The comparison of inflammatory markers in pentylenetetrazole-induced acute epileptic seizure model and chronic epilepsy model in rats

Ahmet Şevki TAŞKIRAN1,2*Yaşar TAŞTEMUR1
1Sivas Cumhuriyet University, School of Medicine, Department of Physiology, Sivas/TURKEY
2Sivas Cumhuriyet University, School of Medicine, Department of Anatomy, Sivas/TURKEY

Abstract
Recent studies have provided important evidence that neuroinflammation has an effective role in epilepsy pathophysiology. However, it is not clear that the occurrence of neuroinflammation is related to one epileptic seizure or repeating seizures. Therefore, we aimed to investigate the comparison of inflammatory markers in pentylenetetrazole-induced acute epileptic seizure model and chronic epilepsy model in rats. In this study, 18 male Wistar albino rats were used. The animals divided into three groups as control, acute epileptic seizure model and chronic epilepsy model. Inflammatory markers (TNF-α, IL-1 β, COX-1, and COX-2) were measured by using ELISA methods in the cortical and hippocampal brain regions after completing the epileptic model procedure. Statistical evaluation of the data was performed by one-way ANOVA and multiple comparisons were determined by the Tukey test. Statistical significance was defined at \( p < 0.05 \). Obtained data show that there was significant increase in inflammatory markers in chronic epilepsy model compared to the control and acute epileptic seizure model (\( p < 0.05 \)). In conclusion, this study may suggest that inflammatory system is related to epileptogenesis process rather than only one epileptic seizure.

1. Introduction
Epilepsy is a neurological disorder characterized by spontaneous recurrent seizures, resulting in cognitive and psychological consequences [1]. About 70 million people are affected by epilepsy in worldwide. Epileptic seizure, which is the unique clinical sign of epilepsy, is seen as a result of increased, rapid and abnormal local electrical discharges of neurons in the cerebral cortex. During the seizure, short-term and temporary behavioral abnormalities appear in consciousness, behavior, emotion, movement or perception functions [2]. Traumatic brain injuries, hypoxia or febrile seizures can lead to neuronal deaths and dysfunctional synaptic modifications that can cause spontaneous recurrent seizures [3]. Since approximately one-third of patients with epilepsy do not respond to current medical treatment, they have progressive cognitive disorders and may need surgical treatment to prevent recurrence of seizures [4].

Neuroinflammation is a complex event involving the activation of microglia, astrocytes and endothelial cells participating in the blood brain barrier, infiltration of plasma proteins and immune system cells into the brain tissue, and the interaction of inflammation-related mediators with the brain tissue [5]. Neuroinflammation findings are found in many central nervous system diseases, and neuroinflammation is often associated with epilepsy. Numerous evidence has been obtained with clinical and experimental research that neuroinflammation increases seizure frequency and severity [6]. Recurrent seizures are also seen in autoimmune diseases and encephalitis patients accompanied by severe and prolonged neuroinflammation, and neuroinflammation findings are frequently encountered in anticonvulsant drug-resistant epilepsies [7]. These findings have demonstrated the importance of neuroinflammation in the pathogenesis of epilepsy and have demonstrated the importance of elucidating these mechanisms in developing antiepileptogenic therapy.

Pentylentetrazzole (PTZ), a tetrazole derivative, is a chemical agent in the class of 'systemic' experimental convulsants. PTZ exerts its effect by blocking the GABAA chloride ionophore complex. Also PTZ, affects various neurotransmitter systems, such as gabaergic and glutamatergic systems [8]. Therefore, it is often used to create an acute or chronic animal epilepsy model. Parenterally administered PTZ has convulsive effects in mice, rats, cats and primates. PTZ
initially produces myoclonic stresses, then causes generalized tonic-clonic seizures [9]. Single-dose (45-80 mg/kg) PTZ causes acute epileptic seizures, while repeated subacute dose PTZ (20-35 mg/kg) produces the chronic epileptic model which is meaning completing epileptogenesis process. Epileptogenesis refers that transform from normal brain to epileptic brain by pathophysiological.

Several studies have shown important evidence that neuroinflammation has an effective role in epilepsy pathophysiology. However, it is not clear that the occurrence of neuroinflammation is related to one epileptic seizure or repeating seizures. Therefore, we aimed to investigate the comparison of inflammatory markers in pentylenetetrazole-induced acute epileptic seizure model and chronic epilepsy model in rats.

2. Materials and Methods

2.1. Animals

The experiments were performed using adult male Wistar rats weighing 230-250 g (n = 18). The animals were fed a standard laboratory diet and water ad libitum, kept at 22 ± 2°C with a 12-h light/dark cycle in a closed room which has lighting system controlled by timers. Animals were acclimatized to laboratory conditions before the test. All experiments were carried out blindly between 09:00 and 17:00 h (n = 6 in each experimental group in the study). The Cumhuriyet University Animal Ethics Committee approved the experimental protocols (Approval Number: 65202830-050.04.04-371).

2.2. Drugs

Pentylentetrazole (PTZ), (Sigma-Aldrich Co., St Louis, MO, USA) was dissolved in physiological saline. Each drug solution was prepared freshly on the days of the experiments.

2.3. Experimental protocol

The animals were divided into three groups as Group 1 (Control; n = 6); rats were treated with 1 ml/kg single dose of physiological saline intraperitoneally (i.p.), Group 2 (Acute epileptic seizure model; n = 6); rats were administered with single dose of i.p. PTZ (45 mg/kg), Group (Chronic epilepsy model; n = 6); rats were given with repeated doses of i.p. PTZ (35 mg/kg) every Monday, Wednesday and Friday day for 12 times. The animals were sacrificed by decapitation after 24 hours. The brain tissue obtained from the animals underwent biochemical assessment (Figure 1).

