Curcumin supplementation improves mitochondrial and behavioral deficits in experimental model of chronic epilepsy

Harpreet Kaur a, Amanjit Bal b, Rajat Sandhir a,⁎

a Department of Biochemistry, Panjab University, Chandigarh 160014, India
b Department of Histopathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

A B S T R A C T

The present study was aimed to investigate the potential beneficial effect of curcumin, a polyphenol with pleiotropic properties, on mitochondrial dysfunctions, oxidative stress and cognitive deficits in a kindled model of epilepsy. Kindled epilepsy was induced in rats by administering a sub-convulsive dose of pentylenetetrazole (PTZ, 40 mg/kg body weight) every alternate day for 30 days. PTZ administered rats exhibited marked cognitive deficits assessed using active and passive avoidance tasks. This was accompanied by a significant decrease in NADH:cytochrome-c reductase (complex I) and cytochrome-c oxidase (complex IV) activities along with an increase in ROS, lipid peroxidation and protein carbonyls. The levels of glutathione also decreased in the cortex and hippocampus. Electron micrographs revealed disruption of mitochondrial membrane integrity with distorted cristae in PTZ treated animals. Histopathological examination showed pyknotic nuclei and cell loss in the hippocampus as well as in the cortex of PTZ treated animals. Curcumin administration at a dose of 100 mg/kg, p.o. throughout the treatment paradigm was able to ameliorate cognitive deficits with no significant effect on seizure score. Curcumin was able to restore the activity of mitochondrial complexes. In addition, significant reduction in ROS generation, lipid peroxidation and protein carbonyls was observed in PTZ animals supplemented with curcumin. Moreover, glutathione levels were also restored in PTZ treated rats supplemented with curcumin. Curcumin protected mitochondria from seizure induced structural alterations. Further, the curcumin supplemented PTZ rats had normal cell morphology and reduced cell loss. These results suggest that curcumin supplementation has potential to prevent mitochondrial dysfunctions and oxidative stress with improved cognitive functions in a chronic model of epilepsy.

⁎ Corresponding author at: Department of Biochemistry, Basic Medical Science Building, Panjab University, Sector-14, Chandigarh, 160014, India. Tel.: +91 172 2534131; fax: +91 172 2541022.
E-mail address: sandhir@pu.ac.in (R. Sandhir).

1. Introduction

Epilepsy is a brain disorder characterized by recurrent unprovoked seizures with prolonged epileptic discharge (Panayiotopoulos, 2005). The type of epileptic seizure depends on the brain region affected and the cause of epilepsy. Complex partial seizures arising from the limbic system (hippocampus) led to a condition known as temporal lobe epilepsy (TLE). TLE is the most commonly studied type of epilepsy because of its high prevalence and severity (Engel et al., 2003). The burst-firing of neurons in an epileptic condition leads to a cascade of events that include increase in oxidative stress, inflammation and activation of cell death pathways (Fujikawa, 2005; Macdonald and Kapur, 1999). Increased oxidative stress is considered as a contributing factor to the pathogenesis of epilepsy (Sudha et al., 2001). Mitochondria are the primary organelles involved in several important metabolic activities and play a central role in energy transduction within the cell (Szewczyk and Wojtaczek, 2002).

Defects in mitochondrial dynamics along with change in energy metabolism generally contribute to generation of superoxide, hydrogen peroxide and hydroxyl radicals which damage cellular components like DNA, lipids and proteins (Van et al., 2013). Mitochondrial dysfunctions have been observed to play a cardinal role in the pathogenesis of various neurodegenerative diseases such as Alzheimer’s disease (Eckert et al., 2012), Huntington’s disease (Sandhir et al., 2014), and Parkinson’s disease (Navarro and Boveris, 2010).

