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Temporal lobe epilepsy (TLE) is a chronic neurological condition characterized by recurrent, unprovoked seizures of neurological origin leads to cell loss in specific regions, most prominently in CA1 and CA3, a pattern termed hippocampal (or mesial temporal) sclerosis progressive neuronal loss and cognitive impairment (Hou et al., 2010). Pentylenetetrazole (PTZ) induced kindling is one of the most commonly used chronic model of TLE, and it is currently used by most anti-epileptic drug (AED) discovery programs (Loscher, 2011). The present study was designed with an aim to study the mechanism and to evaluate the neuroprotective potential of curcumin supplementation in PTZ induced chronic epilepsy. The animals were divided into four groups: Control group [normal saline, intraperitoneally], PTZ treated group [PTZ 40 mg/kg body weight, intraperitoneally for 30 days, (every alternate day)], PTZ + Curcumin treated group [curcumin 100 mg/kg body weight daily for 40 days, 30 min PTZ injection, Curcumin treated group [curcumin 100 mg/kg body weight daily, orally for 40 days]. Neurobehavioral studies along with biochemical studies, mitochondrial dysfunctions, neuroinflammatory studies, blood brain barrier studies, cell death and histopathological studies were carried out in hippocampus and cortex of PTZ treated animals after the completion of treatment paradigm.

4.1 Experimental model of chronic epilepsy induced by PTZ

Kindling is a chronic animal model of epilepsy, extensively studied to understand the process of epileptogenesis and discover novel anti-epileptic compounds. It is a process in which repeated administration of sub-convulsive dose of chemical lowers the seizure threshold which eventually leads to the occurrence of seizures (De Deyn et al., 1992). PTZ is non-competitive GABA antagonist which causes generalized clonic-tonic seizures. This drug is known to interact at the picrotoxin (PTX) binding site of the γ-aminobutyric acid type A (GABAₐ) receptor (Huang et al., 2001; Schroeder et al., 1998).

4.2 Effect of curcumin supplementation on seizure score in PTZ induced model of epilepsy

The repetitive administration of sub-convulsive dose of PTZ for 30 days resulted in severe generalized clonic-tonic seizures in animals (Figure 4.2). The results are in accordance
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Figure 4.2: Effect of curcumin supplementation on PTZ kindling. All values are expressed as mean ± SEM; n= 7/group.

4.3 Effect of curcumin supplementation on body weight of PTZ treated animals.

The effect of PTZ treatment on change in body weight of animals was studied (Table 4.1). Initially, on day zero (start of dosing) no difference in the body weight among different groups was observed. After 40 days, the body weight of control animals was found to be increased by 12 % compared to the weight at the beginning of the study; however, PTZ treated animals showed no difference in the initial and final day body weight (gain of 1 %). The body weight of PTZ animals supplemented with curcumin showed an increase in body...
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weight by 8% when compared to PTZ treated group suggesting curcumin supplementation prevented the decrease in body weight gain in animals (Figure 4.1).

Table 4.1: Effect of curcumin supplementation on body weight of PTZ treated animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>% age weight gain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 40</td>
</tr>
<tr>
<td>Control</td>
<td>225.71 ± 3.68</td>
<td>252.85 ± 2.85</td>
</tr>
<tr>
<td>PTZ</td>
<td>226.25 ± 3.23</td>
<td>230.11 ± 3.77*</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>228.75 ± 3.51</td>
<td>245.21 ± 3.27#</td>
</tr>
<tr>
<td>Curcumin</td>
<td>223.75 ± 3.75</td>
<td>250.13 ± 3.27</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05), #significantly different from PTZ treated group (p<0.05).

It has been documented that the brain opioid system is involved in pathophysiology of seizure disorders and cellular mechanisms of epileptogenesis (Tortella, 1988). Interaction of PTZ with endogenous opioids (which are involved in the regulation of neuronal excitability) might be responsible for inhibition of body weight gain in PTZ animals (Eloqayli et al., 2003; Montaser-Kouhsari et al., 2011). It has been shown that PTZ when administered in single dose or in kindling leads to metabolic alterations in brain of animals which could be responsible for no change in body weight of PTZ treated animals (Carmody and Brennan, 2010). PTZ has also been reported to affect both the glycolysis and TCA cycle turnover in different regions of the brain (Smeland et al., 2012). Curcumin supplementation resulted in improvement in body weight of PTZ animals which could be due to the ability of curcumin to improve the metabolic abnormalities in PTZ animals (Lin et al., 2013). Previous report also showed that curcumin supplementation results in the gain of body weight in rats treated with herbicide (2,3,7,8-tetrachlorodibenzo-p-dioxin) (Ciftci et al., 2010).
4.4 Effect of curcumin supplementation on PTZ induced neurobehavioral deficits

It is well known that neuropsychological deficits are important comorbidity of chronic epilepsy (Elger et al., 2004). Cognitive impairment, behavioural disturbances along with the morphological and neurochemical changes have been observed in experimental model of chronic epilepsy (Hermann et al., 2008). The quantitative MRI volumetric analysis performed in epileptic patients, especially with TLE showed brain structural abnormalities which are associated with cognitive pathology (Bernasconi, 2004; Cendes, 2005). A decline in cognitive performance (memory) of TLE patients was observed when compared to controls (Oyegbile et al., 2004). The beneficial effects of reducing seizures through anti-epileptic drugs (AED) often lead to depression, mood fluctuations, cognitive and behavioural deficits (Ortinski and Meador, 2004). Natural products might be used alone or in combination with AED to reduce cognitive dysfunctions. Therefore, the effect of curcumin supplementation on learning and memory, anxiety, muscle strength, cognitive function (spatial memory) in PTZ induced model of chronic epilepsy was studied.
4.4.1 Rotarod

The grip strength (muscle coordination) of PTZ animals was measured in the terms of time spent by animals on rotating rod (Figure 4.3). On day 0, the average time taken by all the animals to stay on rotating rod was 180 s. After 30 days of treatment, no significant difference in the latency to fall from rotating rod was observed among all the groups suggesting PTZ treatment had no effect on muscle strength. Rotarod test when performed on day 40 i.e. before the challenge dose administration also showed no difference in the time spent on rotating rod among the animals of all groups suggesting PTZ administration had no effect on muscle strength and motor coordination. These results are in accordance to the previous studies performed to investigate the possible effect of PTZ on motor tasks, where no change in motor coordination was observed (de Freitas et al., 2004). Earlier, it has been documented that PTZ does not act on the receptors involved in sedative action and muscle impairment which could be the reason that PTZ animals showed no impairment in motor functions.
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4.4.2 Actophotometer

The locomotor activity of animals were monitored using actophotometer where the animals were individually placed in the chamber and subsequently the total activity counts were recorded for 5 min (Table 4.2). On day 0, all the animals had an average count of 296.75. After 30 days of PTZ treatment, a significant increase (70%) in the number of photo beam counts were observed in PTZ treated animals when compared to controls.

Table 4.2: Effect of curcumin supplementation on locomotor activity of PTZ treated animals assessed using actophotometer.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Locomotor activity (photo-beam counts/5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>Control (Saline)</td>
<td>319.28 ± 34.39</td>
</tr>
<tr>
<td>PTZ</td>
<td>297.12 ± 27.77</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>282.12 ± 32.22</td>
</tr>
<tr>
<td>Curcumin</td>
<td>289.01 ± 16.42</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05).

On day 40 i.e. before challenge dose administration, PTZ animals had a significant increase in photo beam counts (65%) in PTZ animals suggesting impairment in locomotor activity of PTZ animals (Figure 4.4). These results are in accordance with the previous studies performed in experimental model of epilepsy, where repeated electroconvulsive shock to animals resulted in locomotor hyperactivity (Hidaka et al., 2008). Reports are also available showing an increase in locomotor activity along with seizure like stereotypic behaviour in zebrafish on PTZ treatment (Afrikanova et al., 2013). The increase in the locomotor activity reveals the stimulant effect of PTZ on CNS, which could be due to decreased GABA neurotransmitter in brain (Corda et al., 1990). Moreover, kindling process is known to increase the strength of excitatory synaptic connections and decreases the strength of connectivity between inhibitory synapses which could be the reason for an increase in the locomotor activity of PTZ treated animals (Mehta et al., 1993). However, PTZ animals supplemented with curcumin at 100 mg/kg had no improvement in locomotor activity. These results are in accordance with the previous study performed by Bharal et al.,
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(2008), where no effect of curcumin supplementation on the locomotor activity of animals was observed after 21 day of its administration.

![Graph](image)

**Figure 4.4: Effect of curcumin supplementation on locomotor activity of PTZ treated animals assessed using actophotometer.** All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05).

4.4.3 Elevated plus maze (EPM) for spatial learning and memory

The spatial learning and memory of PTZ treated animals was assessed in the term of transfer latency on EPM following PTZ treatment (Figure 4.5). Baseline transfer latency was recorded for each animal before the start of dosing wherein the animals showed an average retention of 69.40%. However, after 30 days of PTZ treatment severe impairment in the memory of PTZ animals was observed as depicted from a significant decrease in the percentage of retention (25.07%) as compared to controls (61.19%). On day 40, the percentage of retention in PTZ treated animal was found to be decreased by 36.57% suggesting memory impairment in chronic epilepsy. The findings are in accordance to the previous reports suggesting deteriorative effect of PTZ kindling on learning and memory of animals (Becker et al., 1992; Marsh et al., 2006). Chronic epilepsy or persistence seizures

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In brain can impair the cognitive and behavioural functions in animals (Sayin et al., 2007; Shannon and Love, 2007).

Figure 4.5: Effect of curcumin supplementation on spatial memory of PTZ treated animals assessed using elevated plus maze. All values are expressed as mean ± SEM. n = 7/group.

On day 30, the transfer latency was remarkably elevated in PTZ animals supplemented with curcumin when compared to PTZ animals (72.23%). An increase percentage of retention (63.57%) was also observed in PTZ animals supplemented with curcumin on day 40, suggesting an improvement in the memory functions (Table 4.3). These findings are consistent with those of previous studies showing the protective effect of curcumin against colchicine-induced cognitive impairment (Kumar et al., 2007b). Previous studies also demonstrated that curcumin when administered at 100 mg/kg, 300 mg/kg or 300 mg/kg had a dose dependent effect on ameliorating cognitive deficit observed in PTZ kindled animals (Mehla et al., 2010). These results indicate the beneficial effect of curcumin supplementation on cognitive impairment in PTZ induced chronic epilepsy. A study performed by (Kumar et al., 2007b) also showed a dose dependent e
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of curcumin in improving the motor and cognitive impairment in 3-Nitropropionic acid treated animals.

Table 4.3: Effect of curcumin supplementation on spatial memory retention in PTZ treated animals assessed using elevated plus maze

<table>
<thead>
<tr>
<th>Groups</th>
<th>%age retention</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0-1</td>
<td>Day 29-30</td>
<td>Day 39-40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Before starting dosing)</td>
<td>(30 days after treatment)</td>
<td>(Before challenge dose)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>67.38 ± 4.3</td>
<td>61.19 ± 0.6</td>
<td>73.61 ±1.3</td>
<td></td>
</tr>
<tr>
<td>PTZ</td>
<td>66.03 ± 2.8</td>
<td>25.07 ± 0.6*</td>
<td>63.57 ± 0.5*</td>
<td></td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>67.79 ± 4.9</td>
<td>72.23 ± 3.5#</td>
<td>43.48 ± 3.9#</td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td>76.41 ± 3.7</td>
<td>75.25 ± 2.6</td>
<td>78.15 ± 2.6</td>
<td></td>
</tr>
</tbody>
</table>

All values are expressed as percentage, n =7/group; *significantly different from control group (p < 0.05), #significantly different from PTZ treated group (p < 0.05).

4.4.4 Elevated plus maze for anxiety

The EPM is also used to assess anxiety-like behavior in rodents. The anxiety was assessed in terms of the time spent in open arms and time spent in closed arms when individual animal was kept in center of the EPM with head facing an open arm. An increase in the proportion of time spent in the closed arms and increase in entries to the closed arms indicate the increase in anxiety. The represented track path followed by the animals is given in Figure 4.6. On day 30, the average number of entries by control animals to open arms was 7.41 compared to 5.5 in closed arms (the %age open arm entries were 57%). The average number entries by PTZ animals to open arms were 3.01 compared to 6.01 in closed arms (the %age open arm entries were 33%). The result clearly shows that control animals had more number of entries to open arm in comparison to the closed arm of maze while PTZ treated animals had more number of entries in closed arm (Figure 4.7).

The anxiety score of animals was measured as the time spent in open arms/total time spent in EPM. PTZ treated animals spent more time in closed arm than open arm when compared to controls as depicted from the anxiety score (Figure 4.8), which suggests increased anxiety in PTZ treated animals. The reduced exploration of open arms i.e. the less time spent and less number of entries in open arm by PTZ animals suggests increases in the
levels of anxiety. Our results are in consistence with the previous reports where anxiolytic effect of PTZ was observed in kindled animals as assessed using EPM (File et al., 1996). Previous studies showed similar effect produced by GABA antagonist in animals assessed using elevated plus maze (Cruz et al., 1994; Pellow et al., 1985).

