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ORIGINAL PAPER

Thymoquinone and Vitamin C Attenuates Pentylenetetrazole-Induced Seizures Via Activation of $GABA_{B1}$ Receptor in Adult Rats Cortex and Hippocampus

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Abstract Epilepsy is a common neurological disorder that leads to neuronal excitability and provoke various forms of cellular reorganization in the brain. In this study, we investigate the anti-convulsant and neuroprotective effects of thymoquinone (TQ) and vitamin C against pentylenetetrazole (PTZ)-induced generalized seizures. Epileptic seizures were induced in adult rats using systemic intraperitoneal injections of PTZ (50 mg/kg) for 7 days. Animals pretreated with either TQ or vitamin C or in combination attenuated PTZ-induced seizures and mortality in rats as well neurodegeneration in the cells. Compared to PTZ, TQ and vitamin C significantly prolonged the onset of seizures ($p > 0.05$) as well decrease the high-grade seizures. Analysis of electroencephalogram (EEG) recordings revealed that TQ or vitamin C supplementation significantly reduced polyspike and epileptiform discharges. Epileptic seizures caused a decline in expression of gamma-aminobutyric acid B1 receptor ($GABA_{B1}R$) ($p > 0.05$), unchanged expression of protein kinase A (PKA), decreased calcium/calmodulin-dependent protein kinase II (CaMKII) ($p > 0.05$) and inhibit the phosphorylation of cAMP response element-binding protein (CREB) ($p > 0.05$) in cortex and hippocampus, respectively, compared with control. Changes in expression of $GABA_{B1}R$, CaMKII and CREB by PTZ were reversed by TQ and vitamin C supplementation. Moreover, PTZ significantly increased Bax, decreased Bcl-2 expression and finally the activation of caspase-3. TQ and vitamin C pretreatment reversed all these deleterious effects induced by PTZ. TQ and vitamin C showed anticonvulsant effects via activation of $GABA_{B1}R$/CaMKII/CREB pathway and suggest a potential therapeutic role in epilepsy.

Keywords Thymoquinone · Pentylenetetrazol · Seizures · Neuroprotection · Antiepileptic · Vitamin C

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Introduction

Epilepsy is one of the common and major neurological disorders characterized by recurrent seizures, behavioral and electroencephalographic (EEG) changes in the brain (Duncan et al. 2006; Haut et al. 2006). This neurological disorder consists of a number of medical complications and affects about 0.5–1.0 % of the human population worldwide (Andrade and Minassian 2007). Generally, the patients suffering from epilepsy have focal seizures and affecting both hemispheres of the brain due to hyper neuronal excitability and excessive hyper-synchronous discharges (Carey and Fuchs 2001; Zeng et al. 2007). Epileptic seizures are complex and involve a number of factors contributing to neuronal cell death. Now, it is widely recognized that seizures increase reactive oxygen species and cause activation of glutamate receptors, changes in γ-aminobutyric acid receptor and finally brain damage (Sudha et al. 2001; Xu and Stringer 2008; Costello and Delanty 2004; Haut et al. 2004; Fujikawa 2005). A variety of signaling pathways are involved in seizure-induced neuronal cell loss, long-term behavioral, cognitive dysfunction and apoptosis (Cendes 2005; Bouilleret et al. 2000; Engel and Henshall 2009).

Pentylenetetrazole (PTZ) a $GABA_A$ receptor antagonist, block chloride ionophore complex of the γ-aminobutyric acid $(GABA)_A$ receptor and is used as an acceptable model for kindling (Patsoukis et al. 2005; Macdonald and Barker 1978; Huang et al. 2001). Gamma-aminobutyric acid is a major inhibitory neurotransmitter in the brain and acts via different types of receptors $GABA_A$, $GABA_B$ and $GABA_C$ (Chebib and Johnston 1999). The $GABA_B$ receptors are composed of two sub-units $GABA_{B1}$ and $GABA_{B2}$, function as a dimmer and are responsible for G-protein coupling and modulate potassium channels and high-voltage calcium channel (Pin et al. 2004; Kulik et al. 2006). $GABA_B$ receptors (R) play an important role in the development of CNS and have a role in epilepsy model of genetic absence seizures (Hosford et al. 1992; Liu et al. 1992). PTZ has a convulsant effect via $GABA_A$ receptors and affecting the main GABAergic and glutamatergic systems (Huang et al. 2001). Alteration in $GABA_B$ receptor (R) expression disturbs the excitatory–inhibitory balance in the brain that may lead to the development of seizures (Leung et al. 2005; Li et al. 2003). However, little is known about the role of $GABA_{B1}R$ in epilepsy and its possible molecular mechanism.

Recently, the use of natural compounds and antioxidant is a major area of research in neuroprotection for different neurological disorders. Thymoquinone, the active constituent of *Nigella sativa* (NS) seeds, is a pharmacologically active quinone, possesses analgesic, anti-inflammatory and strong antioxidant activity (Abdel-Fattah et al. 2000; Houghton et al. 1995; Nagi and Mansour 2000). TQ protects against cell death induced by serum/glucose deprivation in PC12 cells and chronic toluene-induced neurodegeneration in the hippocampus (Mousavi et al. 2010; Kanter 2008). The antiepileptic effect of *N. sativa* oil and TQ has been reported in a PTZ-induced kindling mice model via mechanism involved reduction in oxidative stress and increase in GABAergic tune (Hosseinzadeh and Parvardeh 2004; Ilhan et al. 2005). Treatment with TQ is protective in transient forebrain ischemia and has antiepileptic effects in children with refractory seizures (Al-Majed et al. 2006; Akhondian et al. 2011). The other compound we used, vitamin C, is a powerful water soluble strong antioxidant and have protective effect in pentylenetetrazole and pilocarpine-induced seizures in rats (Gonzalez-Ramirez et al. 2010; Santos et al. 2008).

In this context, we investigate whether TQ and vitamin C are able to prevent the toxic effects of PTZ-induced seizures and brain damage in adult rats. Furthermore, we evaluated both neuroprotective and anticonvulsive profile of these two compounds in terms of seizure control and inhibition of neuronal cell death via modulatory effect of $GABA_{B1}$ receptor and its downstream signaling pathway.

Materials and Methods

Materials

Thymoquinone and vitamin C (Ascorbic acid) were purchased from Sigma Aldrich (3050 Spruce Street, Saint, Louis, MO USA). Fluoro-Jade B was purchased from (Chemicon International, Single Oak Drive Temecula CA, USA), and all other chemicals were of the highest analytical grade and purchased from commercial suppliers.

Animal Treatment

Adult Sprague–Dawley rats (250–300 g average body weight) were used in all experiments. These animals were housed in a room with temperature and humidity-controlled facility (Gyeongsang National University, Neurobiology Laboratory, Jinju, South Korea) under a 12:12 light/dark cycle, with free access to water and food. All the experimental procedures were approved by the animal ethics committee (IACUC ID:26) of the Division of Applied Life Sciences, Department of Biology, Gyeongsang National University, South Korea.