Seizures have also been observed in animals treated with potassium cyanide inhibitor of cytochrome-c oxidase, i.e. complex IV (Yamamoto and Tang, 1996), and 3-nitropropionnic acid, inhibitor of succinate dehydrogenase, i.e. complex II (Urbanska et al., 1998) suggesting that inhibition of mitochondrial respiratory chain components lead to epilepsy. Studies suggest that defects in mitochondrial function might be underlying the pathogenesis of seizures in temporal lobe epilepsy (Azakli et al., 2013). The currently available antiepileptic drugs for treatment of seizure disorder have numerous adverse effects including some degree of cognitive, behavioral or psychiatric reactions (Walia et al., 2004). Therefore, an obvious way to intervene seizure induced mitochondrial damage in epilepsy is through the use of natural antioxidant compounds with wide applicability and therapeutic efficacy with no adverse effects.
Curcumin (diferuloylmethane), a yellow dietary pigment in the rhizome Curcuma longa, belongs to the family Zingiberaeaceae which has been used as Ayurvedic medicine for decades to treat various diseases (He et al., 2010). Numerous studies have shown curcumin as an antioxidant and anti-inflammatory compound with neuroprotective properties in various neurological conditions such as Parkinson’s disease, multiple sclerosis, and Alzheimer’s disease (Hishikawa et al., 2012). Many clinical trials have been completed and some are being undertaken to evaluate the efficacy of curcumin against a wide range of human diseases including neurological disorders. A study was designed to generate tolerability, pharmacokinetics, and preliminary clinical and biomarker efficacy data on curcumin in Alzheimer’s patients which was unable to demonstrate clinical and biochemical evidences of efficacy against Alzheimer’s disease due to a small sample size (30 patients) and short duration of study (Ringman et al., 2012). Moreover, curcumin in the form of capsules, nanoparticles, powder and solution has been in clinical trials to improve cognitive functions and neuropathy in patients (Gupta et al., 2013). Curcumin has been shown to provide protection against amyloid peptide induced mitochondrial dysfunction in Alzheimer’s disease (Huang et al., 2012). Curcumin administration to aluminum-treated animals has been observed to decrease brain mitochondrial damage by normalizing the activities of mitochondrial electron transport complexes affected following aluminum treatment (Sood et al., 2011). A recent study has shown the neuroprotective effect of CNB-001, a pyrazole derivative of curcumin in rotenone induced toxicity (Jayaraj et al., 2013). To the best of our knowledge, this is the first study designed with an aim to evaluate the neuroprotective potential of curcumin in preventing seizure induced mitochondrial dysfunctions and cognitive deficits in a chronic model of epilepsy.

2. Material and methods

2.1. Chemicals

Pentylenetetrazole and curcumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Hi-Media Laboratories (Mumbai, India) respectively. Other chemicals used in the present study were of analytical grade and were procured from either Sigma Chemical Co. (St. Louis, MO, USA) or Sisco Research Laboratories (Mumbai, India).

2.2. Animals and treatment schedule

Adult male Wistar rats weighing 200–220 g were procured from the Central Animal House facility of Panjab University. All the animals were housed under standard housing and acclimatized for a week. They had free access to rat pellet diet (Ashirwad Industries, Ropar, India) and water. The procedures followed were approved by the Institutional Animal Ethics Committee (IAEC) of the university and were in accordance with the guidelines for humane use and care of laboratory animals. Animals were randomly segregated into the following four groups with 8 animals in each group. The schematic representation of the treatment paradigm is provided in Fig. 1 and is based on a report in literature (Rajabzadeh et al., 2012).

Control Animals were administered with vehicle daily for the duration of the treatment.

Curcumin Animals were administered with curcumin (suspended in 1% carboxymethyl cellulose in distilled water) daily at a dose of 100 mg/kg body weight, orally for 40 days.

PTZ Animals were administered PTZ intraperitoneally (dissolved in saline) at a dose of 40 mg/kg on alternate days for a period of 30 days and a single dose of PTZ on the 40th day (challenge dose) to ensure kindling success.

PTZ + curcumin Animals were administered with curcumin daily 30 min before PTZ injection at a dose of 100 mg/kg orally for 40 days while PTZ was administered for 30 days only.

A dose of 100 mg/kg curcumin was selected on the basis of literature indicating the average intake of turmeric of 2–2.5 g per day corresponding to 100 mg of curcumin with no adverse effect (Shah et al., 1999). Curcumin at a similar dose has been found to be neuroprotective in various neurological conditions including epilepsy (Du et al., 2009; Ma et al., 2013; Thiyagarajan and Sharma, 2004; Yadav et al., 2009).

2.3. Kindling procedure

The kindling model is an accepted experimental model for TLE and is generally used for screening of potential antiepileptic drugs (Morimoto et al., 2004). Pentylenetetrazole (PTZ), a GABA_A antagonist commonly used as a convulsant agent, which when administered (repeatedly) in a sub-convulsive dose for a specific period of time, slowly results in the development of a kindled model of epilepsy, also referred to as chronic epilepsy (Hansen et al., 2004). A sub-convulsive dose of PTZ 40 mg/kg in the volume of 1 ml/kg was administered to rats alternatively for 30 days resulting in a permanent change with generalized tonic–clonic convulsions. After every PTZ injection, each animal was placed separately in a cage and the occurrence and intensity of seizures were observed for over 25–30 min. The intensity of seizures was calculated according to Racine’s scale depicted in Table 1 (Racine, 1972). The success of kindling was assessed by giving a challenge dose of PTZ (40 mg/kg) 10 days after the last PTZ injection (on day 40) and seizures were scored. Many investigators have used a similar model (Kumar et al., 2013). The PTZ + curcumin group was administered PTZ for 30 days followed by a single dose of PTZ to access the success of kindling as done in the PTZ treated group. The cumulative kindling score was calculated for each group. Rats that fulfilled the kindling criteria and had tonic–clonic seizures following the challenge dose were included in the study.