![Figure 4.6: Representative track plot of elevated plus maze test for evaluating spatial memory of PTZ treated animals. The track lines reflect the path followed by animals to open and close arms.](image)

However, PTZ animals supplemented with curcumin showed 5.75 entries to open arms compared to 4.16 in closed arm (the percentage open arm entries were 58%), which compared to percentage open arm entries in PTZ treated animals (33.07%) suggesting amelioration of anxiety-like behavior in animals. Previous studies found an increase in levels of serotonin following curcumin administration which is suggested to be associated with its anxiolytic/anti-anxiety effect (Kumar et al., 2007b). Recently, the anxiolytic potential of curcumin has been found in rats with lead induced intoxication, which...
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mediated through the modulation of serotonin levels in the brain (Benammi et al., 2014). The anti-anxiety-like effect of curcumin shown in the present study could also be mediated by the activation of 5-HT receptor that is known to be associated with endogenous neurotransmitter serotonin (Xu et al., 2005).

Figure 4.7: Effect of curcumin supplementation on anxiety expressed in terms of number of entries to open and closed arm by PTZ treated animals. All values are expressed as mean ± SEM.

Figure 4.8: Effect of curcumin supplementation on anxiety score of PTZ treated animals. All values are expressed as mean ± SEM; *significantly different from control group (p < 0.05), #significantly different from PTZ treated group (p < 0.05).
4.4.5 Active avoidance task

The cognitive behaviour was assessed by the number of times the animals respond only to un-conditioned stimulus i.e. electric foot shock (escape trials). Animals generally avoid foot shock and prefer to go to the shock free chamber with light and buzzer stimuli after training. However, 30 days of PTZ treatment resulted in significant increase in the number of escapes as compared to control animals (Figure 4.9). In contrast, PTZ animals supplemented with curcumin showed an improvement in number of escapes i.e. animals entered the safe compartment on getting conditioned stimulus (buzzer). Various reports have indicated that prolonged or recurrent seizures in experimental model of epilepsy may disrupt cognitive behaviour (Rossler et al., 2000; Zhang et al., 2013a). Our findings further provide evidence that animals treated with PTZ exhibited significant cognitive impairment when compared to controls. It has been suggested that PTZ inhibits GABAergic activity (neurotransmission) which results in saturation of endogenous long term potentiation that affects learning and memory in animals (Moser et al., 1998).

Figure 4.9: Effect of curcumin supplementation on short term memory of PTZ treated animals assessed using active avoidance task. All values are expressed as mean ± SEM. n =7/group; *significantly different from control group (p < 0.05), #significantly different from PTZ treated group (p < 0.05).

However, on curcumin supplementation to PTZ animals, an improvement was observed in learning and memory functions as seen from the decrease in escape trials.
compared to PTZ animals. These findings are in line with the previous studies reporting the neuroprotective effect of curcumin by ameliorating the cognitive impairment in PTZ induced kindled rats (Mehla et al., 2010). Similar reports are also available showing the role of curcumin in protecting rats against lead induced memory deficits and attenuating amyloid-beta induced cognitive deficits in animals (Dairam et al., 2007; Frautschy et al., 2001). Furthermore, it has been demonstrated that curcumin supplemented to animals with traumatic brain injury reduces the cognitive impairment by decreasing oxidative damage and normalizing the levels of brain derived neurotropic factor, a member of the neurotrophin growth factor family (Wu et al., 2006).

4.4.6 Passive avoidance task

Passive avoidance task is generally used to measure cognitive alterations after drug administration which is based on the animal ability to remember the previous shock experienced and avoiding the re-entry into the dark chamber. In the acquisition/learning phase, all the animals had an average latency of 30 s to enter into the dark compartment (Figure 4.10). 30 days of PTZ treated resulted in significant impairment in both consolidation as well as long-term memory as observed from their entrance latency to the dark compartment. On day 30, the latency was found to be decreased by 2.1 folds in PTZ treated animals when compared to controls. Similar results were obtained on day 40, the latency was found to be decreased by 8.6 folds in PTZ treated animals as compared to control i.e. PTZ animals took less time to enter the dark compartment.

However, PTZ animals supplemented with curcumin showed prolonged latency to enter the dark compartment (300 s) as compared to PTZ treated animals during consolidation (137 s) and long term memory (34 s) assessment which suggests curcumin could attenuate the PTZ induced long term memory defects in animals. Control and curcumin treated animals avoided entering to the dark compartment and performed better in consolidated as well as long term memory assessment (entrance latency, 300 s).

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 to PTZ animals ameliorated the memory functions as
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observed by the increase in latency of animals to enter into the dark compartment. It has been previously reported that curcumin has the 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 model of Alzheimer’s disease (Pan et al., 2008). The mechanism how curcumin supplementation improves brain cognitive functions is not well understood, but it is suggested to be mediated by regulating the expression of neurotransmitters-related genes such as synaptotagmin (Syt IV and Syt I), complexins (Cplxs), which are known to be associated spatial memory in rats (Dong et al., 2012).

Figure 4.10: Effect of curcumin supplementation on learning and long-term memory of PTZ treated animals assessed using passive avoidance task. All values are expressed as mean ± SEM; n = 7/group.*significantly different from control group (p < 0.05), #significantly different from PTZ treated group (p < 0.05).

4.4.7 Morris water maze (MWM)

The learning and memory performance was evaluated using Morris water maze. In this task, animals learn to locate a hidden platform in a pool of water using distal visual cues following 6-7 days of training (Selcher et al., 1999). During training period, the animals
learned to locate the hidden platform with progressively shorter latencies. After training and before starting dosing, it was observed that animals from all the groups had an average latency of 12.88 s (Figure 4.11). The represented track plot of animals in all the four groups is shown in Figure 4.12 wherein the track line reflects the path followed by the animals to locate the hidden platform. On day 40, PTZ treated animals had longer escape latencies i.e. the time taken by animal to reach the platform was 35.5 s as compared to control animals exhibiting escape latency of 16.18 s. These results are in accordance to the previous studies showing cognitive deficits in TLE (Szyndler et al., 2006). Recent reports also showed cognitive decline in PTZ kindled rats (Aniol et al., 2013; Zhang et al., 2013a). In fact, cognitive impairment has also been observed in epileptic patients also (Hermann et al., 2002). Any lesion in the brain region including hippocampus or cerebral cortex can lead to of spatial learning and memory in animals (D'Hooge and De Deyn, 2001; Veng et al., 2003). Moreover, persistent seizures or longer duration of epilepsy is also suggested to be associated with cognitive decline (Dickinson et al., 2003).
However, curcumin when supplemented to PTZ animals ameliorated the impairment in spatial learning and memory. Previous evidence suggests the role of curcumin supplementation in improving aging-induced and colchicine-induced learning and memory impairment in rats (Dong et al., 2012; Khurana et al., 2012). There are reports available showing the dose-dependent protective effect of curcumin against seizures, oxidative stress and cognitive impairment in rats (Mehla et al., 2010). Therefore, these findings further confirm that curcumin administration can improve the cognitive functions in chronic epilepsy. Curcumin has a potential to improve learning and memory dysfunctions induced in rats with HIV associated dementia assessed by morris water maze (Dong et al., 2008). A recent study has shown that curcumin supplementation decreases the latency to reach the platform in aged female rats and hence suggested improvement of cognitive functions on curcumin supplementation (Belviranli et al., 2013).

A probe trial, in which platform was removed from the pool was carried out on the next day after recoding final readings for accessing the retrieval memory in animals. The computerized video tracking system was used to record the parameters including: the time spent in target quadrant, swimming distance to the target quadrant and the number of platform crossing. The representative track plot for the probe trial is shown in Figure 4.14. PTZ animals spent less time in the target quadrant along with less swim distance to target quadrant as compared to the control animals (Figure 4.13 A, B). The average time spent in target quadrant by PTZ animals was 37.95 s as compared to 60.8 s in control animals and the swim distance was 25.4 m as compared to 40.41 m in control animals. The average number of crossings to the platform was also found to be less i.e. 7.0 in PTZ treated animals as compared to 12.5 in control animals (Figure 4.13 C). However, PTZ animals supplemented with curcumin showed increased time spent (57.25 s) in target quadrant as compared to PTZ animals (37.95 s) along with increased swim distance to target quadrant (34 m) as compared to PTZ animals (25 m). The number of platform crossing in target quadrant by PTZ animals supplemented with curcumin was also found to be increased; it was 12.5 in case of PTZ + curcumin group as compared to 7 in PTZ group, suggesting an improvement in retrieval memory of PTZ animals on curcumin supplementation. Hence, these results suggest the neuroprotective potential of curcumin in ameliorating the cognitive functions (spatial learning and memory) in PTZ treated animals.
Figure 4.12: Effect of curcumin supplementation on acquisition memory of PTZ treated animals assessed using morris water maze test. The track lines represent the path followed by the animals to locate the submerged platform.
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Figure 4.13: Effects of curcumin supplementation on the long term memory expressed in terms of time spent in target quadrant (A) swim distance in target quadrant (B) and the platform crossing (number of entries in the platform zone) (C) by PTZ treated animals assessed using probe trial. All values are expressed as mean ± SEM; n= 7/group.*significantly different from control group (p < 0.05), #significantly different from PTZ treated group (p < 0.05).
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4.4.8 Y-Maze

Y-maze is a hippocampal dependent memory task which is based on the rodent’s innate curiosity to explore novel areas (exploratory behavior). This neurobehavioral test imposes limited stress conditions to the animal (no swimming, no shock etc.). The behavior of animals in Y maze was assessed in terms of total entries and the time spent into the novel arm. The test was carried out 40 days after treatment and the computerized video tracking

Figure 4.14: Effect of curcumin supplementation on the retrieval memory of PTZ treated animals assessed using probe trial in morris water maze test. The track lines represent the path followed by the animals to locate the submerged platform.
system was used to record the total number of entries and the time spent in each of the arms. Represented track plot for each animal is shown in Figure 4.15.

![Track Plots](image)

**Figure 4.15:** Effect of curcumin supplementation on spatial working memory in PTZ treated animals as assessed using Y-maze test. Representative track plots on Y maze. The track line reflects the path followed by the animal to locate novel arm.

PTZ treated animals had less percentage ratio of novel arm entries (48.97%) than observed in control animals (65.38%) (Table 4.4). Moreover, the time spent in novel arm by PTZ animals was also less (25.56s) as compared to the control animals (119s), suggesting PTZ treatment resulted in decrease in exploratory behavior (Figure 4.16). These results are in accordance to the earlier studies showing less exploratory behavior of animals (less exploration for novel environment) after persistent seizures compared to controls, which is suggested to be associated with decreased GABA receptors in the brain of epileptic rat (Koh et al., 2005; Mathew et al., 2012).
Table 4.4: Effect of curcumin supplementation on the number of entries in open arm, novel arm and start arm and the percentage ratio of entries in novel arm of Y maze.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of entries in open arm</th>
<th>Total number of entries in novel arm</th>
<th>Total number of entries in start arm</th>
<th>Total number of entries in open and novel arm</th>
<th>Ratio of entries in novel arms/total entries in open and novel*100 (%age)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9 ± 2.38</td>
<td>17 ± 0.54</td>
<td>44 ± 7.2</td>
<td>26 ± 2.77</td>
<td>65.38</td>
</tr>
<tr>
<td>PTZ</td>
<td>25 ± 2.12</td>
<td>24 ± 3.11</td>
<td>38 ± 4.82</td>
<td>49 ± 3.71</td>
<td>48.97</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>18 ± 2.31</td>
<td>45 ± 1.58</td>
<td>39 ± 3.42</td>
<td>63 ± 3.21</td>
<td>71.42</td>
</tr>
<tr>
<td>Curcumin</td>
<td>16 ± 0.89</td>
<td>23 ± 1.89</td>
<td>26 ± 2.16</td>
<td>41 ± 3.22</td>
<td>59.02</td>
</tr>
</tbody>
</table>

All values are expressed as percentage. n =7/group.

In contrast, PTZ animals supplemented with curcumin increased the percentage ratio of novel arm entries (71.42%) with no difference in the time spent in novel and open arm. These findings suggest that curcumin supplementation with PTZ improved the performance of animals on Y maze by increasing the exploratory behavior of animals towards novel environment. This could be attributed to the potential of curcumin to normalize the cholinergic function which decreases the time for spatial recognition and improving cognitive functions (Peeyush Kumar et al., 2011).

Figure 4.16: Effects of curcumin supplementation on Y maze for evaluating exploratory behaviour towards novel arm expressed in terms of time spent in open and novel arm by PTZ treated animals. All values are expressed as mean ± SEM *significantly different from control group (p < 0.05).
4.5 Effect of curcumin supplementation on acetylcholinesterase activity in PTZ treated animals

The cholinergic system plays a crucial role in modulating cortical, particularly hippocampal functions including, processes such as learning and memory (Winkler et al., 1995). Acetylcholinesterase (AChE) is a membrane bound enzyme that hydrolyse the neurotransmitter acetylcholine, a major regulator of stress response. It act as a neuromodulator at the cholinergic synapses, also plays a major role in synaptic plasticity, specifically in learning and memory (Giacobini, 2003; Lane et al., 2004). The activity of AChE was found to be altered in both hippocampus and cortex of PTZ treated animals (Figure 4.17). The activity was found to be decreased significantly in hippocampal (42%) of PTZ treated animals whereas an increase was observed in cortex (59%) when compared to controls. However, curcumin supplementation resulted in the increase of AChE activity by 55% in the hippocampus and a decrease by 30% in the cortex. These findings suggest brain region specific changes in AChE activity of PTZ treated animals. Previous reports also suggest the region specific and time dependent changes in the brain where the activity was found to be increased in the frontal cortex and decreased in the rest of the brain including hippocampus in traumatic brain injury (TBI) (Valiyaveettil et al., 2012).

