Attenuating effects of pentoxifylline on memory, oxidative stress and monoamine levels in the hippocampus of seizure – induced mice.

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ABSTRACT

Aim : Lithium – pilocarpine (Li–Pc) - induced seizures in rodents serve as convenient animal models to study the mechanisms of epileptogenesis particularly with reference to the development of status epilepticus (SE), memory retention, neurotransmitters and oxidative stress systems in the brain hippocampus which may be involved in the modulation of SE. In the present study, adult male mice were used to study the neuroprotective effects of pentoxifylline (PTX) against Li–Pc induced SE.

Materials and Methods : All SE-induced animals were pre-treated with 0, 20, 40, and 60 mg/kg PTX and were examined for all peripheral cholinergic signs, stereotyped and clonic movements, seizures, tremors, latency to and frequency of SE, and mortality if any within 24h. For assessing the memory retention capacity, the animals were subjected to shuttle-box test. Alterations in the oxidative stress indices like thiobarbituric acid reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) and the monoamine levels of dopamine (DA) and serotonin (5-HT) were determined in the hippocampus of the SE-induced animals.

Results : Pre-treatment with PTX attenuated the severity and frequency of epileptic seizure activities and learning capabilities significantly and dose-dependently in the SE-induced animals. Animals sacrificed 24 h later after SE induction resulted into a significant depletion of the monoamines and oxidative stress in the hippocampus and PTX pre-treatment reversed this condition.

Conclusion : It was concluded that PTX may be having neuroprotective effects through possible involvement of brain monoamines and oxidative stress as prophylactic/therapeutic potentials in seizure mechanisms against SE.

Key words : Pentoxifylline; Seizures; Shuttle-box; Hippocampus; oxidative stress ; Monoamines.

Academic Discipline And Sub-Discipline:

Pharmacology and Biochemistry

SUBJECT CLASSIFICATION

Neurotransmitter, Oxidative stress and Seizures

TYPE (METHOD/APPROACH)

Shuttle-box test; HPLC determination of monoamines and Spectrophotometric determination of Oxidative stress indices.
INTRODUCTION

Seizures and status epilepticus (SE) can inflict harmful effects in the brain of the developing child resulting into cognitive impairment (1, 2). Thus, there is a need of therapeutical agents that can ameliorate the seizure-induced changes and impairments in the developing brain. In spite of extensive research, aetiopathology of SE remains far from clear. Various neurotransmitters like adenosine, norepinephrine (NE), dopamine (DA), serotonin or 5-hydroxytryptamine (5-HT), glutamate, γ-aminobutyric acid (GABA), in different brain areas are known to be affected by seizure and SE activities (3 – 9). Alterations in many other neurochemicals related to seizures, memory retention and oxidative stress have also been detected in seizure-induced rats (10 – 12). Due to high mortality of animals in pilocarpine (alone) model (13), a combination of lithium chloride and pilocarpine (Li-Pc) model is often preferred as an ideal experimental model that has been frequently used to study the pathophysiology and management strategies of SE (14, 15).

Pentoxifylline (PTX) is a methylxanthine derivative and a nonspecific type 4-phosphodiesterase inhibitor. PTX has shown to attenuate ischemic brain injury in rats (16, 17), improve learning and cognitive processes following lesions in the hippocampus of the rats (18) and prevent brain injury in stroke prone rats (19). In recent years, PTX was found to exert neuroprotective activity possibly by improving cerebral functions in various experimental models (18, 19). Furthermore, PTX has been demonstrated of potentiating anticonvulsant effects in various animal models of epilepsy by improving neurotransmitters and neurochemical changes in their brain (20 – 22). In perspective of the association of Li-Pc induced seizure activities with monoamine levels and oxidative stress in different brain regions, the aim of this study was to investigate the effect of PTX on various monoamines and oxidative stress in the hippocampus of mice induced for SE by Li-Pc treatment.

MATERIALS AND METHODS

Animals:

Adult male Swiss-Webster strain mice (8-10 weeks old), housed in the animal facility under controlled conditions with 12 hour light-dark diurnal cycle, temperature 22±1°C, humidity 50-60% and free access to food and water ad libitum were used in the present study. All precautions were taken to minimize animal stress and suffering by following the approved study protocols and animal handling procedures of the Research and Ethics Committee of College of Science, King Saud University, Riyadh, Saudi Arabia.