Drug Treatment and Experimental Groups

In the first set of experiments, a total 36 rats were randomly divided into six groups, six animals ($n = -6$) of each experimental group (1) Control group, consisting of six rats treated with 0.9 % saline intraperitoneally (i.p) (1 ml/kg saline); (2) PTZ group, treated with PTZ daily (50 mg/kg i.p. dissolved in 0.9 % saline for 7 days); (3) TQ group, treated with TQ (40 mg/kg administered orally by gavages for 7 days); (4) PTZ + TQ group, treated with (TQ 40 mg/kg administered orally 2 h prior to PTZ i.p. injection); (5) PTZ + vitamin C group, treated with (vitamin C 250 mg/kg i.p. 2 h before PTZ i.p. injection); and (6) PTZ + TQ + vitamin C group, treated with (vitamin C 250 mg/kg i.p. + TQ 40 mg/kg orally 2 h before PTZ). All the drug treatment was for 7 days and was freshly prepared before use.

Induction of Seizures and EEG Recordings

Seizures were induced using a maximum sub-convulsive dose of PTZ (50 mg/kg) i.p for 7 days. After each PTZ injection, rats were keep in isolated cages and behavior was observed at least for 10–30 min daily observed and data was recorded. All the experimental groups were carefully monitored for generalized seizures every day. The ensuing seizures were scored and classified, and their onset time, latency and mortality were noted. Convulsive behavior started with 3–5 min of PTZ injection. The (50 mg/kg)

dose was enough to generate convulsions in almost 90 % of the rats and intensified with repeated injections. Only the rats fully showed stage 4–5 seizures were included in this study. The resultant seizures were scored according to the Racine's scale of grading of convulsion with some modifications (Racine 1972; Erakovic et al. 2001). The seizures were classified according to some modified Racine scale (0 = immobility; 1 = facial automatism; 2 = head nodding; 3 = forelimb clonus; 4 = rearing; 5 = generalized convulsions, 6 = death). Two types of seizures were noted from grades 1–3 were regarded as low-grade seizures, and seizure grades 4–5 were regarded as high-grade seizures. Latency was defined as the average length of time in minutes between drug administration and seizure onset. Daily after PTZ injection, the seizures onset time was noted, and then, a cumulative data were calculated for 7 days. At the end of 7-day observation, the rats for EEG analysis were anesthetized with ketamine (70 mg/kg i.p.) and pentobarbital (25 mg/kg i.p). A two-channel scalp EEG was performed, and data were recorded for 30 min using amplifiers and a Laxtha digital EEG monitoring device (model no, LXEJ 108) and were digitized at 250 Hz in EEG recording room according to previous methods with some modifications (Greggio et al. 2009; Naseer et al. 2009). Whole EEG samples were analyzed by visual inspection for the presence of epileptiform activity.

Western Blot Analysis

The expression of various proteins was measured in the different groups by Western blot analysis. Animals for Western blot analysis were killed by decapitation after sedation by an i.v. injection of pentobarbital sodium (3 mg/100 g b.w.). After 24 h of last PTZ injection, rats were killed followed by a rapid removal of the brains; the cortex and hippocampus were carefully dissected and frozen on dry ice. The brain tissues were homogenized in 0.2 M phosphate-buffered saline (PBS) containing a protease inhibitor cocktail. The protein concentration was measured using the Bio-Rad protein assay kit (Bio-Red Laboratories, CA, USA). Equivalent amounts of total protein (30 µg per sample) were electrophoresed through a 10–15 % SDS-PAGE gel under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Prestained protein markers, broad range (7–240 kDa, multicolor, Elpis Biotech, Daejeon, Korea) were run in parallel for estimation of the molecular weights of the proteins. The membrane was blocked with 5 % (w/v) skimmed milk. The membranes were incubated with primary antibodies rabbit-derived anti-caspase-3, anti-phospho-CREB (Ser133), anti-CREB, anti-CaMKII (dilution used, 1:500; Cell Signaling Technology Danvers, MA, USA), and rabbit-derived anti-PKAα, anti-$GABA_{B1}R$, anti-Bax, anti-Bcl-2 (dilution used, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-actin (dilution used, 1:2,000; Sigma, St. Louis, MO, USA) were used in immunoblotting experiments and incubated overnight at 4 °C with the mentioned primary antibodies. Membranes were washed, probed with horseradish peroxidase-conjugated secondary antibody, and immunocomplexes were visualized using enhanced chemiluminescence ECL-detecting reagent spray (Westone™, Western blotting detection system, Intron Biotechnology, Arizona USA). The X-ray films were scanned, and the optical densities of the bands were analyzed by densitometry using the computer-based Sigma Gel program, version 1.0 (SPSS, Chicago, IL, USA).

Tissue Collection and Sample Preparation for Histological Staining

The animals were killed after 7 days of PTZ last injection. Brain sections from control rats and rats subjected to PTZ and or TQ pretreated or vitamin C pretreated were used for staining. For tissue analysis ($n = 3$ animals per group), the rats were anesthetized with ketamine (70 mg/kg i.p.) and pentobarbital (25 mg/kg i.p.) and then perfused transcardially with 4 % ice-cold paraformaldehyde followed by 1X PBS; the brains were post-fixed overnight in 4 % paraformaldehyde and then transferred to 20 % sucrose until they sank to the bottom of the tube. The brains were frozen in O.C.T compound (Sakura, CA, USA), and 18-µm sections were cut in the coronal plane using a CM 3050C cryostat (Leica, Germany). The sections were thaw-mounted on probe-on plus charged slides (Fisher, USA).

Fluoro-Jade B Staining

Fluoro-Jade B staining was performed as previously described with some modifications (Sas et al. 2008). Two hours after final PTZ injection, the animals were deeply anesthetized with ketamine (70 mg/kg i.p.) and pentobarbital (25 mg/kg i.p.), and EEG was performed then those rats were transcardially perfused with 0.9 % saline followed by ice-cold 4 % paraformaldehyde in 0.1 M PBS. Brain sections (prepared as described above) were mounted on slides and then air-dried overnight. The slides were washed with PBS twice for 15 min and then permeabilized with 0.1 % triton solution for 2 min followed by PBS washing. Then, slides were immersed in a solution of 1 % sodium hydroxide and 80 % ethanol for 5 min and then in 70 % alcohol for 2 min, followed by 2 min in distilled water. The slides were transferred to 0.06 % potassium permanganate solution for 10 min in shaking condition in dark followed by rinsed in water and then immersed in a solution of 0.1 % acetic acid and 0.01 % FJB (Chemicon

International, USA) for 20 min on shaker. The slides were washed twice in distilled water and allowed to dry for 10 min. Glass cover slips were mounted on the slides using mounting medium. A confocal laser scanning microscope (Fluoview FV 1000, Olympus) was used to detect the green FJB stain using FITC filter. FJB-positive cells in the various regions of each section were counted by observers who were blind to the treatment condition.