2.4. Isolation of mitochondria

Animals were sacrificed by decapitation under mild ether anesthesia; the hippocampus and cerebral cortex were dissected from the brain. Each region was weighed and homogenized separately in ice cold buffer containing 10 mM Tris HCl (pH 7.4), 0.44 M sucrose, 10 mM EDTA and 0.1% BSA and centrifuged at 2100 g for 15 min at 4 °C. The pellet was discarded and the supernatant obtained was further centrifuged at 14,000 g for 15 min at 4 °C. The mitochondrial pellet obtained was washed with the same buffer and again spun at 7000 g for 15 min at 4 °C. The final mitochondrial pellet was re-suspended in a buffer containing 10 mM Tris HCl (pH 7.4) and 0.44 M sucrose.

2.5. Activity of mitochondrial complexes

2.5.1. NADH:cytochrome c reductase (EC1.6.99.3, complex I)

The activity of NADH:cytochrome c reductase was measured as described by King and Howard (1967). The method involves catalytic oxidation of NADH to NAD⁺ with subsequent reduction of cytochrome c on addition of mitochondrial preparation. The increase in absorbance was read at 550 nm for 3 min. Results were expressed as
nmol NADH oxidized/min/mg of protein using the molar extinction coefficient of reduced cytochrome c at 550 nm (1.96 mM⁻¹ cm⁻¹).

2.5.2. Succinate dehydrogenase (EC 1.3.5.1, complex II)

The activity of succinate dehydrogenase was assayed according to the method of King et al. (1976). Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate on addition of potassium ferricyanide that acts as an artificial electron acceptor. The reaction was initiated on addition of a requisite amount of mitochondrial preparation and the change in absorbance was read at 420 nm for 3 min. Results were expressed as nmol of succinate oxidized/min/mg of protein using the molar extinction coefficient of potassium ferricyanide (1000 M⁻¹ cm⁻¹).

2.5.3. Cytochrome-c oxidase (EC 1.9.3.1, complex IV)

The activity of cytochrome oxidase was assayed according to the method of Sottocasa et al. (1967). This assay measures the oxidation of reduced cytochrome c by the enzyme. The cytochrome c was first reduced by adding a few crystals of sodium borohydride and then the oxidation of reduced cytochrome c was measured by a decrease in absorbance at 550 nm after the addition of an appropriate amount of mitochondrial suspension. Results were expressed as nmol of cytochrome c oxidized/min/mg of protein using the molar extinction coefficient of cytochrome c (1.96 mM⁻¹ cm⁻¹).

2.5.4. F₁−F₀ ATP synthase (EC 3.6.3.14, complex V)

The activity of mitochondrial F₁−F₀ ATP synthase was assayed using the method of Griffiths and Houghton (1974). Appropriate amount of mitochondrial sample was added to the ATPase buffer and incubated at 37 °C for 10 min. The reaction was stopped by addition of 10% (w/v) trichloroacetic acid (TCA) and contents were centrifuged at 3000 g for 20 min. The phosphate released was analyzed on the basis of the reaction of the inorganic phosphate with ammonium molybdate as described by Fiske and Subbarow (1927). Results were expressed as nmol of ATP hydrolyzed/min/mg protein.

2.6. Mitochondrial membrane permeability

Mitochondrial transition pore opening leads to swelling of mitochondria. Mitochondrial swelling and contraction were used as a functional test for the mitochondrial membrane integrity which was measured at 520 nm as described by Tedeschi and Harris (1958). Mitochondrial swelling was assessed by adding an appropriate amount of sample to the buffer containing 0.12 M KCl in 0.02 M Tris HCl (pH 7.4) and the absorbance was measured for 6 min at 520 nm. The contraction of mitochondria was initiated by adding Mg⁺²-ATP and absorbance was read at 520 nm.

2.7. Mitochondrial ROS generation

Intracellular generation of ROS was assessed using the method described by Wang and Joseph (1999). Oxidation of intracellular 2',7'-dichlorofluorescein-diacetate (DCFH-DA, nonfluorescent) to highly fluorescent 2',7' dichlorofluorescein (DCF) was measured using a spectrofluorometer with an excitation at 488 nm and an emission at 530 nm after addition of the mitochondrial sample. Results were expressed as pmol DCF/min/mg protein.

2.8. Protein carbonyls

The protein carbonyls were determined using 2,4-dinitrophenylhydrazine (DNPH) assay as described by Levine et al. (1990). The mitochondrial sample was dissolved in 20 mM DNPH prepared in 2 N HCl and kept at room temperature for 1 h in the dark. Respective negative control was prepared in which mitochondria were dissolved in 2 N HCl only. Following 1 h incubation, 20% (w/v) TCA was added for protein precipitation and the mixture was centrifuged at 10,000 g for 10 min. Supernatant was discarded and the pellet obtained was washed three times with an ethyl acetate:ethanol mixture (1:1) to remove excess DNPH. The final pellet was later dissolved in 6 M guanidine HCl solution at 50 °C and the yellow colored complex obtained was read at 370 nm against their negative controls. Negative controls were read at 280 nm to determine protein concentration. The results were expressed as nmol carbonyls/mg protein using the extinction coefficient of DNPH (22 mM⁻¹ cm⁻¹).