Brain cholinergic dysfunctions are well documented in epileptic animals and AChE has a direct relation with cognitive functions (Gnatek et al., 2012). As discussed in previous section that PTZ animals showed marked impairment in the cognitive functions which could directly be correlated with marked decrease in AChE activity. The decrease in AChE activity observed in PTZ animals might be either due to the inhibition of the enzyme synthesis by the altered cellular environment prevailing in the brain, or due to a decrease in the rate of enzyme synthesis (Ahmed and Tarannum, 2009). Various reports suggest that seizures alter the cholinergic system in the hippocampus and some of these alterations are very long-lasting (Mingo et al., 1998). This enzyme also plays a prival role in activation of neuronal signal transduction (Friedman et al., 1996; Kaufer et al., 1998). Earlier reports have shown the neuroprotective efficacy of curcumin against arsenic induced neurotoxicity in rats by increasing cholinergic functions in rats (Yadav et al., 2011). Curcumin has shown to decrease the AChE levels in cortex and hippocampus of aluminium treated rats (Sharma et al., 2009).
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4.6 Effect of curcumin supplementation on PTZ induced oxidative stress

Free radicals are generally produced as a result of aerobic metabolism in the body. Overproduction of these free radical (ROS/RNS) can result in damage to lipids, proteins, DNA in the cells and eventually leading to various neurological disorders (Uttara et al., 2009). Moreover, brain is quite susceptible to oxidative damage as it contains high amount of polyunsaturated fatty acids which can be readily per-oxidized (Floyd and Carney, 1992). To combat the excess production of free radicals, there exist wide varieties of cellular antioxidants (superoxide dismutase, catalase, glutathione) that protect the cells against free radicals produced in CNS. Evidence suggests the involvement of increased oxidative stress and nitrosative stress in the regulation of biological function and pathophysiology of various neurological disorders including Alzheimer’s diseases, Parkinson’s disease, Multiple sclerosis and epilepsy (Barnham et al., 2004). Several studies (animal models and genetic studies) have demonstrated increased oxidative stress and subsequent cell damage following persistent seizures (Bruce and Baudry, 1995). Evidence suggests that antioxidants may reduce the lesions induced by oxidative free radicals in experimental...
models of epilepsy (Aguiar et al., 2012). Thus, the present study was designed with an aim to study the antioxidant potential of curcumin supplementation in PTZ induced chronic model of epilepsy.

4.6.1 Lipid peroxidation (LPO)

Lipid peroxidation is a process in which free radicals formed react with lipids present in cell membranes leading to cell damage (Lobo et al., 2010). The end products of lipid peroxidation are reactive aldehydes such as malondialdehyde (MDA) and hydroxynonenal (HNE), which are commonly used as a marker of lipid peroxidation (Lovell et al., 1995). It is highly complex, self-propagating and destructive reaction which once starts, would lead to oxidation of all lipids in a cell (Niki et al., 2005). MDA levels were observed to be increased remarkably in both the hippocampus (24%) and cortex (40%) of PTZ treated animals on comparison to controls. Curcumin supplementation significantly lowered PTZ induced lipid peroxidation in hippocampus (25%) and cortex (22%) when compared to PTZ treated animals (Figure 4.18). Increasing evidences suggest that the increment in the generation of free radicals or decrease in antioxidants levels in the body could leads to peroxidation of membrane lipids which is suggested to be censoriously involved in epileptic seizures (Maertens et al., 1995).

Present results indicate the involvement of oxidative injury in PTZ treated animals and these findings are in accordance with the previous reports showing increased lipid peroxidation in brain of epileptic rats (Gupta et al., 2003). In contrast, PTZ animals supplemented with curcumin showed reduction in lipid peroxidation in both the regions of brain suggesting the neutralization of lipid peroxide produced. Evidence suggests that curcumin neutralizes the free radicals, inhibits oxidative enzymes such as cytochrome P450 and reduces the lipid peroxidation in rats (Reddy and Lokesh, 1994a; Sreejayan and Rao, 1994). Presence of carbon double bond, β-diketon and phenyl rings in the curcumin structure make it a strong antioxidant and anti-lipidperoxidative agent against a variety of oxidative stress conditions (Aggarwal and Harikumar, 2009; Esatbeyoglu et al., 2012). Previous studies have also shown that curcumin when administrated at a dose of 100 mg/kg body weight significantly prevented lipid peroxidation in kainic acid and PTZ treated animals (Agarwal et al., 2011; Gupta et al., 2009).
Results and Discussion

Figure 4.18: Effect of curcumin supplementation on lipid peroxidation in hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05). #significantly different from PTZ treated group (p<0.05).

4.6.2 Antioxidant defense system

It is considered that increased free radicals or lipid peroxide levels are controlled by a wide range of antioxidants including both enzymatic and non-enzymatic such as glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT). To assess the anti-oxidative potential of curcumin, various antioxidant enzymes such as SOD, catalase, glutathione (GSH), glutathione s-transferase (GST) were measured in both hippocampus and cortex regions of the brain. The results are presented in Table 4.5.

SOD is one of the major antioxidant enzymes that play a role in detoxification of superoxide anions, which are highly reactive radicals in the cell. Dismutation of superoxide results in the formation of molecular oxygen and hydrogen peroxide (H$_2$O$_2$) as the reaction products. In present study, the activity of SOD was found to be reduced by 33% in hippocampus and 29% in cortex of PTZ treatment animals when compared to controls (Figure 4.19). However, curcumin supplementation to PTZ animals resulted in restoration of SOD activity in both the regions where an increase of 37% was observed in hippocampus and 25% in cortex.
Figure 4.19: Effect of curcumin supplementation on superoxide dismutase activity in hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05). #significantly different from PTZ treated group (p<0.05).

Hydrogen peroxide produced as a result of dismutation of superoxide by SOD can damage the cell further converting it to highly reactive hydroxyl radical (·OH). Catalase is a ubiquitously expressed enzyme in CNS which is located in peroxisomes and catalyses the conversion of $\text{H}_2\text{O}_2$ into water and molecular oxygen (non-reactive form). PTZ treated animals showed a significant decrease in the activity of catalase in both the hippocampus (29%) and cortex (27%) region as comparison to controls (Figure 4.20). On the other hand, curcumin supplementation to PTZ animals had no effect on catalase activity in both the regions.

GST is an important enzyme which plays a role in cellular detoxification by catalyzing the addition of tripeptide glutathione to electrophilic functional groups and scavenging the toxic compounds including those produced as a result of oxidative stress (Li et al., 2005). GST activity was observed to be reduced by 19% in hippocampus and 34% in cortex of PTZ treated animals when compared to controls (Figure 4.21). However, curcumin administration restored the GST activity in both the regions where an increase of 19% and 36% was observed in hippocampus and cortex respectively.
Table 4.5: Effect of curcumin supplementation on superoxide dismutase, catalase and glutathione-s-transferase activity in hippocampus and cortex region of PTZ treated animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (units/mg protein)</th>
<th>Catalase (μmole H$_2$O$_2$ decomposed/min/mg protein)</th>
<th>GST (pmole/mg protein)</th>
<th>SOD (μmole H$_2$O$_2$ decomposed/min/mg protein)</th>
<th>Catalase (units/mg protein)</th>
<th>GST (pmole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.49 ± 1.61</td>
<td>2.34 ± 0.22</td>
<td>68.61 ± 2.22</td>
<td>28.38 ± 1.71</td>
<td>3.32 ± 0.34</td>
<td>107.19 ± 10.98</td>
</tr>
<tr>
<td>PTZ</td>
<td>11.67 ± 1.17*</td>
<td>1.66 ± 0.16*</td>
<td>55.73 ± 2.78*</td>
<td>20.26 ± 1.69*</td>
<td>2.44 ± 0.14*</td>
<td>75.99 ± 3.69*</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>15.96 ± 0.67#</td>
<td>1.85 ± 0.38</td>
<td>65.81 ± 2.21#</td>
<td>25.34 ± 1.65</td>
<td>2.72 ± 0.07</td>
<td>105.2 ± 10.08#</td>
</tr>
<tr>
<td>Curcumin</td>
<td>20.45 ± 1.06</td>
<td>2.02 ± 0.11</td>
<td>63.70 ± 4.21</td>
<td>24.76 ± 1.31</td>
<td>3.06 ± 0.23</td>
<td>126.8 ± 11.15</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM, n=7/group.

*significantly different from control group (P<0.05).

#significantly different from PTZ treated group (P<0.05).
The decrease in activities of SOD, catalase and GST in PTZ treated groups could be due to an increased utilization of these enzymes to combat the oxidative stress induced following PTZ treatment. Excess increase of free radicals and lipid peroxide burden in brain could result in the decreased levels of superoxide dismutase, catalase, GST which has already been reported in different animal model of epilepsy (Erakovic et al., 2003; Patsoukis et al., 2004). The increased activity of SOD and GST in PTZ animals supplemented with curcumin could be attributed to the antioxidant property of curcumin which is mediated by indirect scavenging through the up-regulating of endogenous cellular antioxidant devices, including the initiation of cytoprotective nuclear factor erythroid 2-related factor-2 (Nrf2) induced target genes (Dinkova-Kostova and Talalay, 2008; Yang et al., 2009). It has been documented that curcumin induces endogenous antioxidant by modulating the transcription factors such as Nrf2, activator protein-1 (AP-1), and nuclear factor kappa B (NFkB) (Pinkus et al., 1996; Tapia et al., 2012). Both GST and SOD are cytoprotective enzymes that are regulated by the Nrf2 pathway and curcumin is known to modulate this pathway (Gonzalez-Reyes et al., 2013; Kumar et al., 2013; Wenke and Yu,
Moreover, curcumin is very well known to induce the expression of cytoprotective protein including SOD, catalase, GPx, GST and heme oxygenase 1 (HO-1) (Trujillo et al., 2013). Hence, in present study curcumin at molecular levels might be activating signalling pathways Nrf2 and inducing the transcription of genes involved in increased expression of antioxidant enzymes (SOD, catalase and GST). However, curcumin was not able to revert the decreased catalase activity in both the studied region of brain which might be that a dose of 100 mg/kg curcumin was not able to increase catalase activity and was insufficient to combat the free radicals produced in PTZ induced chronic epilepsy.

4.6.3 Thiol redox state

The thiol redox state (TRS) is an essential parameter in biological system which regulates intracellular redox homeostasis (Sies, 1999). It consists of certain components like total thiols, protein thiols (PSH) and non-protein thiols (GSH) (Table 4.6).

The levels of total thiols and protein thiols were decreased considerably by 36% and 40% respectively in hippocampus and were decreased by 19% and 17% respectively in
cortex (Figure 4.22, 4.23). However, PTZ animals supplemented with curcumin showed a reverse trend in cortex where the levels were found to be increased significantly while no change in the levels of total thiols and protein thiols were observed in hippocampus region.

Glutathione (GSH), non-protein thiols is most abundant thiols in brain, also called “master antioxidant and plays a major role as regulator of intracellular redox state. GSH can directly act as anti-oxidant by scavenging ROS and reducing disulfide linkages of protein. The level of GSH was found to be decreased markedly in the hippocampus (13%) and cortex (16%) of PTZ treated animals (Figure 4.24). However, curcumin supplementation resulted in restoration of GSH levels in both the regions of brain.
increase of oxidative stress (Michaelis, 1998; Reynolds and Hastings, 1995). Previous study has demonstrated that reduced levels of GSH induces convulsions in the animals and in contrast, the administration of GSH directly into the brain inhibit PTZ induced convulsions in animals, suggesting protective effect of GSH against seizures (Abe et al., 2000). Low level of GSH can enhance the ROS production and can increase the oxidative stress. In present study, curcumin supplementation to PTZ animals increased the levels of GSH which suggest the role of curcumin as an anti-oxidant in PTZ induced model of chronic epilepsy. Curcumin has been reported to acts as an antioxidant and observed to induce GSH synthesis in vitro (Dickinson et al., 2003). Earlier reports also showed curcumin in enhancing the synthesis and concentration of GSH in astrocytes and neurons by induction of γ-glutamylcysteine ligase (Lavoie et al., 2009).