Cresyl Violet Staining

Cresyl violet was used to stain tissue sections for histological examination and measurement of neuronal loss according to our previous protocol (Ullah et al. 2011). Nissl histology of adult rat brains and the presence or absence of dead and injured neurons was analyzed in 18-µm-thick brain sections mounted on microscope slides. Sections prepared from all five experimental groups of adult rats were defatted in ascending alcohol concentrations (70–100 %), hydrated in descending alcohol concentrations (95–70 %), washed in acetate buffer (pH 5.0) and then stained with 0.25 % cresyl violet for approximately 30–50 min. The sections were then washed with distilled water and dehydrated in graded ethanol. The images were prepared with a fluorescent light microscope, and cells were manually counted and statistically analyzed.

Data Analysis and Statistics

The bands from Western blots were scanned and analyzed by densitometry using the Sigma Gel System (SPSS Inc., Chicago, IL). The density values are expressed as the mean ± SEM. Group differences were analyzed using a one-way analysis of variance (ANOVA) followed by Student's t test using Prism 5 software. Differences with a p value <0.05 were considered statistically significant.

Results

Behavioral Observations and EEGs During Seizures

We investigated whether TQ and vitamin C pretreatment could delay or attenuate generalized seizures induced by a sub-convulsive dose of PTZ. The cumulative data from 7-day treatment were analyzed. Seizure induced by PTZ in adult rats typically started with hind limb kicks, followed by generalized tonic and clonic convulsion of four limbs

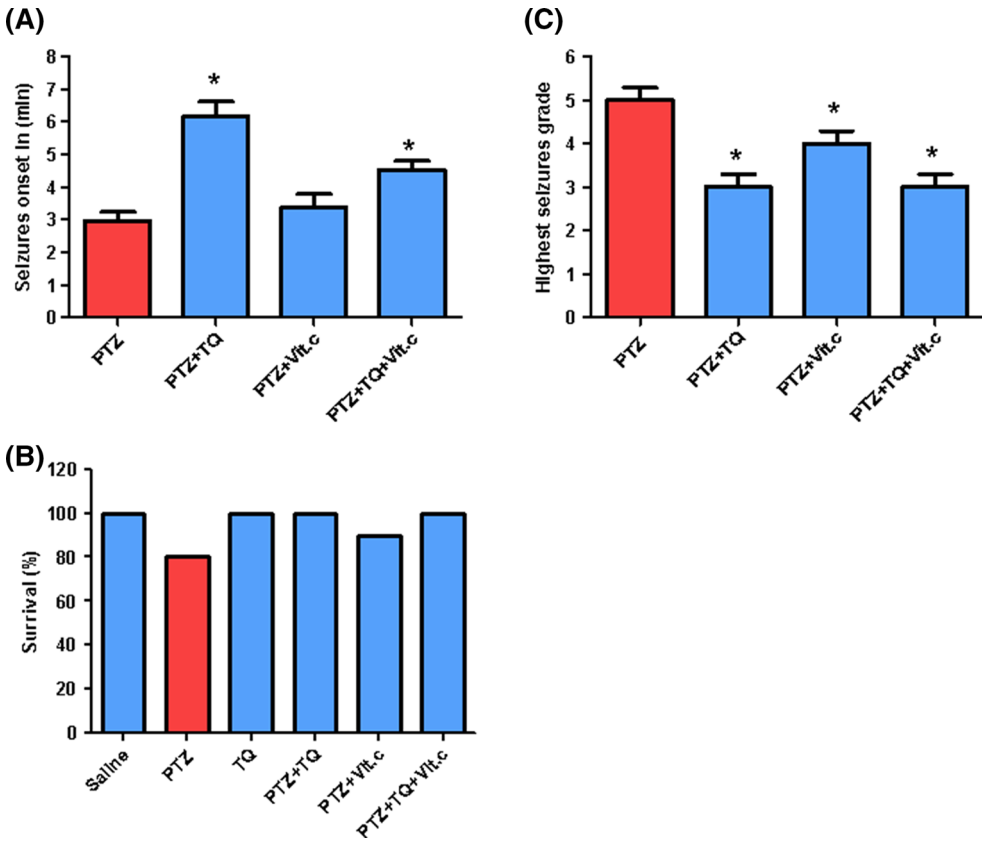

Fig. 1 TQ and vitamin C delay the onset of PTZ-induced seizures. Rats were treated with saline (i.p $n = 6$), PTZ (50 mg/kg for 7 days), TQ (40 mg/kg administered orally for 7 days), TQ + PTZ (TQ 40 mg/kg 2 h before PTZ treatment), vitamin C + PTZ (vitamin C 250 mg/kg 2 h before PTZ treatment), PTZ + TQ + vitamin C (vitamin C 250 mg/kg + TQ 40 mg/kg 2 h before PTZ treatment). **a** Delay in (min) in onset of seizures **b** Mortality induced by PTZ in different groups **c** Severity of seizures and stages according to the Racine scale. Statistics: A cumulative 7-day data were analyzed, and significant difference is denoted ($p > 0.05$)

while lying down and even mortality in some animals. Pretreatment with TQ and vitamin C significantly increased the number of surviving animals compared with PTZ treatment alone. In the PTZ-treated group, two rats die out of six showed almost 33 % mortality where in vitamin C group 16 % mortality (Fig. 1b). The TQ pretreatment increased animal survival to 100 % where no mortality observed (Fig. 1b). The latency of seizures increased from 2.9 min in PTZ-treated group to 7 min in TQ + PTZ-treated group, to 9 min in TQ + vitamin C + PTZ group, and there was no significant difference in vitamin C + PTZ group (Fig. 1a). However, the PTZ-treated group achieved the highest grade seizure (Stage 5), while TQ and vitamin C decrease the frequency of high-grade seizures as shown in (Fig. 1c). To confirm the seizure, EEG was monitored for 30 min started 15 min after the PTZ injection. The results showed that rats subjected to PTZ, bursts of polyspikes and spike wave complexes, while TQ pretreatment with the selected dose 40 mg/kg, vitamin C 250 mg/kg effectively reduced the abnormality in EEG. The results for the evaluation of EEG monitoring are summarized in (Fig. 2).