2.9. Lipid peroxidation

Malondialdehyde (MDA), a measure of lipid peroxidation, was estimated according to the method of Ohkawa et al. (1979). The amount of MDA formed was measured by the reaction with thiobarbituric acid at 532 nm using a spectrophotometer. The results were expressed as nmol MDA/mg protein using the molar extinction coefficient of MDA-thiobarbituric acid chromophore (1.56 × 10⁵ M⁻¹ cm⁻¹).

2.10. Glutathione (GSH)

GSH levels were estimated by the method of Roberts and Francetic (1993). DTNB added during estimation was reduced by free – SH of GSH to form 5-mercapto-2-nitrobenzoate which was read at 412 nm. Results were expressed as μmol GSH/mg protein by using the molar extinction coefficient (13,100 M⁻¹ cm⁻¹).

2.11. Estimation of protein

The protein content was estimated according to the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.12. Electron microscopy

Transmission electron microscopy was done as described by Gao et al. (2013). The hippocampus and cerebral cortex were removed after decapitation under mild anesthesia (ether) and fixed in 3% (w/v) glutaraldehyde (diluted in 0.2 M Sorenson’s buffer) for 24 h at 4 °C. Brain sections were diced into small cubes of 1 mm size and post fixed in osmium tetroxide, dehydrated in a graded ethanol series, cleared in propylene oxide at room temperature and finally embedded in an Epon mixture containing Taab/812. Thick sections were cut, mounted on Nickel grids and were double contrast stained using uranyl acetate and lead citrate. Sections were examined and photographed using Hitachi H–7500 transmission electron microscope (Hitachi, Tokyo, Japan).

2.13. Cresyl violet staining

The brain sections were stained with cresyl violet (0.1% w/v) by the method described by Ogita et al. (2003). Briefly, rats were deep anesthetized and perfused transcardially with saline followed by 0.4% (w/v) paraformaldehyde prepared in phosphate buffered saline. Brains were removed and processed for routine paraffin embedding. Coronal sections

<p>| Table 1 |</p>
<table>
<thead>
<tr>
<th>Stages of seizure</th>
<th>Characterization</th>
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<tbody>
<tr>
<td>Stage 0</td>
<td>No response</td>
</tr>
<tr>
<td>Stage 1</td>
<td>Facial movements, ear and whisker twitching</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Myoclonic convulsions without rearing</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Myoclonic convulsions with rearing</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Clonic-tonic convulsion</td>
</tr>
<tr>
<td>Stage 5</td>
<td>Generalized clonic-tonic seizures with loss of postural control</td>
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</table>
were prepared and mounted on poly-l-lysine coated slides. Paraffin sections were de-waxed in xylene solution twice for 20 min, followed by rehydration in 100% ethanol for 10 min. Then the slides were immersed once in 90% (v/v) ethanol, and once in 70% (v/v) ethanol followed by immersion in 50% (v/v) ethanol. The slides were dipped in 30% (v/v) ethanol for 10 min, rinsed two times in distilled water and finally immersed in cresyl violet staining solution (0.1% w/v) for 5 min. A final rinse in water was given to all slides and later air dried. Two changes of n-butanol were given at a final step followed by cleaning in xylene solution for 5 min. Slides were mounted in DPX and dried overnight. Images were acquired using a Leica DM2500 microscope (Leica microsystems, Wetzlar, Germany) attached with a Leica DFC295 CCD camera.

2.14. Neurobehavioral studies

The animals in treatment groups were assessed for cognitive deficits using active and passive avoidance tests.

2.14.1. Active avoidance test

Active avoidance task was performed according to the method described by Kamboj and Sandhir (2007). The behavior apparatus consisted of a two way shuttle box equipped with a stainless steel grid floor and divided into two equal sized chambers (dark and lit compartments). The chambers were interconnected with an opening enough for the animal to pass through. Acoustic and visual stimulators in the form of buzzer and light were supplied. Each animal was kept inside the shuttle box for 15 min for habituation one day before starting the task during which the animal can explore freely. During the learning session, the animal was placed in the lit compartment where it learned to enter the safe compartment after experiencing light and sound stimulus (conditioned stimulus) for 10 s each and avoiding the electric shock (un-conditioned stimulus). Electric shock (10 s) was given to animal that did not respond to the conditioned stimulus. The total of 10 trials were given to each animal in a day. The number of trials in which the animal responded only to un-conditioned stimulus were recorded as escape trials.

