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Coenzyme Q10 Ameliorates Neurodegeneration, Mossy Fiber Sprouting, and Oxidative Stress in Intrahippocampal Kainate Model of Temporal Lobe Epilepsy in Rat

Tourandokht Baluchnejadmojarad · Mehrdad Roghani

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Abstract Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults and the most resistant type to treatment. Novel treatment approaches are strongly required to prevent or even reverse the cellular and molecular mechanisms of epileptogenesis. In this study, we investigated the possible neuroprotective effect of coenzyme Q10 (CoQ10) in an intrahippocampal kainate model of TLE in rat. Kainate injection caused a higher seizure severity during status epilepticus and spontaneous seizure phases, and CoQ10 pretreatment significantly attenuated its severity and incidence rate. Intrahippocampal kainate also led to elevation of malondialdehyde (MDA) and nitrite, and CoQ10 significantly attenuated the increased MDA and nitrite content. In addition, intrahippocampal kainate induced a significant degeneration of neurons in CA1, CA3, and hilar regions of the hippocampus, and CoQ10 significantly attenuated these changes in CA1 and CA3 regions. Timm's staining data showed a robust mossy fiber sprouting (MFS) in dentate gyrus of kainate-lesioned rats and CoQ10 significantly lowered MFS intensity. These data suggest that CoQ10 pretreatment could attenuate spontaneous recurrent seizures and inhibit hippocampal neuronal loss and aberrant MFS in kainate-induced model of TLE in rat, and part of its beneficial effect is due to its potential to mitigate oxidative stress.

Keywords Coenzyme Q10 · Temporal lobe epilepsy · Kainic acid · Mossy fiber sprouting · Oxidative stress · Hippocampus

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

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

Introduction

Epilepsy is known as a common and chronic brain disorder with recurrent seizures due to excessive activity of the cerebral neurons (Curia et al. 2008). Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults, usually accompanied by hippocampal sclerosis, neurodegeneration, and extensive reorganization of hippocampal circuits (Jokeit and Schacher 2004). In about 40 % of patients with TLE, seizures are refractory to medical therapy (Curia et al. 2008). Although existing medications can symptomatically suppress seizures, there is little evidence that existing antiepileptic drugs could correct the underlying abnormal processes leading to epilepsy or alter its natural history (Dichter 2006; Stefan et al. 2006). Thus, novel treatment approaches are strongly required to prevent or even reverse the cellular and molecular mechanisms of epileptogenesis (Loscher and Schmidt 2006). A well-characterized animal model of TLE has been established through intrahippocampal unilateral injection of the excitotoxic glutamate analog kainic acid (KA) in rodents. This model is a "post status" model in which epilepsy develops after a chemically induced status epilepticus (Loscher 2002). Post-status epilepsy models are best suited for studying the efficacy of potential antiepileptic drugs because the latent period between the status and the first occurrence of spontaneous seizures makes it possible to test neuroprotective and prophylactic drugs against epilepsy (Sharma et al. 2007).

Coenzyme Q10 (CoQ10), also known as ubiquinone, is a fat-soluble vitamin-like substance (Mancuso et al. 2010) with potential efficacy in the treatment of disorders related primarily to suboptimal cellular energy metabolism and oxidative injury (Bonakdar and Guarneri 2005). CoQ10 has a pivotal role in mitochondrial bioenergetics (Littarru and Tiano 2007) and has been accepted as a promising neuroprotective agent in some neurodegenerative disorders including Huntington's and Parkinson's diseases (Mancuso et al. 2010). Neuroprotective effect of CoQ10 has partly been attributed to its free radical

scavenging and anti-apoptotic property (Papucci et al. 2003). In kainate-induced model of epilepsy, the level of lipid peroxidation increases and the level of CoQ10 decreases that per se aggravates the condition (Yalcin et al. 2004) and CoQ10 administration may reverse this condition. In addition, CoQ10 could protect hippocampal neurons against kainate neurotoxicity (Won et al. 2011) and its local application is capable to blunt cellular events leading to apoptosis and cell death in hippocampal CA3 subfield following status epilepticus (Chuang et al. 2009). On this foundation, this compound may be a good candidate for neuroprotection against kainate-induced excitotoxic insult. Until now, no evidence exists on the protective effect of systemic CoQ10 in intrahippocampal kainate model of TLE. Therefore, we decided to investigate its effect in intrahippocampal kainate model of TLE in rat.