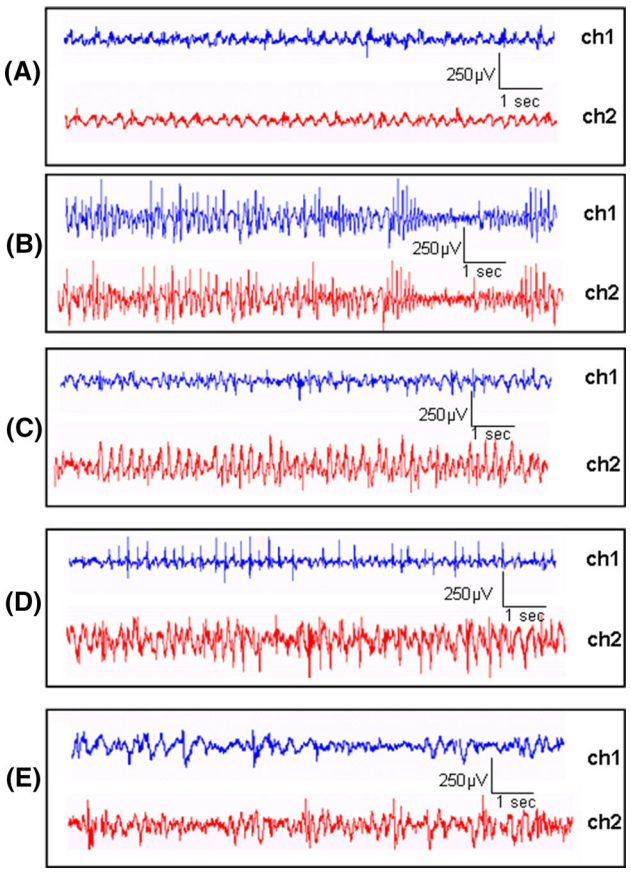

Fig. 2 Electroencephalographic (EEG) recordings from adult rats after a 7-day drug treatment. Representative 30 s EEG samples recorded 15 min after final day seventh injection of PTZ. A two-channel scalp EEG (ch1, channel 1; ch2, channel 2). **a** Showed normal EEG in saline group **b** EEG after seventh PTZ (50 mg/kg) i.p. injection **c** EEG after 7 days for TQ + PTZ (TQ 40 mg/kg 2 h before PTZ treatment) **d** after 7 days for vitamin C + PTZ (vitamin C 250 mg/kg 2 h before PTZ treatment) **e** PTZ + vitamin C + TQ (vitamin C 250 mg/kg + TQ 40 mg/kg 2 h before PTZ treatment)

TQ and Vitamin C Treatment Increases the Expression of $GABA_{B1}$ Receptors, Unchanged PKA-α and Increase CaMKII

Next, we investigated the effects TQ and vitamin C on expression of $GABA_{B1}R$, PKA-α and CaMKII level in cortex and hippocampus. Western blot analysis showed that PTZ dramatically decreased the level of $GABA_{B1}R$, unchanged the expression of PKA-α and decreased the expression of CaMKII in both regions of the brain cortex and hippocampus, respectively (Fig. 3a, b). Pretreatment with TQ or vitamin C or combination of both significantly increased the expression of $GABA_{B1}R$ and CaMKII. Furthermore, from Western blot analysis, no marked difference was observed with regard to the expression of PKA-α among the control, PTZ, TQ and vitamin C-treated groups in both brain regions (Fig. 3a, b).

PTZ-altered the Expression and Phosphorylation of CREB Protein

A number of kinases including protein kinase A (PKA), MAPK and CaMKII activate CREB phosphorylation. The cAMP-responsive element-binding protein (CREB) pathway has been involved in two major cascades of gene expression, as a critical component of the molecular switch that controls long-lasting forms of neuronal plasticity and learning, also involved in neuronal survival and protection (Jancic et al. 2009). The phosphorylation of the CREB protein was examined in all experimental groups using an antibody that detects Ser^{133}-phosphorylated CREB protein. Phosphorylated form of CREB was significantly decrease by PTZ in both hippocampus and cortex. The total CREB protein level was unchanged with PTZ treatment in both cortex and hippocampus. However, animals pretreated with TQ and vitamin C have increased the phosphorylation of CREB at Ser^{133} in both cortex and hippocampus irrespective to the total CREB level as shown in (Fig. 4a, b).

TQ and Vitamin C Regulate Bax and Bcl-2 During Seizures

To confirm that CaMKII-mediated CREB phosphorylation by TQ and vitamin C responsible for the induction of Bcl-2 and may be contributing to cell survival after PTZ-induced seizures. So for that purpose to evaluate the role of pro-apoptotic Bax and anti-apoptotic Bcl-2 Western blot was carried out. The Bcl-2 family of proteins regulates the

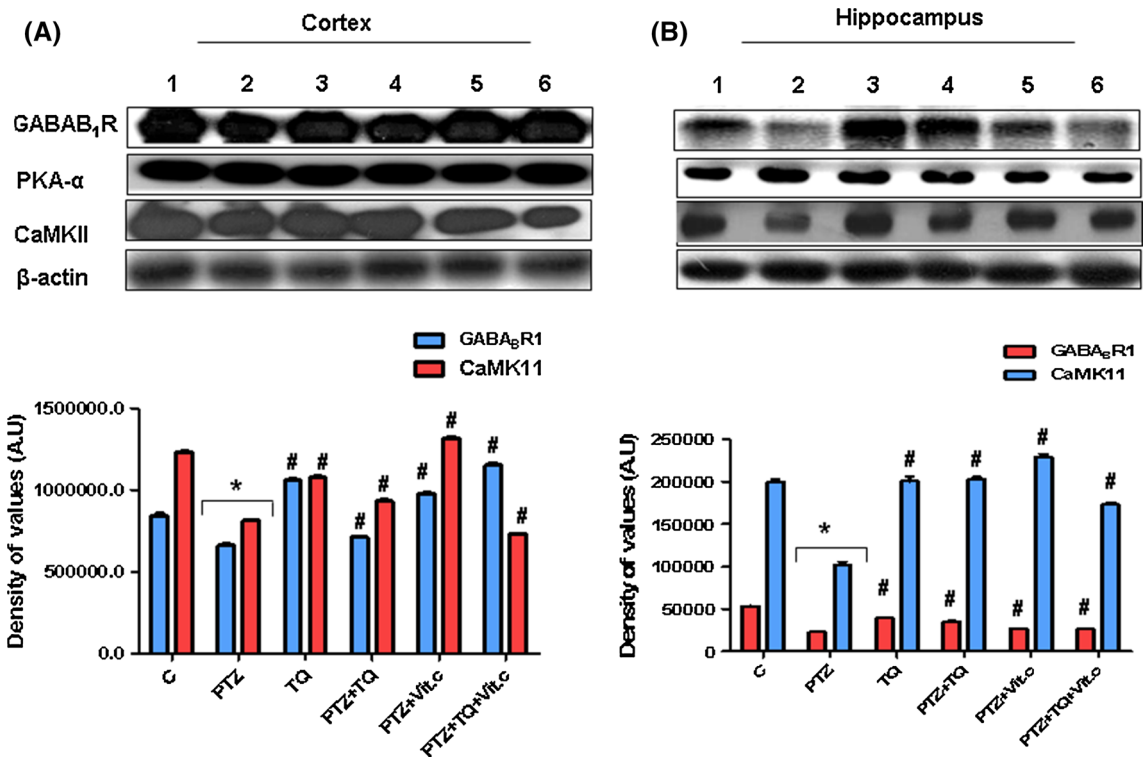

Fig. 3 Effect of TQ and vitamin C on the expression $GABA_{B1}$ receptors, PKA-α and CaMKII in cortex and hippocampus. Rats were treated with saline (1) (i.p n = 3), (2) PTZ (50 mg/kg for 7 days) (3) TQ (40 mg/kg administered orally for 7 days) (4) TQ + PTZ (TQ 40 mg/kg 2 h before PTZ treatment) (5) vitamin C + PTZ (vitamin C 250 mg/kg 2 h before PTZ treatment), (6) PTZ + TQ + vitamin C (vitamin C 250 mg/kg + TQ 40 mg/kg 2 h before PTZ treatment). **a** $GABA_{B1}R$, PKA-α and CaMKII levels were determined by immunoblots analysis with their relevant primary antibodies in cortex represented along with their histograms. The loading was normalized with β-actin as a loading control **b** $GABA_{B1}R$, PKA-α and CaMKII levels in hippocampus along with their histograms. The density values are expressed (in A.U.) as the mean ± SEM. *Significantly different from C (control; #significantly different from PTZ). Significance = $p < 0.05$

intrinsic pathway that control events upstream of mitochondrial dysfunction. Epileptic seizures decrease the expression of Bcl-2 and led to increase the level of Bax both in cortex and hippocampus. Pretreatment with TQ and vitamin C or combination of both reverse the trend by upregulating Bcl-2 and inhibition of Bax (Fig. 5a, b).