Figure 4.23: Effect of curcumin supplementation on non-protein thiols in hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05). #significantly different from PTZ treated group (p<0.05).
<table>
<thead>
<tr>
<th>Groups</th>
<th>Hippocampus</th>
<th></th>
<th>Cortex</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Thiols</td>
<td>Non-protein Thiols</td>
<td>Protein Thiols</td>
<td>Total Thiols</td>
</tr>
<tr>
<td></td>
<td>(μmole /mg protein)</td>
<td>(μmole /mg protein)</td>
<td>(μmole /mg protein)</td>
<td>(μmole /mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>100.52 ± 8.91</td>
<td>9.04 ± 0.29</td>
<td>92.13 ± 9.14</td>
<td>101.68 ± 3.11</td>
</tr>
<tr>
<td>PTZ</td>
<td>63.81 ± 8.01*</td>
<td>7.85 ± 0.17*</td>
<td>55.38 ± 7.94*</td>
<td>81.82 ± 5.22*</td>
</tr>
<tr>
<td>PTZ+ Curcumin</td>
<td>71.05 ± 4.16</td>
<td>8.51 ± 0.18#</td>
<td>62.83 ± 4.54</td>
<td>95.60 ± 1.99#</td>
</tr>
<tr>
<td>Curcumin</td>
<td>90.23 ± 5.27</td>
<td>9.03 ± 0.14</td>
<td>71.01 ± 5.56</td>
<td>96.63 ± 3.94</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM, n=7/group.
*significantly different from control group (P<0.05).
#significantly different from PTZ treated group (P<0.05).
4.7 Effect of curcumin supplementation on PTZ induced mitochondrial respiratory chain dysfunctions

It has been suggested that brain mitochondrial dysfunctions and oxidative stress are associated with pathogenesis of various neurological disorders including epilepsy, however the mechanism by which it happens remained unclear. Mitochondria are organelles found in the neuron, participate in several cellular processes such as generation of ATP, neurotransmitter biosynthesis and cell death that can affect neuronal hyperexcitability. Mitochondrial respiratory chain is also considered to be the main source of free radical (ROS) production. It has been documented that mitochondria has role in epileptogenesis and several key events such as cell loss, inflammation and cell signalling are associated with it. Therefore, further the mitochondrial oxidative stress and mitochondrial enzyme activities were measured to access the role of mitochondrial dysfunction in propagation of seizures and to study the neuroprotective potential of curcumin. The activity of mitochondrial enzymes has been studied in cortex and hippocampus and depicted in Table 4.7.
Table 4.7: Effect of curcumin supplementation on the activity of mitochondrial complexes in hippocampus and cortex of PTZ treated rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hippocampus</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADH dehydrogenase</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>Control</td>
<td>25.62 ± 3.03 (nmole NADH oxidized/min/mg protein)</td>
<td>28.97 ± 2.79 (nmole succinate oxidized/min/mg protein)</td>
</tr>
<tr>
<td>PTZ</td>
<td>14.76 ± 1.77* (nmole NADH oxidized/min/mg protein)</td>
<td>23.21 ± 3.06 (nmole succinate oxidized/min/mg protein)</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>25.97 ± 3.55# (nmole NADH oxidized/min/mg protein)</td>
<td>24.46 ± 3.14 (nmole succinate oxidized/min/mg protein)</td>
</tr>
<tr>
<td>Curcumin</td>
<td>29.87 ± 6.64 (nmole NADH oxidized/min/mg protein)</td>
<td>30.72 ± 2.47 (nmole succinate oxidized/min/mg protein)</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM. n=7/group

*significantly different from control group (P<0.05)

#significantly different from PTZ treated group (P<0.05).
4.7.1 NADH dehydrogenase (EC 1.6.99.3)

NADH dehydrogenase is the first enzyme (complex I) of mitochondrial transport chain that oxidizes NADH to NAD⁺ and generates proton gradient which is used in ATP synthesis. Administration of PTZ to animals resulted in inhibition of NADH dehydrogenase in both the regions of brain (Figure 4.25). The activity was observed to be inhibited by 42% in the hippocampus and 40% in the cortex of PTZ treated animals as compared to controls. However, the activity was found to be increased by 76% in hippocampus and by 68% in cortex on curcumin supplementation to PTZ treated animals.

![Graph showing the effect of curcumin supplementation on NADH dehydrogenase activity in mitochondria isolated from hippocampus and cortex region of PTZ treated animals. All values are expressed as mean ± SEM; n= 7/group. *significantly different from control group (p < 0.05), #significantly different from PTZ treated group (p < 0.05).](image)

4.7.2 Succinate dehydrogenase (EC 1.3.5.1)

Succinate dehydrogenase (complex II) catalyzes oxidation of succinate to fumarate. Activity of this enzyme was found to be remained unaffected in both the regions of brain on PTZ treatment (Figure 4.26). Curcumin supplementation also had no effect on the activity of succinate dehydrogenase.
Hippocampus Cortex

Figure 4.26: Effect of curcumin supplementation on succinate dehydrogenase activity in mitochondria isolated from hippocampus and cortex region of PTZ treated animals. All values are expressed as mean ± SEM; n= 7/group.

4.7.3 Cytochrome oxidase (EC 1.9.3.1)

Cytochrome oxidase receives an electron from cytochrome c molecules and transfer to oxygen in electron transport chain. Animals treated with PTZ exhibited a significant decrease in the activity of cytochrome c oxidase in both the regions of brain (Figure 4.27). The inhibition was found to be 47% in the hippocampus and 62% in the cortex of PTZ treated animals as compared to control animals. PTZ animals supplemented with curcumin showed an increase in cytochrome c oxidase activity by 88% in the hippocampus and 85% in the cortex in comparison to PTZ treated animals.

4.7.4 F1F0 synthase (EC 3.6.3.14)

The activity of F1-F0 ATP synthase was found to be remained unaffected in both the regions of brain on PTZ treatment as compared to controls (Figure 4.28). Administration of curcumin also showed no effect on this enzyme transport chain complex.
Results and Discussion

Figure 4.27: Effect of curcumin supplementation on cytochrome oxidase activity in mitochondria isolated from hippocampus and cortex region of PTZ treated animals. All values are expressed as mean ± SEM; n= 7/group. *significantly different from control group (p < 0.05), #significantly different from PTZ treated group (p < 0.05).

Figure 4.28: Effect of curcumin supplementation on F,F0 synthase activity in mitochondria isolated from hippocampus and cortex region of PTZ treated animals. All values are expressed as mean ± SEM; n= 7/group.
4.7.5 MTT reduction

MTT reduction was used to evaluate the mitochondrial respiration. It is considered as a marker for mitochondrial dehydrogenase activity. The effect of PTZ treatment on mitochondrial respiration in hippocampus and cortex is shown in Figure 4.29. Significant decrease in MTT reduction was observed in both cortex as well as hippocampus of PTZ animals as compared to controls. It was found to be significantly reduced by 68% in hippocampus and by 46% in cortex of PTZ treated animals. However, curcumin administration to the PTZ animals increased the rate of MTT reduction by 133% in hippocampus and 46% in cortex.

In present study, a significant decrease in NADH: cytochrome c reductase (complex I) and cytochrome c oxidase activity (complex IV) was observed in PTZ animals which is in accordance to the study by Kudin et al., (2002). Inhibition of mitochondrial complexes could be due to increased ROS generation following PTZ treatment (Celikyurt et al., 2011). A recent report showed seizures induced dysfunction of mitochondrial complex IV in
animals with pilocarpine induced status epilepticus (Gao et al., 2013). Furthermore, disturbance in glycolytic rates, lactate/pyruvate ratios and loss of mitochondrial N-acetylaspartate have also been observed in human epilepsy which confirms the involvement of mitochondria in TLE (Rowley and Patel, 2013). Inhibition of mitochondrial electron transport components contributes to incomplete electron transport and decrease in intracellular ATP production that disturbs the normal calcium homeostasis thereby affecting neuronal excitability and synaptic transmission (Folbergrova and Kunz, 2012). No significant change was observed in the activity of succinate dehydrogenase and ATP synthase on PTZ treatment in animals which is in accordance to previous experimental reports showing no change in these mitochondrial complexes (Gao et al., 2013; Rahman, 2012). Moreover, results from human studies suggested major changes in complex I and IV of mitochondria whereas complex II and V remained unaltered in epileptic patients (Kang et al., 2007). Therefore, our results provide supporting evidence for the previous observations that mitochondria complex I and IV are major contributors in impaired mitochondrial function in chronic epilepsy (Kunz et al., 2000).

Further, MTT reduction was found to be markedly decreased in both regions of PTZ treated animals when compared to control group. The perturbed activity of mitochondrial enzymes led to decrease in MTT reduction in PTZ treated animals. The amount of formazan reaction product formation in MTT assay depends upon mitochondrial and cytoplasmic enzymatic activity (Huet et al., 1992; Jang et al., 2012). Earlier it has been suggested that cellular MTT reduction is only associated with activity of flavin-containing succinate dehydrogenases however, evidence also indicates the involvement of other dehydrogenase (Berridge et al., 2005). The present results that PTZ treated animals showed decrease in MTT reduction are in agreement with the results of previous study where PTZ treatment decreased MTT reduction in hippocampal astrocytes in a dose dependent manner (Zhu et al., 2012).

On the other hand, curcumin supplementation to PTZ animals restored the activities of these complexes in both the regions. These findings are in agreement with the recent report which showed that curcumin reduces hepatotoxicity by providing protecting against mitochondrial alterations such as decrease in the cellular ATP levels, mitochondrial
Results and Discussion

Permeability transition, calcium homeostasis disruption and oxidative stress (Garcia-Nino and Pedraza-Chaverri, 2014) (109). Pre-treatment of curcumin prevents mitochondrial impairment by directly detoxifying and preventing 3-nitrotyrosine formation in Parkinson’s disease (Mythri et al., 2007). Furthermore, curcumin encapsulated solid lipid nanoparticles have been shown to ameliorate mitochondrial impairments observed in 3-NP induced Huntington’s disease through activation of Nrf2 pathway (Sandhir et al., 2014).

4.8 Effect of curcumin supplementation on PTZ induced mitochondrial membrane permeability (Mitochondrial Swelling)

Mitochondria transition pore opening results in swelling of mitochondria. Mitochondrial swelling and contraction was used as a functional test for the mitochondrial membrane integrity. Mitochondrion swelling was observed to be increased by 8 fold in 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 (Figure 4.30).

Figure 4.30: Effect of Curcumin supplementation on mitochondrial swelling in the mitochondria isolated from hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n= 7/group.*significantly different from control group (p<0.05). #significantly different from PTZ treated group (p<0.05).
Increased oxidative stress can alter the mitochondrial membrane potential that can further increase mitochondrial swelling (Kroemer et al., 2007). Mitochondrial swelling was observed to be increased in cortex as well as hippocampus of PTZ treated animals suggesting the deleterious role of oxidative stress in mitochondria dysfunction in epilepsy. Neuropathological investigations from previous reports suggest seizure associated changes in neurons 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). However, we found that curcumin supplementation to PTZ animals prevented mitochondria swelling and restored other mitochondrial functions which could be because of presence of phenolic as well as β-diketone functional groups in curcumin which scavenge the free radicals and ameliorated PTZ induced oxidant effect (Reddy and Lokesh, 1994b). Moreover, curcumin administration has found to reduce protein carbonyl levels and restored other mitochondrial respiratory functions in oxidant 4-hydroxynonenal treated animals (Raza et al., 2008). Curcumin has been observed to reduce mitochondrial swelling (decreased permeability transition pore opening) and damage in potassium dichromate treated rats and prevented mitochondrial dysfunction (Garcia-Nino et al., 2013).

**4.9 Effect of curcumin supplementation on PTZ induced mitochondrial oxidative stress**

Evidences indicate a link between impaired mitochondria respiratory functions and enhanced free radicals production resulting in mitochondrial oxidative damage, which plays a role in pathogenesis of various neurological disorders (Ankarcrona et al., 1995). In addition, it has been suggested that increased ROS in brain can attack polyunsaturated fatty acids, proteins, enzymes and nucleic acid causing damage to mitochondrial DNA that could results in mitochondrial respiration dysfunctions (Khurana et al., 2013). Brain is rich in mitochondria and has high oxygen consumption; it also has high concentration of polyunsaturated fatty acid and high iron contents which make it more vulnerable to oxidative stress. To measure the mitochondria oxidative damage, two important oxidative stress markers i.e. reactive oxygen species and protein carbonyl were studied in the mitochondria isolated form hippocampus and cortex of PTZ treated animals.
4.9.1 Reactive Oxygen Species

Mitochondria constantly metabolize oxygen thereby producing reactive oxygen species (ROS) as a result of incomplete oxygen metabolism. ROS levels were measured in hippocampus as well as cortex region following 30 days PTZ treatment. ROS production was observed to be increased significantly in hippocampus (94%) and cortex (90%) of PTZ treatment when compared to control animals. However, curcumin administration decreased the levels of ROS by 46% in hippocampus and 36% in cortex when compared to PTZ treated group (Figure 4.31).

![Graph showing ROS levels in control, PTZ, PTZ + Curcumin, and Curcumin groups in hippocampus and cortex](image)

Figure 4.31: Effect of curcumin supplementation on ROS levels in mitochondria isolated from hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05). #significantly different from PTZ treated group (p<0.05).

4.9.2 Protein carbonyls

Protein carbonyls are widely used as a biomarker of oxidative stress. The protein carbonyl levels were measured in both the regions of brain and found to be increased by 188% in hippocampus and 87% in cortex of PTZ treated animals, while curcumin treatment along with PTZ decreased the levels by 91% in hippocampus and 54% in cortex when compared to PTZ treated group (Figure 4.32).

