucidum region of the hippocampus. Kainate administration resulted in marked astrogliosis and TQ attenuated it. *Solid* and *dotted arrows* show normal and reactive astrocytes, respectively. $^{\#}p<0.01$ (versus Kainate)

seizures due to development of recurrent excitatory or inhibitory circuits. The recurrent seizures are associated with aberrant MFS in the dentate region (Epsztein et al. 2005). Kainate injection into the CA3 region of the hippocampus causes development of epileptic seizures (Baluchnejadmojarad and Roghani 2013). These seizures are followed by a pattern of cell loss like that seen in patients suffering from TLE (Sperk 1994). For this reason, kainate-induced brain damage has been routinely used for modeling TLE (Sperk 1994). Enhanced oxidative stress burden also contributes to kainate neurotoxicity (Shin et al. 2008). Out of the different ionotropic glutamate receptors, those kainate receptors containing the GluR6 subunit are important for epileptogenic effects of kainate (Mulle et al. 1998). Therefore, these receptors are expected to be overexpressed following intrahippocampal injection of kainate in the CA3 region and this could promote seizure development and concurrent degeneration of hippocampal neurons and development of an aberrant MFS into the inner molecular layer of the DG (Wu et al. 2009), as was observed in our study.

A massive neuronal loss in the CA1, CA3, and hilar regions and a typical aberrant MFS into the inner molecular layer was observed following intrahippocampal kainate injection in this study which was consistent with previous studies (Wu et al. 2009; Baluchnejadmojarad and Roghani 2013). Kainate injection into the hippocampus led to the degeneration of CA3 pyramidal neurons and dentate hilar cells and granule cell axons (known as mossy fibers) originating from the DG lose their postsynaptic target cells and sprout into the inner molecular layer. These pathologic changes cause the formation of a functional recurrent excitatory circuit between granule cells that contributes to recurrent seizures (Shetty and Hattiangady 2007).

Although antiepileptic and anticonvulsant activity of TQ has been previously reported (Akhondian et al. 2011; Hosseinzadeh and Parvardeh 2004; Hosseinzadeh et al. 2005), but its exact mechanism of action has not been determined in this respect. Part of the beneficial effect of TQ in this study could be attributed to its neuroprotective effect. In this regard, it has been shown that TQ therapy is able to protect the frontal cortex neurons against neurodegeneration after chronic toluene exposure in rats (Kanter 2011) and TQ is also a strong protective agent against ethanol-induced neuronal apoptosis in primary rat cortical neurons (Ullah et al. 2012). In addition, thymoquinone has a strong potential to protect primary dopaminergic neurons against 1-methyl-4-phenylpyridinium and rotenone relevant to Parkinson's disease (Radad et al. 2009). Therefore, TQ could reduce the detrimental action of neurotoxins and/or excitotoxic agents on neurons, thus limiting accumulation of extracellular glutamate and preventing apoptotic death of neurons. In our study, due to neuroprotective effect of TQ10, there were lower degrees of neuronal loss and MFS in the Kainate+TQ10 group than those of kainate. In our study, TQ10 pretreatment of the kainate group attenuated oxidative stress burden as was evident by significantly lower levels of MDA and nitrite and nitrate in the hippocampal tissue, and this has certainly protected hippocampal neurons against oxidative damage with subsequent lower MFS and less severe seizure activity. This study clearly suggests that TQ10 could protect against kainate-induced epilepsy by functioning as an antioxidant. Anti-apoptotic potential of TQ may also be involved in its beneficial effect in this study. Previous reports have shown that in kainate-induced epileptic seizure model in rat, the protective protein Bcl-2 is downregulated and hence apoptosis occurs (Zhang et al. 2011) and TQ treatment is able to inhibit the apoptotic cascade by increasing Bcl-2 expression and to repress the activation of caspase-9 and caspase-3 and to reduce the cleavage of PARP-1 (Ullah et al. 2012). In this way, TQ could prevent kainate-induced apoptotic cell death.

Astrogliosis is also accompanied with neurodegeneration following a variety of insults due to neurotoxins (Ferraguti et al. 2001; Magiatis et al. 2010). It is suggested that astrocyte proliferation and activation could participate in the pathogenic mechanism of epilepsy, since astrocytes themselves contribute to hyperexcitability (Binder and Steinhauser 2006; Miltiadous et al. 2011). A marked astrogliosis was observed in this study which may itself contribute to behavioral changes in the kainate group and TQ pretreatment was capable to attenuate astrogliosis. This issue itself requires further investigation to evaluate the possible direct effect of TQ on this phenomenon.

Kainate-induced epilepsy is also associated with inflammation with enhanced generation of certain prostaglandin such as prostaglandin E2 following an enhancement in mRNA levels of cyclooxygenase 2 and prostaglandin E2 synthase in the brain tissue and anti-inflammatory agents are capable to lower the severity of the condition (Ciceri et al. 2002). Parallel to this fact, it has been proven that kainate-induced excitotoxicity through induction of the matrix metalloproteinases leads to selective neuronal death and neuroinflammation in the hippocampus, and inhibitors of such enzymes could attenuate the ensuing neuronal damage and this could be therapeutically useful in some neurological disorders (Jourquin et al. 2003). On the other hand, TQ in vitro is capable of exerting anti-inflammatory properties via attenuation of interleukin-1 beta, tumour necrosis factor-alpha metalloproteinase-13, cyclooxygenase-2, and prostaglandin E (Vaillancourt et al. 2011) and this may have occurred in our study.

In conclusion, this study confirms the favorable anti-epileptogenic effect of TQ pretreatment via lowering lipid peroxidation, inhibiting hippocampal neuronal loss and aberrant MFS, and its ability to mitigate astrogliosis in the kainate model of TLE. TQ benefits should be further studied as adjuvant therapy with other conventional anti-epileptic drugs such as valproate to reduce their doses and hence, their adverse effects.

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