SE induction and drug treatment:

The animals were randomly divided into seven groups with 10 animals in each. The animal groups 1 to 4 were induced with SE by administering a saline solution of lithium chloride (Li) in 3 mEq/ml/kg concentration i.p., followed by (20 h later) pilocarpine hydrochloride (Pc) administration s.c. in 20 mg/ml/kg concentration. PTX (Sigma, USA) was also dissolved in saline solution and was administered at doses of 0, 20, 40 and 60 mg/mL/kg i.p., 30 min before Pc injection, to all 4 groups of animals. The pups in groups 5, 6 and 7 served as controls and received saline, Li and Pc respectively in same doses as mentioned above.

Behavioral assessment:

Observations for seizure activities

The animals were observed for the behavioral signs of convulsions and seizures in a sequence of peripheral cholinergic signs (PCS), stereotyped movements (STM), clonic movements of forelimbs, head bobbing, tremors and seizures, which later developed progressively into SE within 1 – 2 h (23) and mortality if any, within 24 h, was also recorded.

Shuttle-box test (active avoidance responses)

The learning capabilities of the animals were measured in a shuttle-box (Ugo Basile, Comerio-Varese, Italy) as described elsewhere (24). During the 50 trials session of each animals, the total numbers of avoidance (reinforced crossings) and the latency of avoidance (escape latency in seconds to avoid the shock treatment), were measured.

Biochemical studies:

For biochemical assessments, the animals were sacrificed by decapitation 24 h after SE induction and the brains were dissected on ice and the hippocampus were removed and frozen in liquid nitrogen (-70°C) for determination of monoamines and oxidative stress indices.

Determination of monoamines

The monoamines were estimated using the modified method of Patrick et al. (25). A 10% homogenate of the tissues were prepared by homogenizing for 10 seconds in 0.1M HClO₄ containing 0.05% EDTA, centrifuged at 17,000 rpm at 4°C for 5min. The supernatants thus obtained were micro filtered and analyzed by high performance liquid chromatography (HPLC) to estimate the levels of dopamine (DA) and serotonin or 5-hydroxytryptamine (5-HT) using a calibration curve and the results were expressed as ng /mg tissue weight.
Determination of oxidative stress indices

Lipid peroxides (TBARS)

The lipid peroxides in the form of TBARS was determined spectrophotometrically according to the method of Ohkawa et al. (26) and the activity was expressed as nanomoles of TBARS formed per gram tissue weight.

Glutathione (GS)

Total GS was measured enzymatically (27) and the activity was expressed as nanomoles per gram tissue weight.

Superoxide dismutase (SOD)

The SOD activity was assayed using xanthine and xanthine oxidase to generate superoxide radicals (10) and the results were expressed as U/mg protein. Protein was assayed by the method of Lowry et al. (28).

Catalase (CAT)

CAT activity was determined by the method of Chance and Maehly (29) and the activity was expressed as mmol/min/mg protein. Protein was assayed by the method of Lowry et al. (28).

Statistical analyses:

The mean values of the data of all study groups were compared by ANOVA with respect to the controls and within the experimentally treated groups using post-hoc testing and Student-Newman-Keuls Multiple Comparisons Tests. Results were expressed as means ± SEM and the differences were considered to be statistically significant when p<0.05.

RESULTS

Development of seizures and SE:

After Pc injections, the animals started developing a gradual and significant change in their behavior. The convulsive episode (PCS and STM) consisted of the bobbing of heads with intermittent forelimbs and hindlimbs clonus, loss of posture, hyperextension of tails, falling back and myoclonic jerks. These sequential behavioral changes built up into SE in 100% of the animals. The developed SE lasted for more than one hour on average and only 10% mortality was observed in the SE animals over a period of 24 h following Pc injections. PTX significantly and dose-dependently increased the latency to seizure and reduced the severity, frequency and intensity of seizure, PCS and STM characteristics (Table 1). Furthermore, pre-treatment with PTX caused no mortality at all in all the three doses as compared to the Li-Pc (SE) treated group (Table 1). The control groups that received Li or PTX (20, 40 and 60 mg/kg) alone, did not show any signs of seizure or SE in any of the control groups (data not shown).

Table 1: Effect of pentoxifylline (PtX) on lithium pilocarpine-induced seizures and SE related behavioral changes in young rats.