![Graph showing protein carbonyl levels in control, PTZ, PTZ + Curcumin, and Curcumin groups in hippocampus and cortex](image)
In present study, protein carbonyl and ROS levels were found to be elevated in 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 a main source of ROS production (Vezzani et al., 2011b). Partial inhibition of complex I contribute to increased ROS production which causes cell death (Folbergrova et al., 2010). Proteins are sensitive to oxidation by ROS and RNS, and oxidative damage is a result from such interaction. The protein oxidation leads to loss of protein function and often cell death via necrotic or apoptotic processes (Marchi et al., 2012). Excess ROS formed in present study could have modified proteins leading to increased protein carbonylation. Previous studies also reported increased protein carbonyl levels in rat cortex and hippocampus in experimental status epilepticus (Chuang et al., 2012). Free radicals can cause peroxidation of membrane polyunsaturated fatty acids present in brain. The result of present study clearly shows that curcumin administration to PTZ animals reduced ROS and protein carbonyls which suggest the role of curcumin as potential anti-oxidant in chronic epilepsy (Du et al., 2012). The phenolic hydroxyl groups present in curcumin plays a significant role in ROS scavenging (Anand et al., 2008). Earlier reports have indicated that curcumin act as a free radical scavenger and have antioxidant properties (Aggarwal and Harikumar, 2009). Decrease in the free radical production (oxidative stress) by curcumin could have resulted in reduction of mitochondrial dysfunctions on curcumin supplementation. Taken together, results suggest that curcumin could be a promising therapeutic candidate for protecting epileptic brain against oxidative stress and mitochondrial respiratory chain dysfunctions.

4.10 Effect of curcumin supplementation on PTZ induced ultrastructural changes in mitochondria

The electron micrographs of cortex and hippocampus were examined for ultrastructure changes in mitochondria (Figure 4.33 and 4.34). PTZ administration to animals resulted in severe damage to mitochondrial structure characterized by disruption of mitochondrial membrane integrity, distorted cristae with clearing of matrix density in both the regions. The results are in agreement with the previous findings where seizures are known to be associated with mitochondrial swelling and mitochondrial dysfunctions (Zhang et al., 2013b). Functional defects in mitochondria along with abnormalities in mitochondrial
Figure 4.32: Effect of curcumin supplementation on protein carbonyls levels in mitochondria isolated from hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n= 7/group. *significantly different from control group (p<0.05). #significantly different from PTZ treated group (p<0.05).

Ultrastructure have also been seen in the TLE patients (Folbergrova and Kunz, 2012). These results suggest that abnormalities in the brain mitochondrial activity are associated with seizure induced changes in mitochondrial ultrastructure. Furthermore, curcumin supplementation to PTZ animals protected the brain mitochondria from damage suggesting curcumin can attenuate mitochondrial dysfunctions along with maintaining mitochondrial structure in chronic model of epilepsy. These changes could be directly correlated with impairment in mitochondrial enzyme activities as it has been suggested that respiratory enzymes and mitochondrial membranes are synthesized in a coordinated manner, any changes in respiratory component can affect its membrane structure (King et al., 1972). However, normal morphology of mitochondria was observed in saline and curcumin treated animals. Curcumin supplementation to PTZ animals reversed the morphological (structural) deficits. A recent study has shown that curcumin can prevent ultrastructural changes in brain mitochondria induced by arsenic by modulating the expression of pro-and anti-apoptotic mitochondrial proteins in brain (Srivastava et al., 2014a).
Results and Discussion

Figure 4.33: Transmission electron micrographs of hippocampus showing mitochondrial ultrastructure changes in PTZ treated animals. Left panel shows mitochondrial ultrastructure at 50000X magnification. Right panel shows magnified view of individual mitochondria.

PTZ + Curcumin Control

PTZ / Curcumin

Curcumin
Figure 4.34: Transmission electron micrographs of cortex showing mitochondrial ultrastructure changes in PTZ treated animals. Left panel shows mitochondrial ultrastructure at 50000X magnification. Right panel shows magnified view of individual mitochondria.
4.11 Effect of curcumin supplementation on astrocytes and microglial activation

Neuroinflammation, regarded as inflammation to CNS is characterized by inflammatory molecules, endothelial cell activation and tissue edema in various pathophysiological conditions which results in neuronal damage (Aktas et al., 2007). In brain, the innate immunity is provided by microglial cells and astrocytes which act as a first line of defense against injury (Solito and Sastre, 2012). Activation of microglia and astrocytes releases combinations of bioactive agents including an array of inflammatory molecules (cytokines and chemokines) and proteases which can influence the neuronal survival. Among these inflammatory molecules, cytokines are polypeptides mediate interaction among components of immune system (glia and neurons). In fact, these bioactive agents can have both protective as well as detrimental consequences for the surrounding brain tissues. Previous studies have demonstrated that activated glia cells can play neuroprotective role by releasing various neurotrophic factors and maintaining the CNS homeostasis while its over activation exerts neurotoxic effect by producing excess of neurotoxic factors such as cytokines, chemokines, excitatory neurotransmitters and ROS (Takeuchi and Suzumura). Earlier reports have shown the prominence of reactive gliosis in almost all form of seizure including TLE, the most common form of epilepsy (Maness et al., 1998). In order to evaluate the involvement of glial cells activation in PTZ induced chronic epilepsy, astrocytes (GFAP) and microglial marker (Iba1) were studied in both hippocampus and cortex of PTZ treated animals. Real time PCR (RT-PCR) analysis was performed to examine the mRNA expression and western blotting was performed to access the protein expression of GFAP and Iba1. Immunohistochemistry was performed to access the reactive gliosis in PTZ treated animals.

4.11.1 Immunoblotting and real time-PCR analysis of astrocytes marker (GFAP)

To evaluate the effect of curcumin on the activation of astrocytes in hippocampus and cortex, real time-PCR was performed. Increase in mRNA expression of GFAP was observed in both the brain regions of PTZ treated animals as compared to controls (Figure 4.35). GFAP mRNA expression was up-regulated by 1.7 fold in both hippocampus and cortex when compared to controls. In contrast, curcumin supplementation to PTZ treated animals decreased the mRNA expression of GFAP in both hippocampus and cortex (1.5
fold). In PTZ treated animals, the protein levels of GFAP were found to be increased significantly (75%) in hippocampus region when compared to controls while only 23% increase was observed in cortex region (Figure 4.36). On the other hand, curcumin supplementation to PTZ animals had no effect on GFAP protein expression.

Figure 4.35: Effect of curcumin supplementation on mRNA expression of astrocyte marker (GFAP) in hippocampus and cortex of PTZ treated animals assessed using real time PCR. The relative expression was calculated using the 2-ΔΔCt method. *significantly different from control group (p<0.05). #significantly different from PTZ treated group (p<0.05).

4.11.2 Effect of curcumin supplementation on immunohistochemical staining for astrocytes (GFAP)

To evaluate if reactive gliosis occurs in PTZ treated animals and to study the effect of curcumin supplementation on it, immunohistochemical analysis was performed for astrocytic marker, GFAP in hippocampus and cortex of PTZ treated animals. 30 days of PTZ treatment, resulted in an enhanced immunoreactivity for GFAP in both hippocampus (Figure 4.37) and cortex regions (Figure 4.38). The thresholding and quantification analysis of immunohistochemical staining in hippocampus and cortex was done and results are shown in Figure 4.39, 4.40 respectively. GFAP immunoreactivity was found to be
enhanced by 1.7 fold in hippocampus and by 1.8 fold in cortex. Morphologically, activated astrocytes in hippocampal and cortex showed a hypertrophic form with enlarged cell body and thick processes. The immunoreactivity (activation) was greatly attenuated in PTZ animals supplemented with curcumin.

![Figure 4.36: Effect of curcumin supplementation on protein expression of astrocyte marker GFAP in hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n=4/group*significantly different from control group (p<0.05).]
Figure 3.37: Effect of curcumin supplementation on immunoreactivity of astrocytes stained with anti-GFAP antibodies in hippocampus of PTZ treated animals. Images (a) are taken at 10x magnification and images (b) are taken at 40x magnification. Scale bars 100μm (a) 50μm (b). Inset shows the magnified cell from respective image with scale bar 10μm.
Figure 4.38: Effect of curcumin supplementation on immunoreactivity of astrocytes with anti-GFAP antibodies in cortex of PTZ treated animals. Images (a) are taken at 40x magnification and images (b) are taken at 40x magnification. Scale bars 100μm (a) 50μm (b) shows the magnified cell from respective image with scale bar 10 μm.
Figure 4.39: MATLAB implementation of Ostu’s method (Clustering-based thresholding method) on GFAP stained images to quantify the immunoreactivity of astrocytic cells in hippocampus and cortex region of brain.
GFAP is an astrocyte specific cytoskeletal protein which is a reliable marker of reactive astrogliosis (Kamphuis et al., 2012). Evidence suggests prominence of reactive gliosis in almost all form of seizure including TLE, the most common form of epilepsy (Fellin and Haydon, 2005). The mRNA expression of GFAP was found to be significantly increased in hippocampus as well as cortex of PTZ treated animals while the protein expression of GFAP was found to be increased significantly in hippocampus with small increase in cortex region. The results of our study are in accordance with the previous reports wherein increased GFAP mRNA and protein expression has been observed after seizures (Torre et al., 1993). It has been previously reported that changes in transcription and protein expression of GFAP can affect the morphology of astrocytes in brain (Morgan et al., 1997). PTZ treatment lead to activation of astrocytes as shown by hypertrophied GFAP-labelled astrocytes cells. Quantitative assessment of GFAP suggests activation of astroglial cells throughout the hippocampus and cortex following PTZ treatment. GFAP-
labelled astrocyte had enhanced labelling and thickened processes in PTZ treated group relative to the control group. There are reports available showing activation of hippocampal astrocytes in patients with epilepsy and different animal model of epilepsy (Bechstein et al., 2012; Das et al., 2012) Astrocyte undergoes functional changes including increase in proliferation (i.e. increase in number), biochemical and structural/morphological changes (hypertrophy form) in various stress conditions (Binder and Steinhauser, 2006; Ortinski et al., 2010). Enhanced microglial and astrocyte activation has been seen in the aged brain following traumatic brain injury which is suggested to be linked with poorly controlled inflammatory response during aging process (Sandhir et al., 2008). Increase in abnormal number of astrocytes also known as astrogliosis triggers increase in neuronal glutamine which further lead to hyperexcitability in hippocampus circuits which is suggested to be associated with epilepsy

Reducing inflammation in epileptic brain could be beneficial which can decrease the brain damage and improve neuronal functions. The effect of curcumin supplementation on inflammation in terms of astrocytes activation was studied in PTZ treated animals where a significant decrease in GFAP mRNA expression and moderate decrease in protein levels were observed. Curcumin administration to the PTZ treated animals also exhibited reduced number of activated astrocytes with minimal morphological changes suggesting curcumin attenuates seizure-induced astrogliosis. These results are in line with the recent findings showing curcumin administration decreasing the number of GFAP positive cells along with down-regulation of GFAP gene expression in rat model of Alzheimer’s disease (Wang et al., 2013). Previous studies also reported beneficial effect of curcumin by attenuating the neuronal loss, preventing apoptosis and decreasing astrocytes activation in brain injury (Lin et al., 2011)

4.11.3 Immunoblotting and real time-PCR analysis of microglial marker (Iba-1)

The mRNA expression of Iba1 was increased by 2.8 fold in hippocampus and 4 fold in cortex of PTZ animals with respect to controls (Figure 4.41). However, curcumin supplementation to PTZ animals resulted in significant decrease of Iba-1 mRNA expression in both hippocampus and cortex regions.
The protein expression of microglia marker, Iba-1 was determined using western blot analysis. The results showed a significant up-regulation of Iba1 protein expression in hippocampus (22%) and cortex (15%) of PTZ treated animals when compared to controls (Figure 4.42). On the other hand, curcumin when administered to PTZ animals significantly decreased the Iba1 protein expression in both the regions when compared to PTZ treated animals.

![Graph showing effect of curcumin supplementation on mRNA expression of microglia marker (Iba-1) in hippocampus and cortex of PTZ treated animals assessed using real time PCR.](image)

Figure 4.41: Effect of curcumin supplementation on mRNA expression of microglia marker (Iba-1) in hippocampus and cortex of PTZ treated animals assessed using real time PCR. The relative expression was calculated using the 2-ΔΔCt method. All values are expressed as mean ± SEM; n=6/group. *significantly different from control group (p<0.05), #significantly different from PTZ treated group (p<0.05).

4.11.4 Effect of curcumin supplementation on immunohistochemical staining for microglial (Iba-1)

Activated microglial cells (microgliosis) was observed in hippocampus and cortex of PTZ treated animals, characterized by darkly stained enlarged cell bodies with shorten and thicken processes (Figure 4.43, 4.44). Hypertrophy of cytoplasm with swelling processes was also seen in hippocampus region of PTZ treated animals. The thresholding and quantification analysis of immunohistochemical staining for Iba-1 in hippocampus and cortex was done and results are shown in Figure 4.45, 4.46 respectively. Significant
Results and Discussion

increase in microglial activation was observed in PTZ treated animals which was increased by 1.23 fold in hippocampus and by 1.49 fold in cortex when compared to controls. Control animals showed lightly stained small cell bodies with well-developed thin processes showing ramified form. However, curcumin supplementation to PTZ treated animals led to significant decrease in activation of astrocytes and microglia along with attenuation of morphological changes observed in hippocampus and cortex.