Materials and Methods

All experiments were performed on adult male Wistar rats (280–320 g; n=48) (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 the number of animals used and their suffering.

Experimental Procedure

Rats were randomly divided into equal-sized vehicle-treated sham-operated- (sham), CoQ10-treated sham-operated- (sham + CoQ10), vehicle-treated kainate- (kainate), and CoQ10-treated kainate (kainate + CoQ10) 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 in the skull using the following stereotaxic coordinates according to the atlas of Paxinos and Watson (1986): anteroposterior, 4.3 mm caudal to bregma, 4.1 mm lateral to the midline (right side), and 4–4.2 mm ventral to the surface of the skull. A microsyringe filled with 5 μl of normal saline containing 0.8 μg/μl of kainate was placed over the burr hole and kainate solution was injected at a rate of 1 μl/min in order to induce experimental model of TLE. KA (Sigma-Aldrich, USA) was dissolved in cold normal saline just prior to surgery. The microsyringe was slowly withdrawn after 5 min and the rat scalp was sutured. The sham group received an equivalent volume of normal saline at the same stereotaxic coordinates. The sham + CoQ10 group received CoQ10 (Sigma-Aldrich, USA) i.p. at a dose of 10 mg/kg/day starting 1 week before surgery and the last treatment was 1 h before surgery. CoQ10 was dissolved in corn oil. The dose of CoQ10 was chosen according to previous reports (Rauscher et al. 2001) and our pilot study. The kainate + CoQ10 group received CoQ10 with the same protocol and then lesioned with KA.

Behavioral Assessment of Seizure

All animals were assessed for status epilepticus (SE) during the first 24 h post-surgery. At fifth week post-surgery, all animals were also evaluated for behavioral progression of kainate-induced seizures 4 h/day for five 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

Determination of Hippocampal MDA Concentration

The rats were anesthetized with diethyl ether and decapitated. Hippocampi were isolated and blotted dry, and then weighed and prepared as a 5 % 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 index, was calculated by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant as described previously (Roghani and Baluchnejadmojarad 2009). Briefly, trichloroacetic acid and TBARS reagent were added to aliquots of the supernatant, which were subsequently mixed and incubated at 90 °C for 80 min. After cooling on ice, the samples were centrifuged at 1,000×g for 10 min, and the absorbance of the supernatant was read at 532 nm. The results of TBARS measurements were expressed as MDA equivalents, using tetraethoxypropane as standard.

Assay of Hippocampal Nitrite Concentration

Supernatant nitrite (NO_2^-) content was assayed by the Griess method as described before (Baluchnejadmojarad and Roghani 2011). The compound NO has a short half-life and is rapidly converted to the stable end products nitrate (NO_3^-) and NO_2^-. In the assay used here, NO_3^- is converted to NO_2^- by cadmium, and this is followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium. The absorbance was determined using a spectrophotometer at 540 nm.

Measurement of Hippocampal SOD Activity

Superoxide dismutase (SOD) activity was measured as previously reported (Baluchnejadmojarad and Roghani 2011). Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 °C) for 40 min, and then nitro blue tetrazolium (NBT) was added. Thereafter, blue formazan was monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50 % maximum was defined as 1 nitrite unit of SOD activity.

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).

Histological Studies

Half of the animals in each group (n=4–5) were randomly used for histological assessment. For this purpose, the rats were deeply anesthetized with a high dose of 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 100–150 ml of fixative solution containing 4 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Following perfusion, the brains were removed from the skull and hippocampal blocks were prepared and immersed in 30 % sucrose in PB at 4 °C for 2–3 days. Then, sections were cut at a thickness of 40 μm on a freezing microtome (Leica, Germany) and collected in PB (0.1 M). Every second section was Nissl-stained with 0.1 % cresyl violet (Sigma) and alternate sections were used for Timm's 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.

To visualize mossy fiber sprouting (MFS) in the inner molecular layer of the dentate gyrus (DG) that accompanies epileptogenesis, we employed a modified Timm's histological 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's 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 thiosulfate. The sections were counterstained with 0.1 % cresyl violet, dehydrated, and coverslipped. Assessment of MFS (as Timm index) was obtained from the absolute value of the total area of Timm granules divided by the total length of DG (Wu et al. 2009). The Timm index for each animal was the mean of three sections. All procedures and analyses were done blind to the treatments.