<table>
<thead>
<tr>
<th>PtX (mg/kg)</th>
<th>Seizures (%)</th>
<th>Latency to seizure (min)</th>
<th>SE (%)</th>
<th>Latency to SE (min)</th>
<th>Mortality (%) Within 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>10.74 ± 1.78</td>
<td>100</td>
<td>21.52 ± 1.18</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>17.35 ± 1.53&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.48 ± 1.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>50</td>
<td>26.83 ± 1.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.41 ± 2.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
<td>43.51 ± 2.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.46 ± 2.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviation: SE – status epilepticus; <sup>ns</sup> – non significant;
<sup>a</sup> p<0.001 compared to control (0 mg/kg) by ANOVA followed by Student-Newman-Keuls Multiple Comparisons Test.
Learning capabilities in shuttle-box test:

In the shuttle-box test, the SE-induced animals showed a statistically significant (p<0.001) decrease in the number of avoidances during the reinforced trial period as compared to the control group (Fig. 1A). Furthermore, the total time taken by these animals to enter the other compartment to avoid the shock treatment (latency of avoidance or escape latency response in seconds) was significantly (p<0.001) greater as compared to the controls (Fig. 1B). Animals induced with SE were poor learners and took significant time in avoiding the shock treatment. However, PTX pre-treatment attenuated these effects significantly in a dose–dependent manner (Fig. 1A and B).

![Graph A](image)

![Graph B](image)

Figure 1: Effect of Ptx pretreatment on the cognitive (learning) capabilities of the seizure-induced mice in the shuttle-box test showing the total time taken by the animals (total latency) in avoiding the shock treatment (1A) and the number of reinforced crossing of the chambers by the animals (1B) for avoiding the shock treatment during the light and sound stimuli.

Abbreviations : Ptx – pentoxifylline; Li – lithium; Pc – pilocarpine; SE – status epilepticus.

# shows significantly (p< 0.001) different from control and *, ** and *** show significantly (p<0.05; p<0.01 and p<0.001 respectively) and dose-dependently different as compared to SE group by ANOVA followed by Tukey's and Student's tests.

Biochemical studies in hippocampus tissue:

Levels of monoamines

A significant (p<0.001) depletion in the DA and 5-HT (Fig. 2A and B) of the SE group was noticed as compared to the
controls. Animals pre-treated with PTX, significantly and dose-dependently ameliorated the Li-Pc induced effects (Fig. 2A and B).

![Graph A](image)

![Graph B](image)

**Figure 2:** Levels of dopamine (DA) and serotonin (5-hydroxy-tryptamine or 5-HT) in the hippocampus of male mice (2A and 2B respectively). The levels of DA and 5-HT were significantly (#, p<0.001) depleted in SE groups as compared to controls whereas Ptx pretreatment showed a dose-dependent and significant (*, ** and *** showing p<0.05, p<0.01 and p<0.001 respectively) attenuating effect as compared to the SE groups by ANOVA followed by Tukey’s and Student’s test.

**Abbreviations are the same as in Figure 1.**

**Levels of TBARS**

TBARS in the hippocampus were significantly (p<0.001) increased in the SE-induced animals and pre-treatment with PTX had a significant (P<0.001) and dose-dependent attenuating effect (Fig. 3 A).

**Levels of GSH**

GSH was significantly (p<0.001) depleted in the hippocampus of SE-induced animals and pre-treatment with PTX had a significant (p<0.001) and dose-dependent attenuating effect (Fig. 3 B).
Figure 3: Effect of LiPc induced SE on the oxidative stress depicted by increased level of thiobarbituric acid reactive substances (TBARS) shown in Figure 3A and decreased level of total glutathione (GSH) shown in Figure 3B in the hippocampus of the male mice as compared to the control (# showing p<0.001). Ptx pretreatment showed a dose-dependent and significant (*, ** and *** showing p<0.05, p<0.01 and p< 0.001 respectively) attenuating effect as compared to the SE groups by ANOVA followed by Tukey’s and Student’s test.

Abbreviations are the same as in Figure 1.

Levels of SOD and CAT

The levels of SOD and CAT remained unaltered in the hippocampus of the SE-induced animals and pre-treatment of these animals with PTX increased the activity of SOD and CAT significantly (p<0.01) and dose-dependently (Fig. 4 A and B respectively).
<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Mean value ± SEM</th>
<th>SOD (U/mg protein)</th>
<th>CAT (mmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pc</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Li-Pc (SE)</td>
<td></td>
<td></td>
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<tr>
<td>Ptx 20 mg/kg+SE</td>
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<td>Ptx 40 mg/kg+SE</td>
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<td>Ptx 60 mg/kg+SE</td>
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</table>

Figure 4: Effect of LiPc induced seizures on superoxide dismutase (SOD) and catalase (CAT) activities showing no changes in the level of enzyme activities in the hippocampus of the male mice of SE group as compared to the control as shown in Figure 4A and 4B respectively. However, Ptx pretreatment showed a dose-dependent and significant (*, ** and *** showing p<0.05, p<0.01 and p<0.001 respectively) increase in the level of SOD (4A) and CAT (4B) as compared to the SE groups by ANOVA followed by Tukey’s and Student’s test.