Effect of TQ and Vitamin C on PTZ-Induced the Activation of Caspase-3

Caspase-3 is one of the major proteins and in evident hallmark of apoptosis. The inhibition of caspase-3 by TQ and vitamin C pretreatment was confirmed by Western blot analysis with and with out PTZ. In the PTZ-treated group, the expression of active caspase-3 was significantly elevated both in cortex and hippocampus. Pretreatment with TQ and vitamin C decreased the expression of active caspase-3 in both cortex and hippocampus, respectively (Fig. 6a, b). Taken together, these findings indicate that the activation of caspase-3 induced by PTZ was inhibited by TQ and vitamin C treatment and suggesting that these compounds have the potential to protect adult rat brain from the deleterious effects of seizures.

TQ and Vitamin C Pre-treatment Decreases PTZ-Induced Neurodegeneration

PTZ-induced generalized seizures are known to induce neurodegeneration in brain areas such as cortex and hippocampus. To detect whether PTZ-induced seizures resulted in neuronal death, FJB staining, was performed because it is a reliable marker for neuronal vulnerability (Anderson et al. 2005). The anionic fluorochrome FJB was used to stain degenerating neurons at various time points after PTZ administration. TQ and vitamin C pretreatment attenuated seizures in vivo and protected hippocampal neurons from PTZ-induced neurodegeneration. Animals pretreated with TQ or vitamin C, or combination of both had significantly less neuronal damage in the cortex and CA1 region of hippocampus, respectively

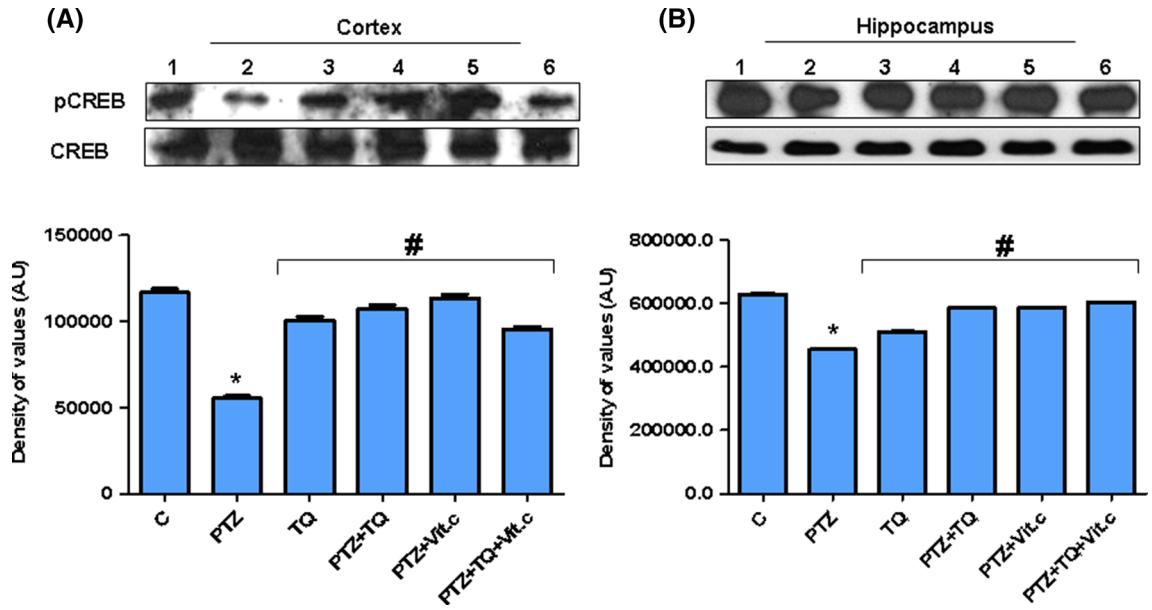

Fig. 4 PTZ-induced seizure altered the phosphorylation of CREB protein. Rats were treated with saline (1) (i.p n = 3), (2) PTZ (50 mg/kg for 7 days) (3) TQ (40 mg/kg administered orally for 7 days) (4) TQ + PTZ (TQ 40 mg/kg 2 h before PTZ treatment) (5) vitamin C + PTZ (vitamin C 250 mg/kg 2 h before PTZ treatment), (6) PTZ + TQ + vitamin C (vitamin C 250 mg/kg + TQ 40 mg/kg 2 h before PTZ treatment). **a** pCREB and total CREB levels were determined by immunoblots analysis with their relevant primary antibodies in cortex represented along with their histograms. The loading was normalized with total CREB as a loading control **b** pCREB and total CREB levels in hippocampus along with their histograms in hippocampus. The density values are expressed (in A.U.) as the mean ± SEM. *Significantly different from C (control; #significantly different from PTZ). Significance = $p < 0.05$