Statistical Analysis

All statistical analysis was performed using SigmaStat software (version 3.5). Values 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 nonparametric Kruskal–Wallis test. Percentage of rats with spontaneous seizure was examined by x^2 test. In all analyses, the null hypothesis was rejected at a level of 0.05.

Results

All rats except for one rat from kainate + CoQ10 group well tolerated the experimental procedure until the end of the study.

Seizure Activity and Behavior

Sham and sham + CoQ10 groups showed no signs of seizure activity during the first 24 h post-surgery and/or after 4 weeks. In contrast, all rats (100 %) in kainate group exhibited high scores of seizures (status epilepticus) and 80 % of them had spontaneous seizures. In addition, rats injected with KA and pretreated with CoQ10 exhibited only mild behavioral signs (lower seizure scores) as compared to the kainate group. In this respect, only 40 and 22.2 % of such rats had signs of SE and spontaneous seizures and this difference was statistically significant versus kainate group (p<0.05 and p<0.01, respectively) (Table 1).

Oxidative Stress Markers

CoQ10 pretreatment of sham group did not cause a significant change in hippocampal level of MDA and nitrite and activity of SOD as compared to sham group. In contrast, kainate group showed a significant elevation of MDA (p<0.05) and nitrite content (p<0.01) and nonsignificant reduction of SOD activity. Meanwhile, pretreatment of kainate group with CoQ10

Table 1 Numbers and rates of animals with spontaneous seizures (at fifth week) and status epilepticus (during the first 24 h post-surgery) in experimental groups

	Number of animals with status epilepticus	Rate (%)	Number of animals with spontaneous seizures	Rate (%)
Sham	0/10	0	0/10	0
Sham + CoQ10	0/10	0	0/10	0
Kainate	10/10	100	8/10	80
Kainate + CoQ10	4/10	40*	2/9	22.2**

Animals were treated with CoQ10 at a dose of 10 mg/kg/day before intrahippocampal kainate injection

*$p<0.05$; **$p<0.01$ (as compared to kainate group)

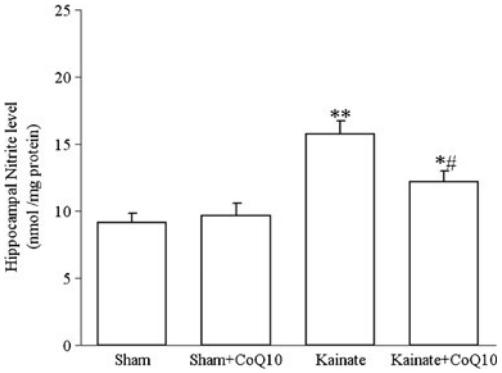

Fig. 2 Nitrite content in hippocampal homogenate from different groups. Animals were treated with CoQ10 at a dose of 10 mg/kg/day before intrahippocampal kainate injection. *$p<0.05$, **$p<0.01$ (in comparison with sham); #$p<0.05$ (in comparison with kainate) (means±SEM, $n=5–6$)

significantly attenuated the increased MDA and nitrite content ($p<0.05$). However, the level of SOD activity was slightly and nonsignificantly higher in kainate + CoQ10 as compared to kainate group (Figs. 1, 2, and 3).

Cytoarchitecture of the Hippocampus in Nissl Staining

In this study, the number of neurons per unit area in the CA1, CA3, and hilar regions was counted and compared among groups (Fig. 4). Our results showed that CoQ10 pretreatment of sham group did not produce any significant change in this regard. In contrast, intrahippocampal kainate induced a dramatic and significant degeneration and reduction of neurons in CA1 ($p<0.05$), CA3 ($p<0.01$), and hilar ($p<0.05$) 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 showed granule cell dispersion and displacement, and it was two- to threefold broader as compared to the contralateral side (non-injected side) in the upper border. Of interest, a few epileptic rats clearly showed greater neurodegeneration with more considerable loss of CA1 and CA3 pyramidal cell layers at certain anteroposterior levels. Furthermore, CoQ10 pretreatment of kainate group significantly attenuated these changes in CA1 and CA3 regions ($p<0.05$) and not in hilar region as compared to kainate group. These data suggest that CoQ10 pretreatment can protect and rescue the neurons of CA1 and CA3 regions against kainate neurotoxicity.