Abbreviations are the same as in Figure 1.

DISCUSSION

The present results demonstrate that pharmacological intervention using PTX, significantly attenuated SE induced seizure activities and learning capabilities and also ameliorated the levels of monoamines and oxidative stress in the hippocampus area of the brain. The mechanism of neuroprotective effect of PTX is not fully understood, however, it has been shown to exert neuroprotective effects in many seizure models (18, 21, 22). PTX is a dimethylxanthine derivative and is known to exert its pharmacological effects by different mechanisms (20, 30). cAMP plays a major role as a second messenger in biochemical processes regulating the cognitive and memory processes (31, 32). Alteration in the cAMP content in the cerebral cortex has been reported in the chemically induced epilepsy (33, 34). Like rolipram, PTX also has the ability to increase cAMP levels that may also exert neuroprotective effect (22, 35). Furthermore, PTX is an adenosine receptor antagonist and it plays an important role in the progression of seizures and SE (36). Furthermore, adenosine is a natural neuroprotectant with anticonvulsant action (37). Impairment of learning capabilities in rodents with SE has been reported by several investigators (38, 39). In the present study, pre-treatment of mice with PTX dose-dependently attenuated the Li-Pc – induced seizure activities and neurobehavioral abnormalities.

Several neurotransmitters have been associated with the pathogenesis of SE (8, 9). Altered brain monoamine contents have been reported following experimentally induced SE in rats (6, 9) and in human epilepsy (40). The results of the present study showed that DA level reduced significantly in the hippocampus of the SE-induced animals (Fig. 1A). Role of DA mediated inhibition of D2 type receptors has been reported in depressing the hyperexcitability of hippocampus neurons.
which are considered to be involved in SE (40, 41). Furthermore, in the present study, a decrease in 5-HT level in the hippocampus (Fig. 1B) is consistent with the hypothesis suggesting a significant decrease in synthesis and release of 5-HT during SE (9). A definite role of 5-HT has also been suggested in myoclonic epilepsy (42). In spite of numerous studies, a definite role of monoamine neurotransmitters in SE is yet to be established.

The significant increase in TBARS and decrease in GSH in the hippocampus of mice with SE in the present study clearly suggests for a high level of oxidative stress through increased free-radicals production. Moreover, the neuroprotective effect of PTX may be due to reduction in the formation of free-radicals during Li-Pc – induced seizures. On the contrary, there was no alteration in the SOD and CAT activities suggesting that these enzymes were not activated or affected during the phase of seizures development. It has further been demonstrated that PTX pre-treatment increased these antioxidant enzymes in the hippocampus which was probably a necessary consequence due to inhibition in the formation of free-radicals by PTX during convulsion process. Such increase in antioxidant enzymes activities in presence of PTX, while reduces free-radicals formation, also produces a significant decrease in the susceptibility to seizures induced by Li-Pc. Earlier study with buspirone also supports this finding (43).

CONCLUSION

The result of the present study showed antiepileptic effect of PTX accompanied by attenuation of convulsions and learning capabilities in the SE-induced animals. Furthermore, attenuating effects on the SE related brain monoamines and oxidative stress indices suggest for evidence that free-radical formation has a relevant role in the propagation of seizure activities and PTX could be used as anti-convulison drug. Although it is evidenced from the present study that monoamines and oxidative stress have a definite role in epileptic activity, the exact mechanism of epileptogenic activity of PTX warrant further studies.

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REFERENCES


**Author' biography with Photo**

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Dr. Mohammad Ahmad completed his Ph.D. from Aligarh Muslim University, India, and visited Queen's University of Belfast, UK, as a Visiting Fellow under the sponsorship of British Council to persuade his Post Doctoral studies. He has a vast experience of research and teaching. He is involved in applied research on various neurological diseases of human health importance. At present, he is an Assistant Professor in Medical Surgical Department of King Saud University, Riyadh and has published more than 45 papers in International Journals of repute and of high impact factors and is member of many scientific societies.

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