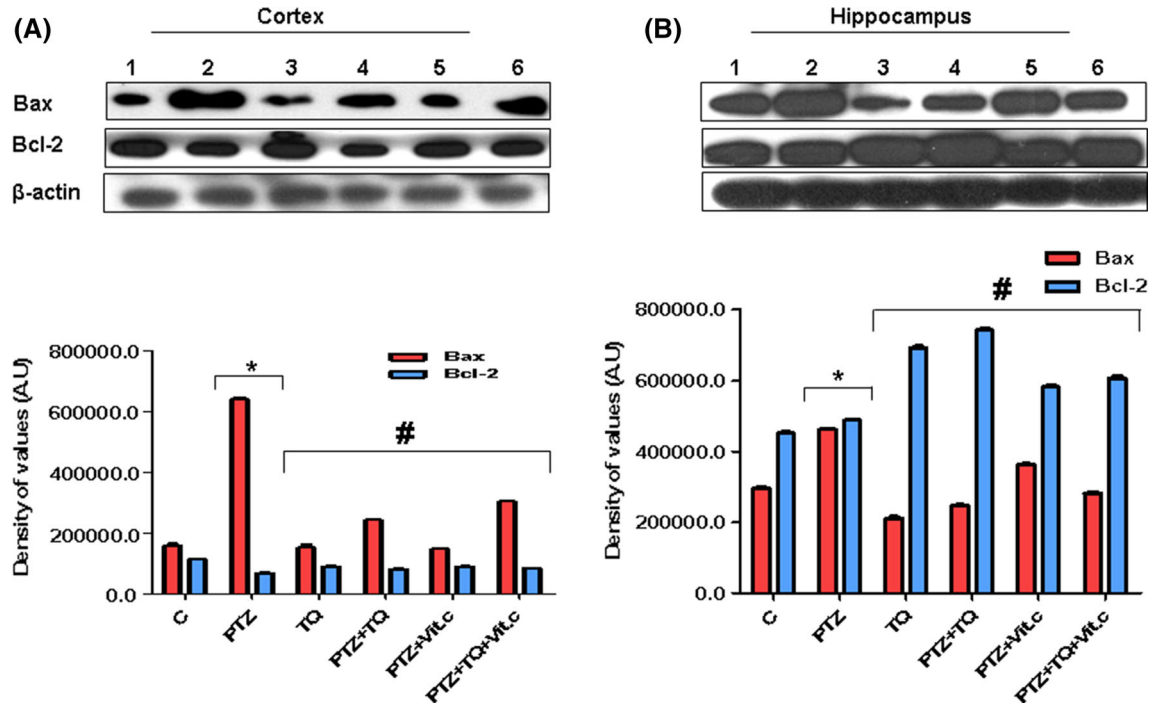

Fig. 5 Effect of TQ and vitamin C on the expression of Bax and Bcl-2 in PTZ-induced seizures in cortex and hippocampus. Rats were treated with saline (1) (i.p n = 3), (2) PTZ (50 mg/kg for 7 days) (3) TQ (40 mg/kg administered orally for 7 days) (4) TQ + PTZ (TQ 40 mg/kg 2 h before PTZ treatment) (5) vitamin C + PTZ (vitamin C 250 mg/kg 2 h before PTZ treatment), (6) PTZ + TQ + vitamin C (vitamin C 250 mg/kg + TQ 40 mg/kg 2 h before PTZ treatment). **a** Bax and Bcl-2 levels were determined by immunoblots analysis with their relevant primary antibodies in cortex represented along with their histograms. The loading was normalized with β-actin as a loading control **b** Bax and Bcl-2 levels along with their histograms in hippocampus. The density values are expressed (in A.U.) as the mean ± SEM. *Significantly different from C (control; #significantly different from PTZ). Significance = $p < 0.05$

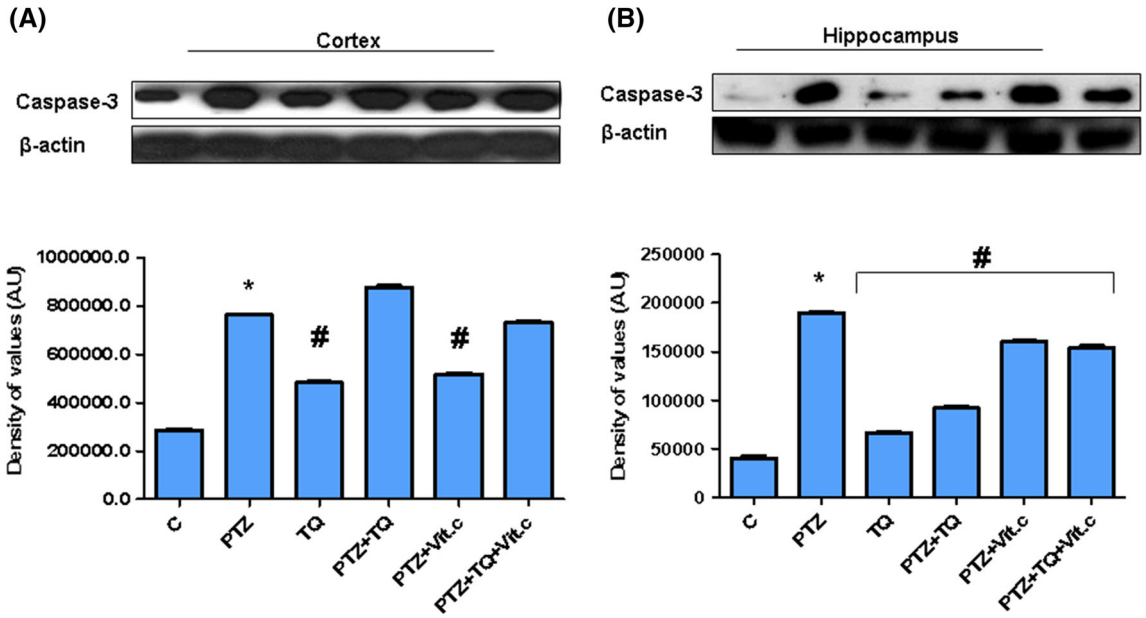

Fig. 6 Effect of TQ and vitamin C on PTZ induced the activation of caspase-3. Rats were treated with saline (1) (i.p n = 3), (2) PTZ (50 mg/kg for 7 days) (3) TQ (40 mg/kg administered orally for 7 days) (4) TQ + PTZ (TQ 40 mg/kg 2 h before PTZ treatment) (5) vitamin C + PTZ (vitamin C 250 mg/kg 2 h before PTZ treatment), (6) PTZ + TQ + vitamin C (vitamin C 250 mg/kg + TQ 40 mg/kg 2 h before PTZ treatment). **a** Caspase-3 level was determined by immunoblots analysis with their relevant primary antibodies in cortex represented along with their histograms. The loading was normalized with β-actin as a loading control **b** caspase-3 level along with their histograms in hippocampus. The density values are expressed (in A.U.) as the mean ± SEM. *Significantly different from C (control; #significantly different from PTZ). Significance = $p < 0.05$

compared with PTZ group. A very few cells were FJB positive in saline group while PTZ-treated animals have extensive cell damage in hippocampus and cortex (Fig. 7 Panels c, d). Rats pretreated with TQ and vitamin C along with PTZ had a significant decrease in number of FJB-positive cells both in cortex and hippocampus (Fig. 7 Panels e–j). The number of FJB-positive cells was quantitatively analyzed in each group and statistically shown in histogram (Fig. 7).

Seven days after treatment, Nissl staining was performed to examine the extent of neuronal cell death induced by PTZ and to assess protection by TQ and vitamin C with in the cortex, hippocampus of the adult rat brain. We found clear indications of dead neuronal cells (i.e., cells containing small or large condensed, fragmented, dark-blue nuclei and apoptotic bodies) in the cortex and hippocampus of PTZ-treated group. There was a significant increase in the extent of neuronal cell loss, vacuolization and tissue breakdown in these brain regions in PTZ-treated animals (Fig. 8 Panels c, d), whereas these regions appeared morphologically normal in control animals. Pretreatment with TQ and vitamin C in the selected doses with PTZ led to significantly less vacuolization and neuronal loss in these brain regions as well as showed normal cells morphology (Fig. 8 Panels e–j).

Discussion

The main findings of our present study demonstrated that PTZ-induced epileptic seizures in adult rats initiate a chain of events associated with generalized seizures, abnormal EEG discharges, biochemical and morphological evidence of neuronal apoptosis. Further, PTZ decreases the expression of $GABA_{B1}R$, calcium/calmodulin-dependent protein kinase II (CaMKII) and phosphorylation of CREB. So these events open a window for a neuroprotective approach in such kind of scenario. We observed that thymoquinone (TQ) and vitamin C administration protects against PTZ-induced seizures via a mechanism involved the $GABA_{B1}R$/CaMKII/pCREB signaling pathway. We have shown that TQ and vitamin C are effective in regulating $GABA_{B1}R$ and phosphorylation of CREB, which in turn induce the expression of Bcl-2 and play a major role in neuronal survival.