2.14.2. Passive avoidance test

Passive avoidance task was performed to evaluate the learning and long-term memory in animals as described by Uzum et al. (2010). The apparatus consisted of two chambers (a dark and an illuminated chamber), with a stainless steel grid floor to deliver foot-shocks. The rat was placed in the illuminated compartment and had free access to all parts of the apparatus for 300 s for habituation. One day after the last PTZ injection, the animal was placed in the illuminated chamber and allowed to explore for 30 s. After 30 s, the door between the two chambers was lifted up and thereafter its latency to enter the dark compartment was recorded. The interval between the placement of rat in the light chamber and its entry into the dark chamber was measured as entrance latency. Immediately, the door was closed once the animal enters the dark compartment and a single electric foot shock (65 V AC, 50 Hz) for 5 s was delivered. Consolidation and long-term memory were assessed. One day after this learning session, the animal was placed in the illuminated chamber and the latency to re-enter the dark chamber was measured. This was known as consolidation memory. The same procedure was used for the long-term memory test which was performed three days after the learning/training session. Foot shocks were not administered during the retention tests. Rats that did not enter the dark compartment during the given 5-min period was removed from the apparatus and the latency was considered to be 300 s.

2.15. Statistical analysis

Values are expressed as mean ± standard error mean (SEM). The statistical significance was analyzed by one-way analysis of variance followed by Newman–Keuls test using SPSS 16 software. Values with p < 0.05 were considered as statistically significant.

3. Results

3.1. PTZ kindling score

Fig. 2 shows the effect of curcumin supplementation on seizure score. 30 days of PTZ treatment in animals resulted in generalized tonic–clonic seizures. It was observed that curcumin administered at a dose of 100 mg/kg had no significant effect on the severity and progression of seizures in PTZ treated animals.

3.2. Mitochondrial respiratory chain enzymes

The activities of mitochondrial electron transport chain components in the cortex and hippocampus are presented in Table 2. NADH:cytochrome c reductase activity was inhibited by 40% in the cortex and 42% in the hippocampus of PTZ treated animals as compared to controls. However, the activity was found to be increased by 68% in the cortex and by 76% in the hippocampus on curcumin supplementation to the PTZ treated animals. The activity of cytochrome c oxidase was found to be significantly lowered by 62% in the cortex and by 47% in the hippocampus of PTZ treated animals as compared to controls. Supplementation with curcumin increased cytochrome c oxidase activity by 85% in the cortex and 88% in the hippocampus in comparison to PTZ treated animals. The activity of succinate dehydrogenase and F1–F0 ATP synthase was found to be unaffected in both the regions of brain on PTZ treatment as compared to controls. Administration of curcumin also had no effect on these electron transport chain complexes.

3.3. Mitochondrial swelling

Mitochondrion swelling was observed to be increased by 8 fold in the hippocampus and by 3 fold in the cortex of PTZ treated animals when compared to controls. However, mitochondrial swelling was observed to be decreased by 2 fold in the hippocampus and cortex of animals co-administered with curcumin when compared to PTZ treated animals (Fig. 3).

3.4. Reactive oxygen species, lipid peroxidation and protein carbonyls

ROS production was found to be significantly increased in the cortex (90%) and hippocampus (94%) of PTZ treated animals in comparison to controls. However, curcumin administration to PTZ animals decreased the levels by 36% in the cortex and by 46% in the hippocampus when compared to the PTZ treated group (Table 3). Malondialdehyde, a byproduct of lipid peroxidation, was observed to be increased
Effect of curcumin administration on the activity of mitochondrial complexes in cortex and hippocampus of PTZ treated rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>NADH:cytochrome c reductase (nmol NADH oxidized/min/mg protein)</th>
<th>Succinate dehydrogenase (nmol succinate oxidized/min/mg protein)</th>
<th>Cytochrome oxidase (nmol cytochrome c oxidized/min/mg protein)</th>
<th>F$<em>{1}$F$</em>{0}$ synthase (nmol ATP hydrolyzed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>cortex: 42.55 ± 4.41; hippocampus: 25.62 ± 3.01</td>
<td>cortex: 43.24 ± 10.53; hippocampus: 28.97 ± 2.79</td>
<td>cortex: 3.16 ± 0.65; hippocampus: 5.63 ± 1.08</td>
<td>cortex: 161.39 ± 6.56; hippocampus: 231.36 ± 25.07</td>
</tr>
<tr>
<td>Curcumin</td>
<td>cortex: 29.71 ± 3.96; hippocampus: 29.87 ± 6.64</td>
<td>cortex: 25.96 ± 3.03; hippocampus: 30.72 ± 2.47</td>
<td>cortex: 4.08 ± 0.94; hippocampus: 3.23 ± 0.29</td>
<td>cortex: 153.37 ± 15.99; hippocampus: 288.94 ± 42.94</td>
</tr>
<tr>
<td>PTZ</td>
<td>cortex: 25.39 ± 4.04*; hippocampus: 14.76 ± 1.77*</td>
<td>cortex: 41.68 ± 6.52; hippocampus: 23.21 ± 3.06</td>
<td>cortex: 1.21 ± 0.34*; hippocampus: 2.99 ± 0.38*</td>
<td>cortex: 155.65 ± 11.09; hippocampus: 312.31 ± 40.66</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>cortex: 42.56 ± 5.38*; hippocampus: 25.97 ± 3.37*</td>
<td>cortex: 32.59 ± 9.04; hippocampus: 24.46 ± 8.29</td>
<td>cortex: 2.24 ± 0.78; hippocampus: 5.64 ± 0.79*</td>
<td>cortex: 175.95 ± 17.15; hippocampus: 225.46 ± 27.22</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; n = 8/group.