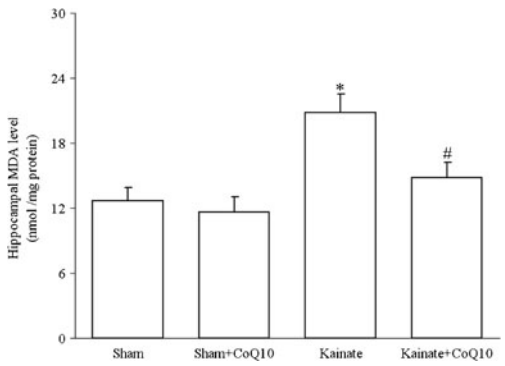

Fig. 1 Malondialdehyde (MDA) concentration in hippocampal homogenate from different groups. Animals were treated with CoQ10 at a dose of 10 mg/kg/day before intrahippocampal kainate injection. *$p<0.05$ (in comparison with sham); #$p<0.05$ (in comparison with kainate) (means±SEM, $n=5–6$)

Timm's Histochemistry

In this study, KA lesion-induced aberrant MFS was shown by Timm's method at the sixth week post-lesion. In the sham groups, little sprouting was present in the DG molecular layer. On the contrary, in the kainate group, Timm's staining showed robust MFS that extended into the dentate supragranular layer, and in the CoQ10-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's staining density (as indicated by Timm's index) between kainate and CoQ10-pretreated kainate groups and found that CoQ10 pretreatment could significantly reduce MFS width and staining density in the upper and lower blades of dentate gyrus ($p<0.05$). These data indicate that CoQ10 could restrain KA-induced aberrant MFS (Fig. 5).

Discussion

Temporal lobe epilepsy is a chronic and intractable neurological disorder with recurrent seizures due to the development of

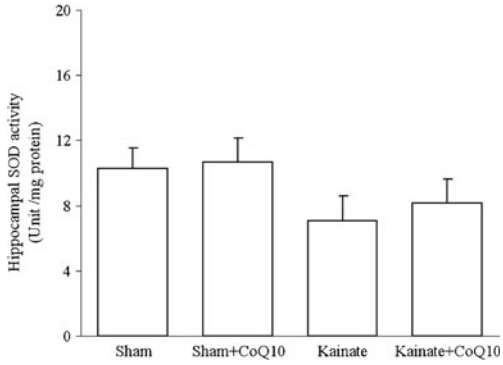

Fig. 3 Superoxide dismutase (SOD) activity in hippocampal homogenate from different groups. Animals were treated with CoQ10 at a dose of 10 mg/kg/day before intrahippocampal kainate injection (means±SEM, n=5–6)

recurrent excitatory or inhibitory circuits. Recurrent excitation and the development of seizures have also been associated with aberrant mossy fiber sprouting in the hippocampus (Sharma et al. 2007). Of the animal models used to investigate TLE, post-status epilepticus models have received the greatest acceptance because they are characterized by a latency period, the development of spontaneous motor seizures, and a spectrum of lesions like those of mesial TLE (Sharma et al. 2007). To this objective, intracerebral injection of kainate into the CA3 region of the hippocampus causes development of epileptic seizures. These seizures are followed by a pattern of cell loss that is similar to that seen in patients suffering from TLE (Ben-Ari and Cossart 2000). For this reason, KA-induced brain damage has been routinely used for modeling TLE and excitotoxic neurodegenerative disorders (Sperk 1994). Accumulating evidence indicates that hippocampal oxidative stress is also involved in KA-induced neurotoxicity (Shin et al. 2008). KA in general acts through three classes of ionotropic receptors, i.e., *N*-methyl-D-aspartate, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, and kainate receptors. Out of the different ionotropic glutamate receptors, those kainic acid receptors (KAR), containing the GluR6 subunit, are important in synaptic transmission as well as in the epileptogenic effects of kainate (Mulle et al. 1998). Therefore, these receptors are expected to be overexpressed in intrahippocampal kainate-induced TLE in the CA3 region, and this could promote seizures development and the ensuing pathology including degeneration of hippocampal neurons and an aberrant MFS into the inner molecular layer of the DG (Wu et al. 2009). Therefore, downregulation of KARs may inhibit the epileptogenesis by potent antiepileptic agents.