Figure 4.42: Effect of curcumin supplementation on protein expression of microglia marker (Iba-1) in hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n=4/group. *significantly different from control group (p<0.05), #significantly different from PTZ treated group (p<0.05).
Figure 4.43: Effect of curcumin supplementation on immunoreactivity of microgli stained with anti-Iba-1 antibodies in hippocampus of PTZ treated animals. Images taken at 10x magnification and images (b) are taken at 40x magnification. Scale bars 100 50μm (b). Inset shows the magnified cell from respective image with scale bar 10 μm.
Figure 4.44: Effect of curcumin supplementation on immunoreactivity of microglial cells stained with anti-Iba-1 antibodies in cortex of PTZ treated animals. Images (a) are taken at 10x magnification and images (b) are taken at 40x magnification. Scale bars 100 µm (a) 50 µm (b). Ins shows the magnified cell from respective image with scale bar 10 µm.
Figure 4.45: MATLAB implementation of Ostu's method (Clustering-based Im thresholding method) on Iba1 stained images to quantify the immunoreactivity of micro cells in hippocampus and cortex region of brain.

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Iba1, a calcium binding cytosolic protein, is a marker of activated microglia (Drago et al., 2014). A marked increase in Iba1 mRNA and protein expression in hippocampus as well as cortex of PTZ treated animals suggests seizure-induced microglial activation. This increase in protein and mRNA levels of Iba1 in PTZ treated animal correlates with enhanced immunoreactivity for Iba1 observed in both the brain regions. The intensity of Iba1 staining was found to be more in the case of PTZ treated animals. An up-regulation of Iba1 expression has been suspected of contributing to several neurodegenerative diseases via production of cytotoxic molecules, free radicals, pro-inflammatory prostaglandins and cytokines (Luo and Chen, 2012; Smith et al., 2012). Microglia present in high density under pathological conditions affects synaptic transmission (Bessis et al., 2007). Increase in the number of microglial processes (more branching) suggests the microglial activation that has been observed in rat hippocampus after seizures (Shapiro et al., 2008).

Figure 4.46: Graph represents the quantification of immunoreactive microglial (Iba-1) in hippocampus and cortex region. All values are expressed as mean ± SEM; n=4/group. *significantly different from control group (p<0.05), #significantly different from PTZ treated group (p<0.05).

Microglia responds more quickly than astrocytes as found in different neurological conditions which could be the reason that an increase in Iba-1 expression was observed in
cortex region with no significant increase in GFAP expression (Matsumoto et al., 1992). Moreover, astrocytes require a high level of stress to be activated and are generally activated at a later recovery stage (Reali et al., 2005). This might be the reason that no significant increase in protein expression of GFAP (astrocyte marker) was observed while an increase in Iba-1 protein expression was observed in cortex region after PTZ treatment. Curcumin supplementation to the PTZ animals, on the other hand, was found to decrease the Ibal mRNA and protein expression and maintain the morphology of microglia in both the brain regions suggesting anti-inflammatory potential of curcumin in chronic model of epilepsy that maintains microglia in the quiescent state. Previous reports have shown curcumin as a potent immune-regulatory molecule that modulates the activation of other cells including T-cells, B-cells and natural killer cells (Jagetia and Aggarwal, 2007). Curcumin acts as a strong regulator of microglia transcriptome and is reported to reduce Ibal immunoreactivity in mice model of Parkinson’s disease (Tripanichkul and Jaroensuppaperch, 2013).

4.12 Effect of curcumin supplementation on PTZ induced cytokines and chemokine activation

It has been well documented that unrestrained glial-mediated immunity can cause sustained inflammatory changes that facilitate epileptogenesis (Devinsky et al., 2013). Cytokines released by the activated astrocytes further induce transcriptional and post-translational signalling in the astrocytes itself or in the neighbouring cells that promotes the activation of transcription factors which regulating pro-inflammatory gene expression (Maroso et al., ; Vezzani et al.). To correlate the activation of glial cells and the release of cytokines and chemokines in PTZ treated animals, real time PCR and ELISA were performed in hippocampus and cortex region of brain.

4.12.1 mRNA expression of pro-inflammatory cytokines and chemokine

It has been documented that activated microglia and astrocytes can affect the concentration of pro-inflammatory cytokines and chemokines in CNS. Real time PCR was performed to quantitatively measure the mRNA expression of cytokines (TNF-α, IL-1β, IL-6) and chemokine (MCP-1) in hippocampus and cortex. PTZ administration significantly increased the expression of cytokines and chemokine in hippocampus as well as in cortex.
Results and Discussion

(Table 4.8). The IL-1β mRNA expression was increased by 14.2 fold in hippocampus and 3 fold in cortex. The expression of TNF-α mRNA was increased by 2.4 fold in hippocampus and 3.2 fold in cortex along with the expression of IL-6 mRNA which was increased by 4.5 fold in hippocampus and by 3.7 fold in cortex. MCP-1 is most commonly expressed chemokine in the inflamed brain, the expression of which was found to be increased by 2.2 fold in hippocampus and 2.6 fold in cortex of PTZ treated animals. In contrast, curcumin supplementation to PTZ animals significantly decreased the expression of respective genes i.e., IL-1β by 3.2 fold, TNF-α by 2.8 fold, IL-6 by 1.8 fold and MCP-1 by 2.1 fold in hippocampus region. The mRNA expression of TNF-α, IL-1β, IL-6 and MCP-1 was decreased by 1.5 fold, 2.1 fold, 4.9 fold and 2.02 fold respectively in the cortex region which reflects the anti-inflammatory potential of curcumin in PTZ induced chronic model of epilepsy.

4.12.2 Protein levels of pro-inflammatory cytokines and chemokine

ELISA was performed to analyse the protein levels of cytokines and chemokine in hippocampus and cortex regions of the brain and the results are presented in Table 4.9. PTZ administration significantly increased the levels of TNF-α (34%), IL-1β (43%), IL-6 (46%) and MCP-1 (84%) in hippocampus and the levels were increased by 60%, 40%, 54% and 33% respectively in cortex when compared to control animals. The standard curves for each cytokines and chemokine are shown in Figure 4.47.

Supplementation of curcumin with PTZ showed a significant reduction in the levels of cytokines and chemokine suggesting curcumin decreased the inflammatory response. The levels of TNF-α, IL-1β, IL-6 and MCP-1 were found to be decreased by 33%, 22%, 60% and 42% respectively in hippocampus and by 47%, 27%, 55% and 38% respectively in the cortex on curcumin supplementation to PTZ animals.

In recent years, increasing evidence has indicated that immune activation within the central nervous system is a common feature of various neurodegenerative diseases, immune-mediated disorders, infections and trauma which may contribute to neuronal damage. Immune (Amor et al., 2010). Significant increase in mRNA and protein expression of cytokines and chemokine after PTZ treatment suggests increased brain inflammation following seizures.
Table 4.8: Effect of curcumin supplementation on mRNA expression for cytokines (TNF-α, IL-1β, IL-6) and chemokine (MCP-1) in hippocampus and cortex of PTZ treated animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hippocampus</th>
<th></th>
<th></th>
<th></th>
<th>Cortex</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1β</td>
<td>TNF-α</td>
<td>IL-6</td>
<td>MCP-1</td>
<td>IL-1β</td>
<td>TNF-α</td>
<td>IL-6</td>
<td>MCP-1</td>
</tr>
<tr>
<td>Control</td>
<td>1.29 ± 0.03</td>
<td>2.57 ± 0.46</td>
<td>0.99 ± 0.19</td>
<td>2.03 ± 0.38</td>
<td>2.26 ± 0.17</td>
<td>1.38 ± 0.14</td>
<td>2.49 ± 0.54</td>
<td>2.13 ± 0.31</td>
</tr>
<tr>
<td>PTZ</td>
<td>18.14 ±1.65*</td>
<td>6.19 ± 0.56*</td>
<td>4.55 ± 0.81*</td>
<td>4.52 ± 0.44*</td>
<td>6.51 ± 0.72*</td>
<td>4.52 ± 0.59*</td>
<td>9.42 ± 0.37*</td>
<td>5.61 ± 0.69*</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>5.66 ± 0.31#</td>
<td>2.17 ± 0.45#</td>
<td>2.48 ± 0.44#</td>
<td>1.65 ± 0.17#</td>
<td>3.85 ± 0.23#</td>
<td>2.11 ± 0.32#</td>
<td>1.91± 0.01#</td>
<td>2.77 ± 0.36#</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1.71 ± 0.08</td>
<td>2.02 ± 0.99</td>
<td>1.14 ± 0.21</td>
<td>1.97 ± 0.49</td>
<td>1.63 ± 0.21</td>
<td>1.34 ± 0.03</td>
<td>0.97 ± 0.04</td>
<td>2.97 ± 0.21</td>
</tr>
</tbody>
</table>

Relative Expression (ΔΔCt)

The relative expression was calculated using the 2-ΔΔCt method.
All values are expressed as mean ± SEM; n=6/group.
*significantly different from control group (p<0.05)
#significantly different from PTZ treated group (p<0.05).
Results and Discussion

Figure 4.47: Standard curve generated for protein levels of TNF-α, IL-1β, IL-6 and MCP-1 in ELISA assay. R-squared value is the square of the correlation coefficient which gives a measure of the reliability of the linear relationship between the x and y values (Values close to 1 indicate excellent linear reliability).

These observations are in agreement to the previous findings showing a strong implication of cytokine involvement in modulating acute seizures, wherein increased expression of TNF-α and IL-6 during epilepsy is suggested to be associated with increased seizure frequency and knockout mice lacking the receptors for these cytokines showed reduced seizure frequency (Kirkman et al., 2010). Cytokines can modulate the levels of various neurotransmitters such as serotonin, GABA, acetylcholine etc. in brain which could be associated with severity the seizures (Fann and Patterson, 1993). Moreover, various secondary messengers including cAMP, proteinase kinase, and nitric oxide are known to be affected by the increased brain cytokine levels (Rao et al., 2009). Evidence are also
available demonstrating over expression of natural antagonist of IL-1β could significantly
delay the onset of generalized seizures in mice implying an important role of astrocytic IL-
1β in regulating seizure susceptibility (Vezzani et al., 2000). IL-1β and IL-6 are considered
as pro-convulsant cytokines in the brain and increased expression of these cytokines affect
the severity of seizures as observed in various experimental model of epilepsy (Balosso et
al., 2008). Indeed, a number of animal studies indicates that epileptic seizure induce TNF-α
expression in brain, which has recently be confirmed in TLE patients, where a marked up-
regulation of TNF-α was observed, which corroborates with chronic hippocampal
inflammation (Li et al., 2011; Teocchi et al., 2013). Hence, these findings support the
existence of a link between epileptic seizure and production of pro-inflammatory mediators.

In contrast, curcumin supplementation to PTZ animals decreased IL-6, TNF-α, IL-
1β and MCP-1 protein levels as well as and mRNA expression when compared to PTZ
treated animals. These results are in accordance to the previous reports showing anti-
inflammatory effect of curcumin, mediated through suppression of transcription of pro-
inflammatory cytokine genes (Jin et al., 2007). The intricate mechanism action of curcumin
involves various targets that include transcription factors including NF-κB, AP-1, mitogen
activated protein kinases, and subsequent inflammatory pathways (Srivastava et al., 2011).
Curcumin significantly inhibits neuroinflammation by blocking the pro-inflammatory gene
expression and is considered as a novel anti-inflammatory targets in microglia (Karlstetter
et al., 2011). It also reduces the secretion of pro-inflammatory cytokines and cytotoxic
mediators such as NO, IL-1β, IL- 6 and TNF-α in dose dependent manner which suggests
curcumin mediated neuroprotection is attributed to its anti-inflammatory properties (Lee et
al., 2007). Curcumin is reported to have anti-proliferative effect on microglia where a small
dose of curcumin (mM concentration in glioma cell line) inhibited the proliferation of
neuroglia cells (Mishra and Palanivelu, 2008). Curcumin has shown to protect neurons
from inflammation and neuronal damage by reducing the production of ROS and cytokines
by microglia (Guo et al., 2013). Curcumin has free radical scavenging property, two
electrophilic carbonyl groups present on it are involved in neutralization of hydroxyl
radicals, ROS and RNS generated (Scapagnini et al., 2010).
Table 4.9: Effect of curcumin supplementation on cytokines (IL-1β, TNF-α, IL-6) and chemokine (MCP-1) levels in the hippocampus and cortex of PTZ treated animals assessed using ELISA.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hippocampus</th>
<th></th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1β</td>
<td>TNF-α</td>
<td>IL-6</td>
</tr>
<tr>
<td></td>
<td>(pg/mg protein)</td>
<td>(pg/mg protein)</td>
<td>(pg/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>5.66 ± 0.49</td>
<td>160.79 ± 28.13</td>
<td>1.07 ± 0.23</td>
</tr>
<tr>
<td>PTZ</td>
<td>8.07 ± 0.74*</td>
<td>265.91 ± 24.04*</td>
<td>1.56 ± 0.21*</td>
</tr>
<tr>
<td>PTZ + Curcumin</td>
<td>6.31 ± 0.36#</td>
<td>177.13 ± 8.92#</td>
<td>0.63 ± 0.08#</td>
</tr>
<tr>
<td>Curcumin</td>
<td>6.39 ± 0.31</td>
<td>111.39 ± 36.12</td>
<td>0.13 ± 0.04</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM. n=6/group
*significantly different from control group (p<0.05)
#significantly different from PTZ treated group (p<0.05).
4.13 Effect of curcumin supplementation on transcription factor NF-κB in PTZ treated animals

The transcription factor NF-κB has an important role in immunity, generally regulated by intracellular redox state and known to be involved in pathogenesis of various autoimmune and inflammatory diseases (Rong and Baudry, 1996). Electrophoretic mobility shift assay (EMSA) was employed to investigate NF-κB DNA binding activity in hippocampus and cortex regions (nuclear extract) of the rat brain following PTZ treatment. 30 days of PTZ treatment resulted in no change in the NF-κB/DNA binding activity in hippocampus and cortex as no significant difference was observed in the intensity of bands (Figure 4.48).