* Significantly different from control group (p < 0.05).

3.5. Glutathione (GSH)

GSH, a first line of defense, was observed to significantly decreased after PTZ treatment in both the hippocampus (13%) and cortex (16%) when compared to controls. However, curcumin supplementation resulted in restoration of GSH levels in both regions of the brain.

3.6. Electron microscopy

The electron micrographs of the cortex and hippocampus were examined for ultrastructure changes in mitochondria (Figs. 4 and 5). PTZ treatment to animals resulted in severe damage to mitochondrial structure characterized by disruption of mitochondrial membrane integrity and distorted cristae with clearing of matrix density in both regions. However, normal morphology of mitochondria was observed in saline and curcumin treated animals. Curcumin supplementation to PTZ animals was able to reverse the detected structural abnormalities.

3.7. Cresyl violet staining

PTZ treated animals showed shrunken nucleus (pyknotic) and cytoplasm in both the cortex and hippocampus when compared to control animals (Fig. 6). However, in the PTZ animals supplemented with curcumin, a decrease in the number of pyknotic neurons was observed. Curcumin supplementation preserved the normal morphology and integrity of cells in both the regions (increase in viable cells). The curcumin treated animals also exhibited normal morphology with well-rounded cells devoid of pyknosis in the cortex and hippocampus similar to that of the control animals.

3.8. Active avoidance test

Cognitive behavior was assessed by the number of times the animals avoid foot shock after training (Fig. 7a). Animals avoid foot shock and prefer to go to the shock free chamber with light and buzzer stimuli. In the present study, the PTZ treated animals showed a significant increase in the number of escapes as compared to control animals. However, curcumin co-administration with PTZ proved to be beneficial as in this group the animals entered the safe compartment on conditioned stimulus (buzzer).

3.9. Passive avoidance test

This test is generally used to measure cognitive alterations after drug administration which is based on the animal’s ability to remember the previous shock experience and to avoid the re-entry into the dark chamber. In the acquisition/learning phase, the latency to enter into the dark compartment was observed to be the same for all groups. However, a significant impairment in both consolidation as well as long-term memory occurred after PTZ treatment as a significant decrease in the entrance latency was observed. The animals treated with PTZ took less time to enter the dark compartment than the controls. On the other hand, the PTZ treated animals supplemented with curcumin showed prolonged latency to enter the dark compartment when compared to animals in the PTZ treated group which depicts that curcumin attenuate PTZ induced decrease in entrance latency. Control and curcumin treated animals avoided entering the dark compartment and performed better in consolidated as well as long term memory assessment (Fig. 7b).

4. Discussion

In the present study, repeated administration of a sub-convulsive dose of PTZ on alternative days resulted in the development of kindling in animals and these results are in accordance with the earlier studies where repetitive administration of a sub-convulsive dose of PTZ results in the appearance and progressive increase in convulsant activity resulting in generalized seizures (Corda et al., 1990). On the other hand, curcumin administration with PTZ had no significant effect on...
the seizure score suggesting that curcumin at a dose of 100 mg/kg could not prevent epileptic seizure. This is in accordance with the study performed by Sumanont et al., (2007) wherein curcumin failed to delay the onset of kainic acid induced seizure and had no effect on seizure scores.

A significant decrease in NADH:cytochrome c reductase and cytochrome c oxidase activity has been observed in PTZ animals which is in accordance with the study by Kudin et al. (2002). Inhibition of mitochondrial complexes could be due to ROS generation following PTZ treatment (Celikyurt et al., 2011). A recent report shows seizures induced dysfunction of mitochondrial complex IV in animals (Gao et al., 2014). Furthermore, disturbance in glycolytic rates and lactate/pyruvate ratios and loss of mitochondrial N-acetylaspartate have also been observed in human epileptic tissue which confirms the involvement of mitochondria in TLE (Rowley and Patel, 2010). Inhibition of mitochondrial electron transport components contributes to incomplete electron transport and decrease in intracellular ATP production that disturb the normal calcium homeostasis thereby affecting neuronal excitability and synaptic transmission (Folbergrova and Kunz, 2012). Curcumin supplementation to PTZ animals restored the activities of these complexes in both the regions possibly because curcumin scavenges ROS because of its antioxidant properties (Barzegar and Moosavi-Movahedi, 2011). These findings are in agreement with the recent report which showed that curcumin reduces hepatotoxicity by protecting against mitochondrial alterations: oxidative phosphorylation disruption, decrease in the cellular ATP levels, mitochondrial permeability transition, calcium homeostasis disruption and oxidative stress (Garcia-Nino and Pedraza-Chaverri, 2014). Furthermore, curcumin encapsulated solid lipid nanoparticles have been shown to ameliorate mitochondrial impairments induced in 3-NP induced Huntington's disease through activation of the NRF2 pathway (Sandhir et al., 2014).