The balance between excitatory and inhibitory neurotransmitters in the central nervous system is one of the important ways to keep the normal brain function. The disruption of this balance leads to abnormal neuronal activity and finally epileptic episodes. Gama-aminobutyric acid is the main inhibitory neurotransmitter in mammalian brain acts on GABA type A ($GABA_A$) and type B

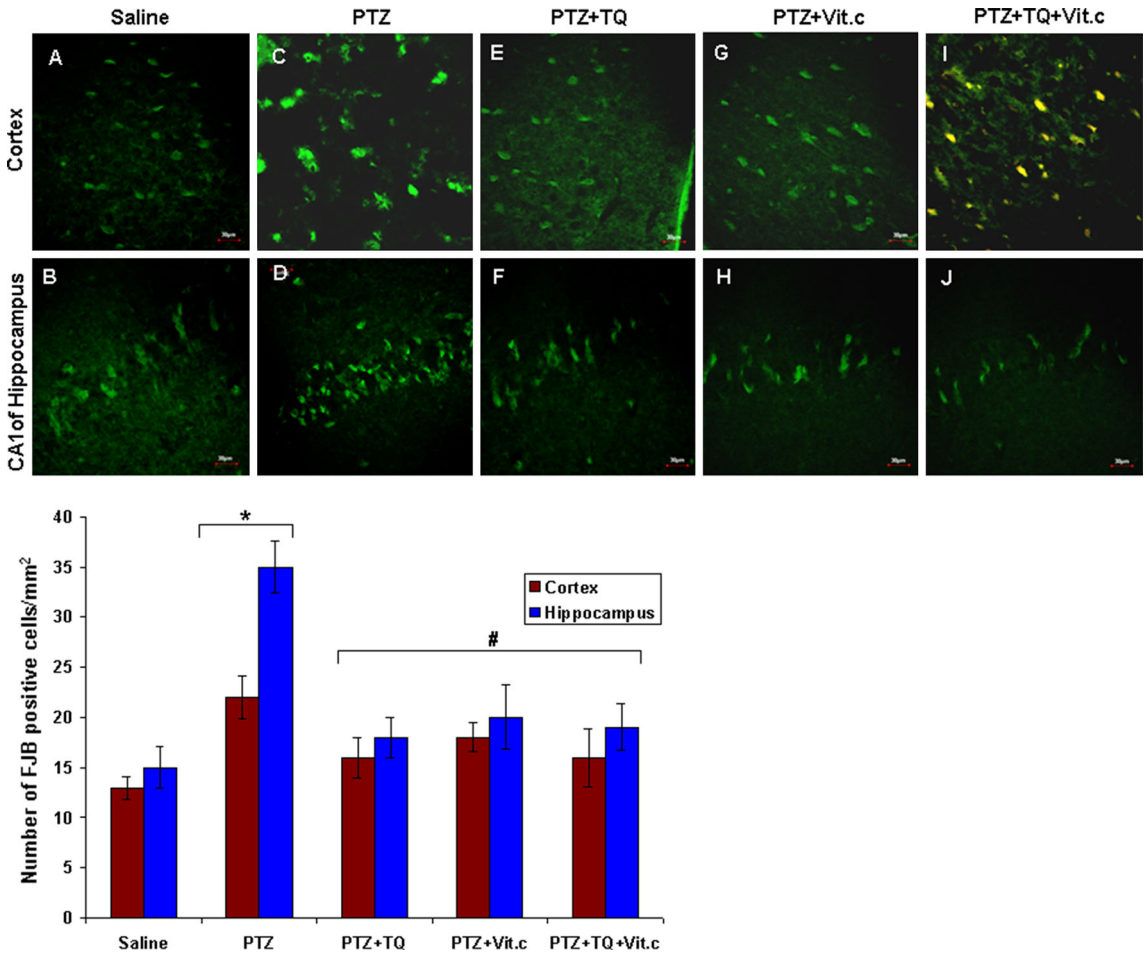

Fig. 7 The effect of TQ and vitamin C pretreatment on PTZ-induced neurodegeneration. The representative photomicrographs of FJB staining in cortex and hippocampus for damaged and dead neuronal cells after PTZ treatment with and/or with out TQ and vitamin C. The images are representative of staining obtained in sections prepared from at least three animals/group. Panels (**a**–**b**) saline group (with a 60× objective), Panels (**c**–**d**) PTZ group with a 60× objective, Panels (**e**–**f**) TQ + PTZ group (40 mg/kg TQ 2 h before PTZ treatment), Panels (**g**–**h**) PTZ + vitamin C group (250 mg/kg vitamin C 2 h before PTZ treatment for a total 7 days), Panels (**i**–**j**) Combine group TQ + PTZ + vitamin C. The *scale bar* = 30 µm. Down is the quantitative analysis of histopathology expressed as a mean of degenerating neurons and number of FJB-positive cells in CA1 of hippocampus and cortex. Statistics: *Significantly different from C (control; #significantly different from PTZ). Significance = $p < 0.05$

($GABA_B$) receptors (Chebib and Johnston 1999; Vollenweider et al. 2006). GABAB receptors, heterodimeric G-protein-coupled receptors composed of $GABA_{B1}$ and $GABA_{B2}$, act presynaptically by inhibiting Ca^{2+} channels and postsynaptically by regulating inwardly rectifying K^+ channels (Pin et al. 2004; Kulik et al. 2006). It is demonstrated that mice lacking functional GABAB receptors develop complex generalized epilepsies (Brown et al. 2003; Schuler et al. 2001). The current study demonstrates significant reduction in $GABA_{B1}R$ expression in both cortex and hippocampus of PTZ-treated rats. On the other hand, TQ and vitamin C treatment maintain the expression of $GABAB_1R$ in both brain regions and showed neuroprotection via phosphorylation of CREB (Fig. 3a, b). A similar pattern of neuroprotection through $GABAB_1R$ is demonstrated in a number of animal models using GABAB receptor agonist and a number of natural products (Naseer et al. 2013). Decreased expression of $GABA_B$ receptors have been observed in autism, cerebral ischemia and activation of these receptors provide neuroprotection through different signaling pathways (Oblak et al. 2010; Cheng et al. 2010; Tu et al. 2010).