Figure 4.48. EMSA analysis of NF-κB with nuclear extracts from hippocampus and cortex of PTZ treated animals using NF-κB consensus sequence 5'‐AGT TGA GGG GAC TTT CCC AGGGC-3'. 15μg/ml of protein was used.

Moreover, curcumin administration to PTZ animals also had no effect on NF-κB activity. Intense bands of complex p65/p50 were observed in nuclear extract of the cell line.
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used as positive control. These findings suggest that the transcription factor NF-κB alone may not be a major inducer for gene expression of immune related molecules there must be the role of other transcription factors such as AP-1 or Sp-1 in the activation of genes associated with seizure activity. Earlier study performed on PTZ induced model of epilepsy reported a dramatic increase of AP-1 DNA binding activity in hippocampus and other regions of brain assessed using EMSA, suggesting a role of AP-1 in PTZ induced seizure activity (Lukasiuk and Kaczmarek, 1994). Moreover, curcumin has been reported to regulate the expression of various inflammatory cytokines, growth factors, cell cycle protein and can modulate the activity of AP-1 transcription factor and its signalling pathways (Shishodia, 2013). Reports also indicate that increased concentration of IL-1 and TNF-α induces AP-1 expression in bone marrow stromal cell which was found to be blocked on supplementation of curcumin (Xu et al., 1997).

4.14 Effect of curcumin supplementation on blood brain barrier (BBB) permeability

BBB plays a major role in the pathophysiology of various neurological disorders. Immune-mediated damage to CNS and BBB breakdown is considered as a major contributor for epileptogenesis (Fabene et al., 2008). It has been suggested that CNS inflammation is associated with breakdown of the BBB, and BBB leakage has been implicated both in the induction of seizures and in the progression to epilepsy (van Vliet et al., 2007). Increase in neuroinflammation and production of pro-inflammatory cytokines/chemokines by astrocytes can affect BBB permeability through interacting with their cognate receptors overexpressed on microvessels of brain without even entering the brain (Morin-Brureau et al., 2011). Moreover, histological studies have shown increased albumin accumulation in the human epileptic brain which is a sign for increased BBB permeability (Raabe et al., 2012). The molecular mechanism of increased BBB is not fully understood, however, earlier reports showed the role of leukocyte-vascular interaction (VCAM, leukocyte mucin p-selectin glycoprotein ligand 1 and leukocyte integrin) in BBB damage and seizure generation (Fabene et al., 2008). BBB permeability can be demonstrated by intravascular infusion of exogenous tracers and subsequent detection of extravasated molecules into the brain tissue. The permeation of two most commonly used tracers, namely Evans blue dye, sodium fluorescein were measured in PTZ treated animals.
4.14.1 Sodium fluorescein

Sodium fluorescein (NaFlu, 376 Da) was used to access BBB permeability following PTZ treatment. The brain sodium fluorescein levels were found to be markedly increased in PTZ animals when compared to controls (Figure 4.49). It was observed to be increased by 5.8 fold in hippocampus and 6.6 fold in cortex when compared to their respective controls suggesting that the passage of sodium fluorescein across the BBB is enhanced in PTZ animals. Gurses et al., (2009), has reported increased blood brain permeability after PTZ administration in animals. The enhanced permeation of sodium fluorescein in the present study seems to be consistent with the previous reports from various experimental and human studies showing increased BBB permeability following epilepsy (Hildebrandt et al., 2008). BBB damage is associated with perivascular haemorrhage, disruption of tight junctions and increased pinocytic activity of endothelial cells (Grange-Messent et al., 1999).

Figure 4.49: Effect of curcumin supplementation on sodium fluorescein extravasation in hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05), #significantly different from PTZ treated group (p<0.05).

The inflammatory response in brain cells such as glia and neurons can increase BBB permeability (Huber et al., 2001). Moreover, it has been demonstrated that astrocytes play a
major role in mediating the effect of albumin extravasation (due to BBB leakage) into the brain environment and contribute to induce pathologic events associated with epileptogenesis (Kim et al., 2012b).

In contrast, PTZ animals supplemented with curcumin had decreased sodium fluorescein extravasation to hippocampus and cortex regions indicated attenuation of seizure-associated BBB damage. Curcumin is known to be a potent antioxidant which might be decreasing the BBB damage by scavenging oxygen free radicals that are known to contribute to increased BBB permeability (Zhu et al., 2000). The exact mechanism how curcumin decreases the BBB damage is not fully understood. Curcumin supplementation to PTZ animals in present study significantly reduced the expression of pro-inflammatory cytokines/chemokines in brain suggesting decrease in brain inflammation which may be resulting in decreased BBB damage. These results are in accordance to the previous studies showing curcumin in protecting human intestinal epithelial cells against blood brain barrier dysfunctions via heme oxygenase-1 pathway (Wang et al., 2012). The distribution of tight junction inhibited by curcumin suggests that curcumin might protect the integrity of the BBB following intracerebral hemorrhage, partly by inhibiting the MMP mediated tight junction gap formation (Sun et al., 2011).

4.14.2 Evans blue

A second molecule with higher molecular weight, Evans blue (961 Da) was also used to access the BBB permeability. An increase in permeability of evans blue was observed in hippocampus and cortex region of PTZ treated animals when compared to controls suggesting BBB disruption (Figure 4.50). The levels were increased by 1.8 fold in hippocampus and by 3 fold in cortex of PTZ treated animals when compared to controls. Increased permeability to evans dye could be due to the factors such as increase in excitatory amino acids, disturbance in the calcium and potassium homeostasis along with increased oxidative stress (Kozler and Pokorny, 2003). It has been suggested that alteration in the neural activity takes place when electrolytes levels changes or intravascular protein (albumin) enters into the brain (Seiffert et al., 2004; Uva et al., 2008). In contrast, curcumin supplementation to PTZ animals reduced BBB permeability to evans dye as depicted from decreased levels of evans dye in hippocampus and cortex of PTZ animals. The level of
evans dye was decreased by 3.2 fold in hippocampus and 2.4 fold in cortex when compared to PTZ treated animals. The decreased oxidative stress and increased anti-oxidant levels in the brain on curcumin supplementation in PTZ animals could be considered responsible for the decreased BBB permeability. Curcumin administration had earlier shown to decrease the BBB permeability by reducing nitric oxide overproduction via inhibition of iNOS expression in astrocytes and scavenging oxygen free radicals thereby preventing endothelial cell damage (Jiang et al., 2007).

4.14.3 Brain edema

BBB is essential for maintaining the appropriate ion, protein, and water levels in the brain. It has been widely stated that BBB disruption alone will lead to increase in water entry into the brain resulting in brain edema (Donkin and Vink, 2010; Unterberg et al., 2004).

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**Figure 4.50: Effect of curcumin supplementation on Evans dye extravasation in hippocampus and cortex of PTZ treated animals.** All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05), #significantly different from PTZ treated group (p<0.05).
Regional brain edema (regional tissue water content) was quantitated by the wet-dry weight method following PTZ treatment. Brain water content was significantly increased after PTZ treatment in hippocampus (84.78 % vs 79.13 % in the control group, p<0.05) and cortex (83.14 % vs 79.53% in the control group p<0.05) (Figure 4.51). Inflammatory molecules in the brain could cause the adherence and migrations of leukocytes to brain endothelial cells, which further contributes to the structural modification of tight junction proteins and induce edema (Coisne and Engelhardt, 2011). Enhanced expression of cytokines and chemokine in the present study affirms the role of brain inflammation in the disruption of BBB and increase in edema. However, curcumin supplementation to PTZ animals resulted in significant reduction of water content in hippocampus (80.14 % vs 84.78 in PTZ treated group) and cortex (78.32 vs 83.14% in PTZ treated group). The decrease in brain edema could here be correlated with decrease in cytokine and chemokine expression in both the regions of brain on curcumin supplementation. Our results are in consistence to the previous studies showing curcumin in attenuating cerebral edema and glial activation following traumatic brain injury by decreasing the expression IL-1β and aquaporin-4 expression (Laird et al., 2010).

Figure 4.51: Effect of curcumin supplementation on brain edema (water content) in hippocampus and cortex of PTZ treated animals. All values are expressed as mean ± SEM; n=7/group. *significantly different from control group (p<0.05), #significantly different from PTZ treated group (p<0.05).
4.14.4 Effect of curcumin supplementation on blood vessel changes in PTZ treated animals

The astrocytic endfeet cover blood vessels and release the molecules that regulate tight junctions between the endothelial cells, they also have chemicals that cause blood vessels to expand or contract, thereby regulating blood flow in the brain (Rajkowska et al., 2013). Several structural, molecular, and functional changes have been observed in brain microvasculature following seizures in epilepsy (Marcon et al., 2009; Rigau et al., 2007). Evidence suggests that BBB damage increases excitability and promotes epileptogenesis (Heinemann et al., 2012). Electron micrographs of blood vessels in hippocampus and cortex of PTZ treated animals showed swelling in endothelial and sub-endothelial zone (edema) (Figure 4.52). The decrease in the number of mitochondria along with disruption in mitochondrial structure has been seen in the blood vessels of PTZ treated animals. Endothelium plays a crucial role in the regulation of vascular function and the development of physiological and pathophysiological inflammation (Hartge et al., 2007).

It has been documented that oxidative stress damages the endothelial cells in BBB and contributes to vasogenic edema (Rosenberg, 2012). Increased oxidative stress and enhanced superoxide free radicals (O$_2^-$) are involved in increased vascular permeability and edema formation. Earlier reports showed that a mice overexpressing superoxide dismutase enzyme have decreased proteases activation and reduced BBB injury and vasogenic edema (Kim et al., 2003). Hence it could be stated that increase in oxidative stress could have resulted in increase in proteases expression and disruption of BBB along with changes in blood vessels morphology in PTZ animal. On the other hand, curcumin supplementation to PTZ animals resulted in the decreased edema in endothelial cells and hence decreased the damage to mitochondria in blood vessels. The mechanism how curcumin protect the endothelial cells from damage is not completely known, however, it can be speculated that curcumin protected the cells by decreasing oxidative stress (quenching the brain damaging free radicals) and ameliorating the inflammatory response induced in PTZ animals, thereby decreasing BBB damage. Previous report provides evidence that curcumin protects the brain against high glucose-induced endothelium-dependent vasodilator dysfunction by a mechanism involving heme oxygenase (HO)-1 (Fang et al., 2009).
Results and Discussion

Figure 4.52: Transmission electron micrographs showing blood vessels ultrastructural changes in hippocampus and cortex of PTZ treated animals. Images are taken at 15000X magnification.

Figure 4.52: Transmission electron micrographs showing blood vessels ultrastructural changes in hippocampus and cortex of PTZ treated animals. Images are taken at 15000X magnification.
4.15 Effect of curcumin supplementation on expression of MMP-9 and MMP-2 in PTZ treated animals

Matrix metalloproteinase (MMP’s) constitute a family of enzymes that mediate the degradation of extracellular matrix proteins. MMP-9 is a member of this family and a zinc dependent endopeptidase that degrades collagen IV, a major component of the basement membrane of the cerebral epithelium responsible for the integrity of the BBB (Suenaga et al., 2008). Its structure is similar to MMP-2, another member of this family. It has been documented that MMP-9 up-regulation could play a detrimental role by fostering abnormal synaptic plasticity and increasing degenerative changes (Nakamura and Lipton, 2007). Several other studies support the role of MMP-9 in the development of epilepsy in animal models (Jourquin et al., 2003; Zhang et al., 2000). A common final pathway which is suggested to be involved in BBB disruption is through neuroinflammatory response induced by the free radicals and proteases (MMP’s), which affects the tight junctions in endothelial cells (Rosenberg, 2012). Moreover, recent studies also indicates the role of cytokines in activation of MMP-9 through various pathways such as ERK1/2, p38 MAPK and JNK 1/2 dependent NF-κB pathways contributing to pathogenesis of various neurological disorders (Li et al., 2014). To determine the role of MMP-9 and MMP-2 in altered BBB permeability in PTZ treated animals, zymography and real time PCR was performed.