The activities of succinate dehydrogenase and ATP synthase were unaffected in both brain regions of PTZ treated animals. The results are in line with previous studies on animal models of epilepsy where no significant change was observed in the activity of succinate dehydrogenase and ATP synthase (Gao et al., 2013; Rahman, 2012). Clinical profiles of patients with intractable childhood epilepsy have shown major changes in NADH:cytochrome c reductase and cytochrome c oxidase activity whereas the activity of other complexes remained almost unchanged (Kang et al., 2007). Therefore, our results provide supporting evidence for previous observations that mitochondrial complexes NADH:cytochrome c reductase and cytochrome c oxidase are major contributors in impaired mitochondrial function in chronic epilepsy (Kunz et al., 2000).

Impairment in mitochondrial respiration can accentuate oxidative stress (Ankarcrona et al., 1995). In this study, protein carbonyl, ROS and LPO levels were found to be elevated in the hippocampus and cortex of PTZ treated animals suggesting an increase in oxidative stress. Increased ROS levels might be due to decreased mitochondrial complex I activity which is considered to be the main source of ROS production (Kudin et al., 2004). It has been found that partial inhibition of complex

### Table 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cortex ROS (pmol/min/mg protein)</th>
<th>Hippocampus ROS (pmol/min/mg protein)</th>
<th>Cortex LPO (nmol MDA/mg protein)</th>
<th>Hippocampus LPO (nmol MDA/mg protein)</th>
<th>Cortex Protein carbonyls (nmol/mg protein)</th>
<th>Hippocampus Protein carbonyls (nmol/mg protein)</th>
<th>Cortex GSH (μmol/mg protein)</th>
<th>Hippocampus GSH (μmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.22 ± 7.79</td>
<td>40.88 ± 3.55</td>
<td>1.62 ± 0.21</td>
<td>3.04 ± 0.11</td>
<td>0.25 ± 0.09</td>
<td>0.23 ± 0.08</td>
<td>8.96 ± 0.44</td>
<td>9.04 ± 0.29</td>
</tr>
<tr>
<td>Curcumin</td>
<td>46.88 ± 4.82</td>
<td>50.66 ± 6.11</td>
<td>1.77 ± 0.18</td>
<td>2.98 ± 0.21</td>
<td>0.11 ± 0.01</td>
<td>0.12 ± 0.04</td>
<td>8.77 ± 0.61</td>
<td>9.03 ± 0.14</td>
</tr>
<tr>
<td>PTZ</td>
<td>64.88 ± 9.61⁎</td>
<td>79.28 ± 14.24⁎</td>
<td>2.26 ± 0.09⁎</td>
<td>3.77 ± 0.24⁎</td>
<td>0.47 ± 0.07⁎</td>
<td>0.65 ± 0.01⁎</td>
<td>7.53 ± 0.11⁎</td>
<td>7.85 ± 0.17⁎</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>41.77 ± 9.58⁎</td>
<td>42.66 ± 11.99⁎</td>
<td>1.77 ± 0.15⁎</td>
<td>2.80 ± 0.18⁎</td>
<td>0.22 ± 0.03⁎</td>
<td>0.05 ± 0.01⁎</td>
<td>8.73 ± 0.28⁎</td>
<td>8.51 ± 0.18⁎</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; n = 8/group.

⁎ Significantly different from control group (p < 0.05).

# Significantly different from PTZ treated group (p < 0.05).

![Cortex](image1.png)

![Curcumin](image2.png)

![PTZ](image3.png)

![PTZ + Curcumin](image4.png)

Fig. 4. Transmission electron micrographs of cortex showing mitochondrial ultrastructure changes in PTZ treated rats. Upper panel shows mitochondrial ultrastructure at 50000× magnification. Lower panel shows magnified view of individual mitochondria.
I contributes to increased ROS production which causes cell death (Folbergrova et al., 2010). Excess ROS formed can modify the protein leading to increase in protein carbonyl content. Previous studies also reported increased protein carbonyls in rat cortex and hippocampus after status epilepticus (Chuang et al., 2012). Curcumin administration to PTZ animals resulted in a decrease in ROS, protein carbonyl and LPO levels which demonstrates the role of curcumin as anti-oxidant in epilepsy (Du et al., 2012). The presence of hydroxyl moiety and carbonyl groups in curcumin is considered to be a major contributor for its direct free radical scavenging ability (Anand et al., 2008).

Fig. 5. Transmission electron micrographs of hippocampus showing mitochondrial ultrastructure changes in PTZ rats. Upper panel shows mitochondrial ultrastructure at 50000× magnification. Lower panel shows magnified view of individual mitochondria.