A major factor in our current hypothesis is the decreased expression of $GABA_{B1}R$ during PTZ-induced seizures leads to neuronal cell death. So to explore the downstream targets of $GABA_{B1}R$ like calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA) and the phosphorylation of CREB were investigated using Western blot analysis. The level of CaMKII was profoundly increased in TQ and vitamin C pretreated rats

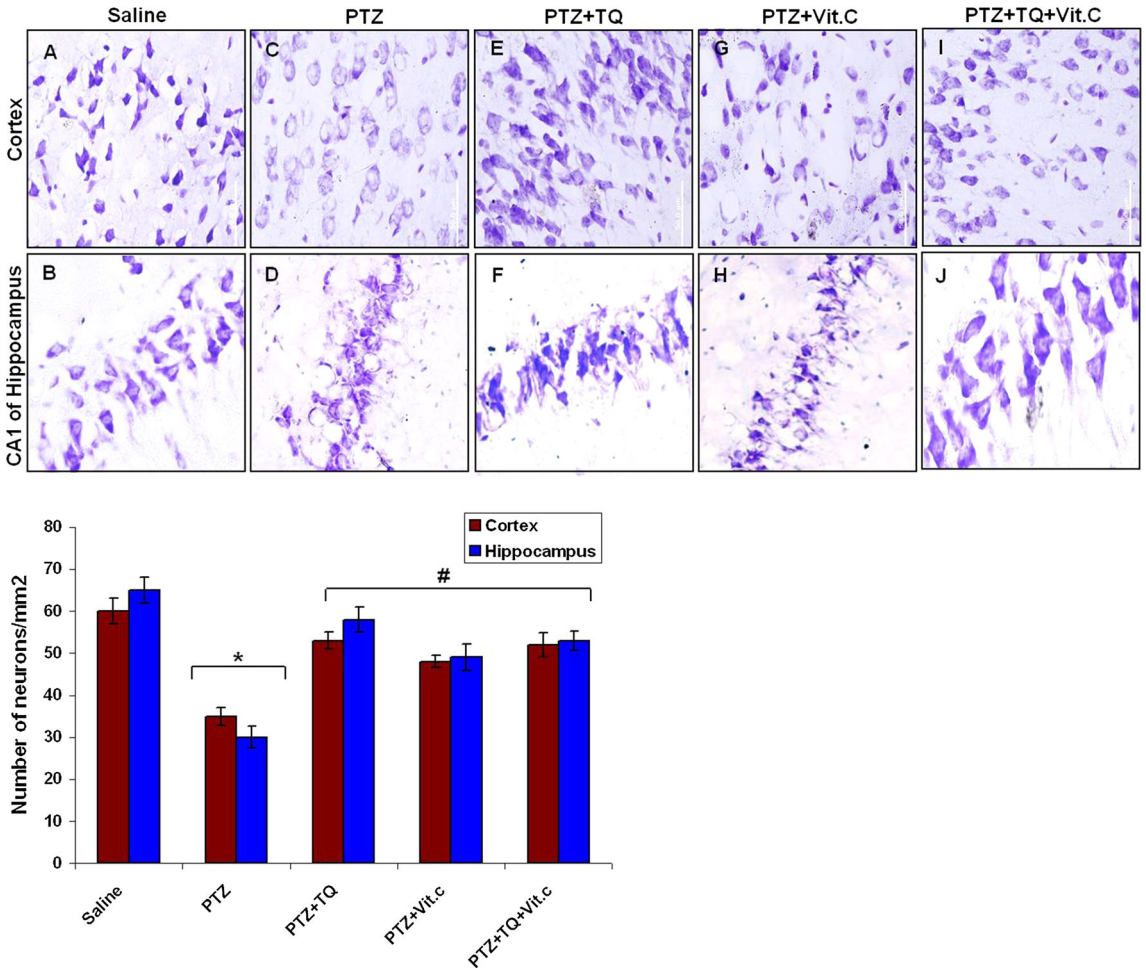

Fig. 8 The effect of TQ and vitamin C pretreatment on PTZ-induced neurodegeneration. The effect of TQ and vitamin C pretreatment on PTZ-induced neurodegeneration. The representative photomicrographs of cresyl violet-stained slices, showing damaged and dead neuronal cells after PTZ administration with and/or without TQ and vitamin C or combination of both. The images are representative of staining obtained in sections prepared from at least three animals/group. Nissl-stained brain tissue at higher magnification with a 40× objective; *scale bar* = 40 μm. The data are also shown statistically with their respective histogram. Statistics: *Significantly different from C (control; #significantly different from PTZ). Significance = $p < 0.05$

compared with PTZ alone, and there was no significant change in all groups in terms of total PKA expression. Our findings showing that TQ and vitamin C-induced CREB phosphorylation is mediated by CaMKII and may be independent of PKA. These results are consistent with previous findings demonstrating that febrile seizures impair memory, CREB activation and PTZ decreased the expression of CaMKII and CREB in kindling rat model (Chang et al. 2003; Dong and Rosenberg 2004; Pi et al. 2004). CREB plays an important role in memory and neuronal survival. Although current antiepileptic drugs have the capability to suppress seizures, it lacks neuroprotection and memory enhancement properties. Therefore, we feel a need for that and we used two compounds TQ and vitamin C which have antiepileptic as well neuroprotective potential. The antiepileptic effect of *N. sativa* oil and TQ has been reported in a PTZ-induced kindling mice model via mechanism involved reduction in oxidative stress and increase in GABAergic tune (Hosseinzadeh and Parvardeh 2004; Ilhan et al. 2005). Treatment with TQ is protective in transient forebrain ischemia and has antiepileptic effects in children with refractory seizures (Al-Majed et al. 2006; Akhondian et al. 2011). The other compound we used was vitamin C a powerful water soluble strong antioxidant and has protective effect in pentylenetetrazole and pilocarpine-induced seizures in rats (Greggio et al. 2009; Santos et al. 2008).

The cAMP-responsive element-binding protein (CREB) pathway has been involved in two major cascades of gene expression, controls long-lasting forms of neuronal plasticity and involved in neuronal survival (Jancic et al. 2009). Here, we addressed that EEG activity or PTZ-induced

prolonged seizures are responsible for the activation of intrinsic apoptotic pathway in the adult rat brain in the present 7-day treatment paradigm. The role of cell death regulatory genes, particularly the bcl-2 family of proteins in the control of epilepsy and seizures is a burning issue. The seizures model use in the current study a 7 days PTZ treatment showed upregulation of Bax, downregulation of Bcl-2 and activation of caspase-3 in cortex and hippocampus, respectively. The pretreatment of TQ and vitamin C inhibit the expression of Bax and caspase-3 induced by PTZ in cortex and hippocampus. There is clear evidence that prolongs or repeated seizures activate the apoptosis related signaling (Engel and Henshall 2009). A number of researchers have documented that seizures induced the activation of caspase-3 both in PTZ and kainic acid models (Wei et al. 2012; Pavlova et al. 2004). These results were further supported by morphological studies. PTZ-induced pronounced damage in the CA1 area of hippocampus and as well in cortex as evaluated by Nissl and FJB staining. On the other hand, TQ and vitamin C pretreatment reversed these changes and showed neuroprotection.

Conclusion

In conclusion, the present study demonstrates that prolong epileptic seizures in vivo suppress $GABA_{B1}R$ in cortex and hippocampus of adult rat brain. The suppression of $GABA_{B1}R$ may be partially involved the inactivation CaMKII and phosphorylation of CREB. These data further demonstrate the functional influence of Bcl-2 family of proteins during prolong seizures and neuronal cell death. Our results support the hypothesis that TQ and vitamin C have antiepileptic and neuroprotective potential in vivo via activation of $GABA_{B1}R$/CaMKII/pCREB pathway and provide a potential way in the treatment of epileptic seizures.

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Conflict of interest The authors have no competing interest to declare.

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