4.15.1 MMP-9 activity and mRNA expression in PTZ treated animals

In present study, a significant increase in the MMP-9 mRNA expression was observed in hippocampus (1.45 fold) and cortex (1.45 fold) of PTZ treated animals when compared to controls as accessed using real time PCR (Figure 4.53). Zymography performed to access the MMP-9 activity showed no change in hippocampus and cortex regions (Figure 4.55). The increased relative mRNA expression has been previously reported in PTZ induced model of epilepsy (Mizoguchi et al., 2011). It has been evidenced that formation of synaptic network and synaptogenesis depend on the MMP-9 levels and alteration in MMP-9 levels affects the susceptibility to epileptogenesis (Wilczynski et al., 2008). An increase in synaptic processes contributes to increase in severity of seizures in epilepsy. (Wilczynski et al., 2008). Similar results have been observed in an experimental
study showing increased MMP-9 mRNA levels in hippocampal dentate gyrus during seizures (Konopacki et al., 2007). Moreover, it has been documented that BBB dysfunction and up-regulation of MMP-9 can enhance the frequency of seizures (Ichiyama et al., 2007). Furthermore, it has been shown that MMP-9 inhibitor or MMP-9 gene knock out in mice prevents axonal sprouting and synaptogenesis in hippocampus region of kainate induced status epilepticus (Wilczynski et al., 2008). On the other hand, curcumin supplementation to PTZ animal significantly attenuated MMP-9 mRNA levels (1.6 fold) in hippocampus and cortex (1.52 fold) when compared to PTZ treated animals. Earlier results provide evidence that curcumin attenuate MMP-9 activity and expression which results in healing of endometriosis (Swarnakar and Paul, 2009). Curcumin has been shown to suppress MMP-9 activity through activation of mitogen-activated protein kinase/NF-κB (Hwang et al., 2005; Woo et al., 2005). The decrease in pro-inflammatory cytokines and decreased glial cell activation on curcumin supplementation could have resulted in decreased MMP-9 activity as matrix metalloproteases (MMP-9, MMP-2, MMP-9) are known to be regulated by cytokine and chemokine expressions (Richardson, 2010).

Figure 4.53: Effect of curcumin supplementation on mRNA expression of MMP-9 in hippocampus and cortex of PTZ treated animals. The relative mRNA expression of the target gene was normalized with β-actin housekeeping gene and was calculated using the 2-ΔΔCt method. All values are expressed as mean ± SEM; n=6/group. *significantly different from control group (p<0.05), #significantly different from PTZ treated group (p<0.05).
4.15.2 MMP-2 activity and mRNA expression in PTZ treated animals

Real time PCR showed no change in MMP-2 expression in both hippocampus and cortex of PTZ treated animals and controls (Figure 4.54). No significant difference in MMP-2 activity among all the groups was observed in hippocampus and cortex regions of brain (Figure 4.55). These results are in accordance to the earlier studies showing no change in activity of MMP-2 on repeated PTZ administration in animals (Mizoguchi et al., 2011). A recent study with epileptic patients also found an increase in MMP-9 concentrations in seizure patients with no change in MMP-2 when compared to controls and suggested that MMP-9 plays a role in BBB dysfunction (Li et al., 2013). Hence, it could be stated that higher MMP-9 levels and not MMP-2 levels are associated with increased BBB permeability and seizure insults. Curcumin supplementation to PTZ animals also showed non-significant effect on MMP-2 activity and mRNA levels in both the regions of brain.

Figure 4.54: Effect of curcumin supplementation on mRNA expression of MMP-2 in hippocampus and cortex of PTZ treated animals. The relative mRNA expression of the target gene was normalized with β-actin housekeeping gene and was calculated using the 2-ΔΔCt method. Values are expressed as mean ± SEM; n=6/group.
4.16 Effect of curcumin supplementation on PTZ induced histopathological changes

Epileptic discharge for longer period, mitochondrial dysfunctions, increase in oxidative stress, neuroinflammation and tissue edema could result in morphological changes in the brain.
tissue. Hippocampal sclerosis which is characterized by increase in neuronal cell loss in the
dentate hilus, CA1 and CA3 subfields is a hallmark of TLE (Morimoto et al., 2004). Extensive
investigations of animal models suggest that cell loss or cell damage varies from one model to
other model of epilepsy. Status epilepticus (severe and continuous seizures) induced by
pilocarpine results in cell loss in different brain areas with increased rate of neurogenesis and
mossy fiber sprouting in the CNS (Winawer et al., 2007). Neuronal cell death has also been
observed after kainate induced status epilepticus, where the excess of glutamate levels
following seizures triggers an increase in the intracellular calcium that results in increased
oxidative stress, activation of proteases and necrosis (Meldrum, 1993, 2000). In kindling, after
generalized seizures with tonic–clonic convulsions (later stage), a mild to moderate cell death
has been observed in DG region of hippocampus but the findings remain controversial
(Cavazos et al., 1994; Cavazos and Sutula, 1990; Crandall et al., 1979). Hence, to investigate
whether PTZ induced seizures results in apoptotic cell death or necrotic with specific
neuropathology, different markers were used to detect neuronal loss or damage. Cresyl violet,
Hematoxylin-eosin (H&E), Fluoro-jade B and TUNEL staining was performed to evaluate the
morphological changes in hippocampus and cortex following PTZ treatment.

4.16.1 Cresyl violet staining

Cresyl violet staining was carried out after the completion of PTZ treatment to access the
neuronal loss in hippocampus and cortex of PTZ treated animals. Pathological changes in
hippocampus and cortex are shown in Figure 4.56, 4.57 respectively. PTZ treated animals
showed shrunken nucleus (pyknotic) and cytoplasm in hippocampus and cortex when
compared to control animals where normal morphology with well-rounded cells and no
pyknosis were seen. The cells were irregularly distributed and exhibited abnormal structures,
as well as wider interspaces were observed in PTZ treated animals. Curcumin supplementation
with PTZ resulted in prevention of neuronal damage/loss by preserving the normal
morphology and integrity of cells in both the regions (increase in viable cells). 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.
Figure 4.56: Effect of curcumin supplementation on neuronal cell damage as assessed using cresyl violet stain in hippocampus of PTZ treated animals. (a) magnification at 20 X and magnification at 40 X.
Figure 4.57: Effect of curcumin supplementation on neuronal cell damage as assessed by cresyl violet stain in cortex of PTZ treated animals. (a) magnification at 20 X a magnification at 40 X.
4.16.2 H & E staining

H & E staining was performed to the morphological changes induced in hippocampus and cortex of PTZ treated animals. In control animals, regular morphological integrity (round and clear nuclei) was observed. Moderate cell loss with irregular distributed cells were seen in PTZ treated animals in hippocampal and cortex when compared to controls (Figure 4.58, 4.59). In contrast, curcumin supplementation to PTZ animals indicated partially decreased cell injury in both the regions.

Figure 4.58: Effect of curcumin supplementation on morphological changes in hippocampus of PTZ treated animals assessed using H & E staining (a) magnification at 20 X and (b) magnification at 40.
Figure 4.59: Effect of curcumin supplementation on morphological changes in cortex of treated animals as assessed using H & E staining. (a) magnification at 20 X and magnification at 40.
14.16.3 Fluoro-Jade B staining

Fluoro-Jade B staining was performed to label the degenerating neurons in the PTZ treated animals. The result of the present study showed no Fluoro Jade B stained cells in hippocampal and cortex of control animals (Figure 4.60). Moreover, 30 days of PTZ administration had no significant difference in the Fluoro Jade B staining. Curcumin supplementation to animals also had no effect on neuron staining.

Figure 4.60: Effect of curcumin supplementation on neuron degeneration in hippocampus and cortex of PTZ treated animals as assessed using Fluoro-Jade B staining.
4.16.4 TUNEL staining

TUNEL staining was performed in brain sections of animals to detect DNA fragmentation, which is a hallmark of apoptosis following PTZ treatment. In present study, no significant difference in the TUNEL positive cells was observed among all groups (Figure 4.61). Very few TUNEL positive cells were observed in the PTZ animals when compared to controls in hippocampus and cortex regions. Curcumin supplementation also showed no change in the TUNEL staining. However, when the sections were treated with DNAase I to induce the DNA break, many TUNEL positive cells were observed. This was done to rule out the possibility of methodological flaw and the images are shown in Figure 4.61.

In present study, increased neuronal cell damage (loss) was observed in both the regions of PTZ treated animals as observed from Cresyl violet staining, and mild damage was seen in H & E staining. In present study, increased free radical production or decreased antioxidant enzymes levels could have resulted in the neuronal damage as seen after kainate induced seizures (Baik et al., 1999). Similar findings that seizure induced oxidative stress led to increased cell loss have been reported previously (Liang et al., 2000). It has also been documented that seizures result in excessive increase of excitatory amino acid (glutamate) levels, which leads to neuronal cell death via the activation of NMDA receptors (Rothman and Olney, 1986). Morphological changes in the mitochondria including mitochondrial swelling and disruption of mitochondrial membrane may contribute to cell damage as observed from clinical studies of epilepsy (Chen et al., 2010). Curcumin supplementation attenuated PTZ-induced neuronal cell damage in both the regions which could be due to increase in antioxidants and decrease in oxidative stress parameters such as LPO, ROS and protein carbonyls on curcumin administration. Study performed by Shin et al. (2007) has also shown the neuroprotective role of curcumin in protecting against neuronal cell death in kainic acid induced model of epilepsy.

Fluoro Jade B staining revealed no neurodegeneration in PTZ treated animals (no significant bright cells were seen) in hippocampus and cortex region as revealed from Fluoro-Jade B staining which is a high affinity fluorescent marker for degenerating neurons. These results are in accordance to the previous studies showing no degenerating neurons in the
Figure 4.61: Light microscopic images of hippocampus and cortex of PTZ treated animal with TUNEL staining. Images are taken at 40X and scale bar=1 μm.
chronic epileptic animals experiencing around 10 seizures per day (Pitkanen et al., 2002). PTZ administration also caused mild neuronal loss in both the studied brain regions as found in H&E staining. These results are in consistent to the previous reports suggesting that cell loss due to seizure in kindling is not always confirmed when cell densities are taken into account and when seizures are generalized with restricted duration (on average, 1–2 min) (Mikati, 2004). Evidence also indicates that kindling model do not necessarily lead to cell loss (Henshall and Meldrum, 2012). Moreover, the extent of injury to the neurons caused by generalized tonic clonic seizures in kindling is less severe than status epilepticus (Deshpande et al., 2007). The reduced neuronal density and the change in neuronal morphology after kindling is generally attributed to change in tissue volume (expansion) which has been reported by numerous researchers (Bertram and Lothman, 1993; Guillery and August, 2002; West, 2002). In similar study performed by Brandt et al., (2004) showed that kindling induced reduction of hilar neuronal density was not due to change in tissue volume (increase) and not because of neuronal death. Previous reports also demonstrated PTZ induced kindled animals had mossy fiber sprouting but no cell damage in the brain (Wilczynski et al., 2008).

In addition, no significant difference in the number of apoptotic cells were observed in PTZ animals when compared to control as assessed using TUNEL assay. These results are in accordance to the earlier studies showing no TUNEL positive cells in the kindled animals, due to interaction of Bax molecule with Bcl protein that inhibits the release of cytochrome c from the mitochondria and blocks caspase activity and the onset of apoptosis (Akcali et al., 2005). From these results it can be suggested that the mode of cell death in kindling animals is not through apoptotic pathway but rather they could be undergoing necrotic cell death as demonstrated in previous reports (Chuang et al., 2007). Cells death following recurrent seizures and excitotoxic mechanisms leading to cell degradation occurs through necrosis (Olney, 1986; Wasterlain et al., 1993). Necrosis is usually characterized by loss of cluster of cells, loss of membrane integrity of cells with cellular swelling along with activation of brain microglia and astrocytes (astrocytic gliosis) (Bengzon et al., 1997). Interesting study done by Gallyas F et al., (2008) has shown that neuronal cell death in epilepsy is not of apoptotic origin as no characteristics of apoptosis were observed, furthermore they also demonstrated that the pathobiochemical processes in excitotoxic environment undergo necrotic-like removal process on already dead “dark” neurons.
Clinical data shows that epileptic patients generally experience learning and memory impairment along with psychological problems and cell death (Pitkanen and Sutula, 2002; Thom et al., 2002). The results are in accordance to these findings as cognitive deficits have been observed in animals on PTZ treatment.

Taken together, the findings demonstrate that PTZ induced kindling resulted in increase of oxidative stress, impairment in mitochondrial functions which together contributed to increase in blood brain barrier disruption that caused cerebral edema. Moreover, an increase in microglia and astrocytes activation along with increase in cytokine/chemokines production was observed leading to cell loss in PTZ animals (Figure 4.62). This study further shows that curcumin when supplemented to PTZ animals improved cognitive functions, lowered oxidative stress, increased mitochondrial functions, decreased glial cells activation, decreased cytokines/chemokines production, decreased BBB disruption and reduced cell damage. Neuroprotective potential of curcumin against PTZ induced behavioral dysfunctions, oxidative stress, mitochondrial dysfunctions, neuroinflammation accompanied by cell loss and morphological changes. Therefore, it could be suggested that curcumin may be used as an adjuvant therapy in chronic epilepsy.
Results and Discussion

Curcumin supplementation

Altered mitochondria respiratory chain functions, mitochondrial structural changes

Mitochondria dysfunction

Increased LPO, ROS, Protein carbonyls and Decreased SOD, catalase, GST and thiols

OXIDATIVE STRESS

Increased GFAP and Iba-1

Microglial activation (gliosis)

Increased sodium fluorescein and Evans dye extravasation into brain, increased cerebral edema

Pro-inflammatory cytokin & chemokine release

Impaired BBB permeability

Neurobehavioral Impairment and Histological changes

Figure 4.62: Hypothetical model for PTZ induced damage in chronic epilepsy and potential neuroprotective effect of curcumin supplementation.
Results and Discussion