In this study, a massive neuronal loss was found out in the CA1, CA3, and hilar regions in the kainate group. Also, typical aberrant mossy fibers invading into the granule cell layer and granule cell inner molecular layer in the hippocampus of these rats were noticed. In this regard, mossy fibers originating from granule cells in the dentate gyrus undergo reorganization of their terminal projections in both human epilepsy and animal models of epilepsy (Wu et al. 2009). Timm's staining that selectively labels synaptic terminals of mossy fibers due to their high zinc content is an accepted method for the visualization of zinc-containing neuronal elements. Our observation concerning zinc histochemistry in the hippocampus was in agreement with the literature (Liu et al. 2012). These pathologic changes cause the formation of a functional recurrent excitatory circuit between granule cells that may have contributed to recurrent seizures in our study (Shetty and Hattiangady 2007; Wu et al. 2009).

CoQ10 is an important component of the mitochondrial electron transport chain and also a potent antioxidant that afford neuroprotection in some neurodegenerative diseases (Mancuso et al. 2010). CoQ10 is able to cross the blood–brain

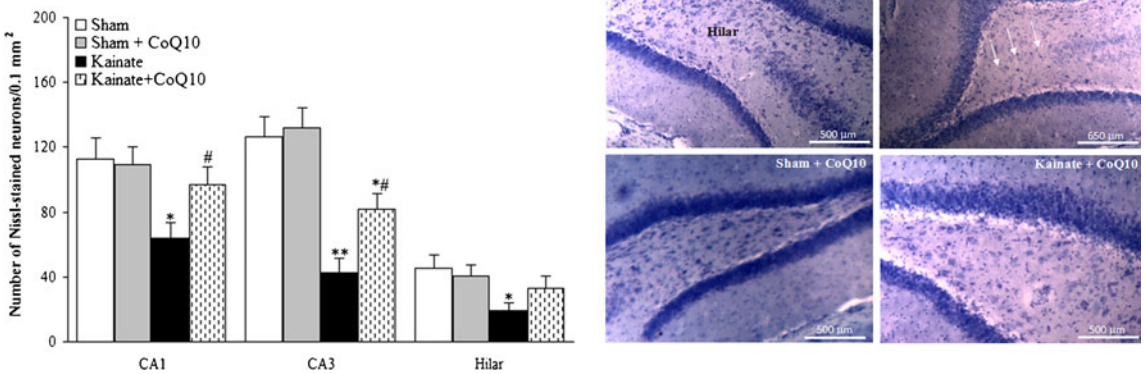

Fig. 4 Number of Nissl-stained neurons per unit area in different areas of the hippocampus (*left panel*) and a photomicrograph of coronal sections through the hippocampus (dentate region) showing Nissl-stained neurons in experimental groups (*right panel*). Animals were treated with CoQ10 at a dose of 10 mg/kg/day before intrahippocampal kainate injection. Cell dispersion is clearly observed on the upper border of dentate gyrus. *p<0.05, **p<0.01 (in comparison with sham); #p<0.05 (in comparison with kainate) (means±SEM, n=4–5)