Fig. 6. Effect of curcumin supplementation on neuronal cell death assessed after cresyl violet staining of cortex and hippocampus of animals with PTZ induced chronic epilepsy. (a) magnification at 20× and (b) magnification at 40×.
Glutathione is the most abundant non-enzymatic antioxidant which provides the first line of defense against free radicals. The data from the present study showed a significant decrease in GSH levels in both regions of the brain following PTZ treatment; however, curcumin supplementation restored GSH levels in both brain regions indicating curcumin in increasing endogenous antioxidant defense of brain. These results are in agreement with the previous findings where curcumin has been shown to provide neuroprotection both in vivo and in vitro by restoring the depleted GSH levels and protecting the neuronal cells against protein oxidation and mitochondrial dysfunctions (Jagatha et al., 2008).

Increased oxidative stress can alter the mitochondrial membrane potential that can contribute to mitochondrial swelling (Kroemer et al., 2007). Mitochondrial swelling was observed in the cortex as well as hippocampus of PTZ treated animals. Previous reports suggest seizure associated changes in neurons which were characterized by swollen and often disrupted mitochondria (Kudin et al., 2009). Severe degenerative damage including mitochondrial swelling had been seen in animals administered with PTZ (Asadi-Shekaari et al., 2012). The mitochondria swelling might be an effect of increased calcium concentration due to PTZ administration (Papp et al., 1987). It has been documented that excessive intracellular calcium results in an increase in free radicals which cause mitochondria pore to open leading to mitochondrial swelling (Brustovetsky et al., 2003). However, we found that seizures result in excessive increase in excitatory amino acid (glutamate) levels, which leads to neuronal cell death via the activation of NMDA receptors (Rothman and Olney, 1986). Morphological changes in mitochondria including swelling and disruption of mitochondrial membrane may also contribute to cell damage as observed in a study conducted in epileptic patients (Chen et al., 2010). Curcumin supplementation attenuated PTZ-induced neuronal cell death in both regions which could be due to an increase in GSH levels and decrease in oxidative stress parameters such as LPO, ROS and protein carbonyls on curcumin administration.

An impaired memory performance is considered to be a hallmark of TLE (Dietl et al., 2004). In the present study, cognitive impairment was observed in PTZ treated animals when compared to controls as assessed using passive avoidance task. PTZ administration resulted in disturbance of both consolidation and long term memory. These results are in accordance with the previous studies where memory deficits were observed on PTZ treatment (Genkova-Papazova and Lazarova-Bakarova, 1995). However, curcumin supplementation ameliorated memory functions as seen by the increase in latency of animals to enter into the dark compartment. It has been reported that curcumin has potential to reverse aluminium-induced cognitive dysfunctions and oxidative damage in rats (Kumar et al., 2009). Reports are also available where curcumin significantly enhanced learning and memory in mouse models of Alzheimer’s disease (Pan et al., 2008). Curcumin is found to improve cognition by regulating the expression of neurotransmitter-related genes such as synaptotagmin (Syt IV and Syt I) and complexins (Cplx1) which are known to be associated with spatial memory in rats (Dong et al., 2012).

Various reports have indicated that prolonged or recurrent seizures disrupt cognitive behavior in kindled models of epilepsy (Rossler et al., 2000; Sutula et al., 1994; Zhang et al., 2013). Our study further provides evidence that animals treated with PTZ exhibit significant cognitive impairment.

**Fig. 7.** Effect of curcumin supplementation on cognitive behavior as assessed using active avoidance task (a) and passive avoidance task (b). Values are expressed as mean ± SEM; n = 8.

*Significantly different from the control group (*p < 0.05), **significantly different from the PTZ treated group (p < 0.05).
imperfections. This alteration in memory function could be because PTZ administration inhibited GABAergic activity which leads to saturation of endogenous long term potentiation (Moser et al., 1998). However, curcumin supplementation to PTZ animals, resulted in improvement in learning and memory. This finding is in line with the previous studies reporting the neuroprotective effect of curcumin where its administration ameliorates cognitive impairment in PTZ induced kindled rats (Mehla et al., 2010). Similar reports showing the role of curcumin in protecting rats against lead induced memory deficits and attenuating amyloid-beta induced cognitive deficits in animals are also available (Dairam et al., 2007; Frautschy et al., 2001).

In conclusion, our results demonstrate that PTZ induced kindling results in impairment of mitochondrial functions contributing to elevation of oxidative stress that leads to mitochondria ultrastructure changes accompanied by cognitive impairment in animals. This study shows that curcumin supplementation has a potential to prevent mitochondrial dysfunction, oxidative stress and cognitive impairment in chronic models of epilepsy which might involve multiple mechanisms. Therefore, curcumin could be used as an adjuvant therapy in preventing mitochondrial deficits and cognitive impairment in chronic epilepsy.

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