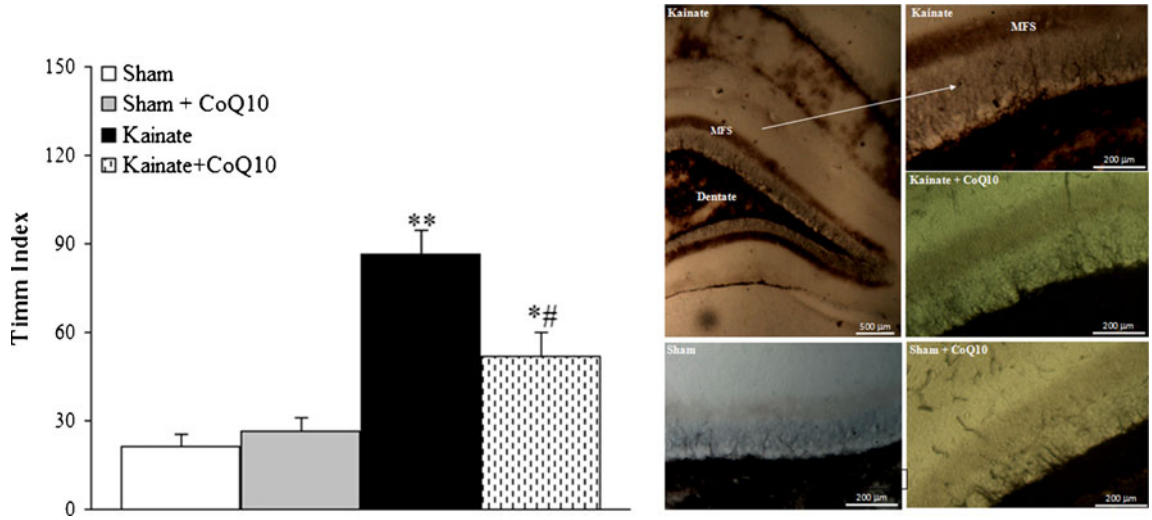

Fig. 5 Timm's index as an indicator of mossy fiber sprouting (MFS) intensity (*left panel*) and a photomicrograph through the hippocampus (dentate region), which shows MFS (*right panel*) in different groups $*p<0.01$, $**p<0.005$ (in comparison with sham); $\#p<0.05$ (in comparison with kainate) (means±SEM, $n=4–5$)

barrier to achieve effective concentrations in the brain to exert its neuroprotective effect (Abdin and Hamouda 2008). The neuroprotection afforded by CoQ10 may be far greater than that provided by antioxidants like vitamin E (Russo et al. 2008). Thus, it can be presumed that free radical scavenging ability of CoQ10 may play a minor role in its beneficial effect. In support of this idea, CoQ10 also exert neuroprotection in experimental models of neurodegenerative disorders including Alzheimer's and Parkinson's diseases whose pathogenesis implicates failure of mitochondrial energy metabolism (Abdin and Hamouda 2008; Orsucci et al. 2011; Yang et al. 2009; Young et al. 2007). Neuroprotective and anti-apoptotic effects of CoQ10 have somewhat been attributed to its ability to scavenge free radicals and to the inhibition of the mitochondrial permeability transition pore (PTP), a channel whose opening causes the mitochondrial membrane potential collapse that leads to apoptosis (Papucci et al. 2003). Compounds like CoQ10 could reduce the detrimental action of neurotoxins and/or excitotoxic agents on the mitochondrial energy metabolism, cellular bioenergetics (Yang et al. 2009), and the functioning of glutamate transporters (Sandhu et al. 2003), thus limiting accumulation of extracellular glutamate and preventing apoptotic death of neurons. In this respect, excessive activation of glutamate receptors via the excitotoxic cascade leads to the PTP formation and the release of cytochrome c, a member of the mitochondrial electron transport chain, from the mitochondrial inter-membrane space into the cytosol, where it also functions as a pro-apoptotic factor, leading to subsequent neuronal death (Kroemer and Reed 2000). Thus, CoQ10 could inhibit apoptosis by maintaining these pores in a closed conformation via a mechanism independent from its free radical scavenging property (Papucci et al. 2003).

Kainate-induced seizure model in the rat also accompanies inflammation with increased production 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 could reduce the severity of the condition (Ciceri et al. 2002). In parallel with this fact, it has been shown that kainate-induced excitotoxicity through induction of 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 relevant in related neurological disorders (Jourquin et al. 2003). On the other hand, CoQ10 is capable to exert anti-inflammatory properties via modulation of NFkappaB1-dependent gene expression (Schmelzer et al. 2008) and this may have occurred in our study which itself needs further investigation.

In our study, due to neuroprotective effect of CoQ10, there was lower degree of neuronal loss and MFS in the kainate + CoQ10 group than those of kainate one. Previous studies have shown that CoQ10 could ameliorate pilocarpine-induced seizure severity and protect against seizure-induced oxidative damage due to enhanced oxidative stress (Tawfik 2011). In addition, the neuroprotective effect of CoQ10 regarding hippocampal neurons against oxidative stress induced by kainate in hippocampal slice culture has also been shown (Won et al. 2011). In our study, CoQ10 pretreatment of kainate group attenuated oxidative stress burden as was evident by significantly lower levels of MDA and nitrite in the hippocampal tissue, and this have certainly protected hippocampal neurons against oxidative damage with subsequent lower MFS and less severe seizure activity. This study clearly suggests that CoQ10 could

protect against kainate-induced epilepsy by functioning as an antioxidant.

In conclusion, CoQ10 pretreatment could attenuate spontaneous recurrent seizures and inhibit hippocampal neuronal loss and aberrant MFS in kainate-induced model of TLE in rat, and part of its beneficial effect is due to its potential to mitigate oxidative stress burden.

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