![Experimental Design](created by BioRender).
2.4. Biochemical assessment

2.4.1 Preparation of brain tissue homogenates

After mixing the brain tissue samples (both cortex and hippocampus area of the brain by separating) of the animals with a cold phosphate-buffered saline solution, the tissue samples were homogenized using a mechanical homogenizer (Analytik Jena speedmill plus, Jena, Germany). The homogenates were centrifuged at 4000 rpm for 10 min at a temperature of 4°C. Then, the supernatants were obtained and stored in ice until biochemical analysis. Bradford protein assay kit (Merck, Germany) was used to determination of total protein levels in samples [10].

2.4.2 Measurement of Tumor Necrosis Factor Alpha (TNF-α), Interleukin-1 Beta (IL-1 β), Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2)

The levels of TNF-α, IL-1 β, COX-1 and COX-2 from brain supernatants were measured using rat ELISA commercial kits (Shanghai Sunred Biological Technology, Shanghai, China). The operation protocols were according to manufacturer’s instructions. In brief, standard and tissue samples were added in plate and incubated for 60 min at 37°C. After washing step, staining solutions were added and incubated for 15 minutes at 37°C. Stop solution was added and read at 450 nm. There were standard curves used to calculate for all these kits. The coefficients of variation within and between plates were less than %10.

2.5. Statistical analysis

The data are expressed as mean ± standart error of mean (SEM). The one-way ANOVA followed by Tukey posthoc test was used to compare the study data. The p-value of < 0.05 indicated a statistical significance.

3. Results and Discussion

3.1. Proinflammatory cytokines in acute and chronic PTZ models

Cytokines are proteins that regulate the inflammatory process. They are produced by neurons and glial cells during brain inflammation [11]. In a clinical study, IL-1β and TNF-α levels in febrile seizures have been shown to increase [12]. TNF-α increases α-amino-3-hydroxy-5-ethylisoxazole-4-propionic acid (AMPA) receptors, which increase glutamatergic transmission. Increased AMPA receptors cause excessive calcium intake and neurotoxicity in neurons. In addition, TNF-α induces GABA receptor endocytosis. Through these mechanisms, TNF-α causes an increase in excitation and a decrease in inhibition. This means a tendency to seizure [13,14]. In this study, we found that there is increase in TNF-α levels chronic PTZ models in cortex and hippocampus compared to control (Figure 2 A-B; p<0.01). However, the acute PTZ-induced seizures did not affect TNF-α levels in both cortex and hippocampus compared to control (Figure 2 A-B; p>0.05). Therefore, there was statical different in TNF-α levels between acute and chronic model in hippocampus (Figure 2 A-B; p<0.05). These findings show that TNF-α raises after epileptogenesis and is not related to acute epileptic seizures.

Our findings of TNF-α levels in acute PTZ model is coherent one of the previous study [15]. On the other hand, some studies have shown the increase in TNF-α levels after a single PTZ induced seizures dose in total brain and serum in contrast to our study [16,17]. It can be related to using the different doses of PTZ or collecting different parts of the tissues such as total brain and serum in these studies. Moreover, our findings of TNF-α levels in chronic PTZ models is consistent of the previous study performed in both mice and rat chronic PTZ models in hippocampus [18,19]. However, in contrast to our study, one of the study have suggested that there is no changing in TNF-α levels in hippocampus after chronic PTZ model [20,21]. It can be explained the collecting time of tissues after last PTZ injection.

Proinflammatory cytokine IL-1β is expressed in active microglia and astrocytes. IL-1β increases glutamate release from astrocytes and reduces the reuptake of glutamate. By these ways, IL-1β increases glutamate levels in neuronal synapses and causes neuronal hyperexcitability. It has been suggested that IL-1β gives rise to seizures by activation of the GluN2B subunit of the N-methyl D-aspartate (NMDA) receptor and increasing regulation of NMDA receptors on postsynaptic cells [22]. In this study, we show that there is increase in IL-1β levels chronic PTZ models in the cortex (Figure 2 C; p<0.05) and hippocampus (Figure 2 D; p<0.001), also in hippocampal region of acute PTZ model (Figure 2 D; p<0.05) compared to the control. However, the acute PTZ-induced seizures did not affect IL-1β levels in the cortex region compared to the control (Figure 2 C; p>0.05). Moreover, there was statical different in IL-1β levels between acute and chronic model in only hippocampal region (Figure 2 D; p<0.05). These findings show that IL-1β raises after acute epileptic seizures and chronic. It can be
Show that IL-1β may be associated with both epileptic seizure and epileptogenesis. Our findings of IL-1β levels in acute and chronic PTZ models is consistent one of the previous studies which are carried out in rat and mice brain [15,20,23]. According to our findings, it may be suggested that TNF-α is associated with epileptogenesis, while IL-1β is related to both acute seizure and epileptogenesis. This also may explain the relationship between TNF and AMPA, and also between IL-1β and NMDA. Because recent studies have shown that AMPA is the dominant role in epileptogenesis, while NMDA has an important role in both acute seizure and epileptogenesis [24,25].

3.2. Inflammatory enzymes in acute and chronic PTZ models

COX-1 and COX-2 are isoenzymes that catalyze the conversion of arachidonic acid into prostaglandins. It has been reported that COX-1 and COX-2, are induced in humans and experimental animals after seizures. COX-1 studies in animal models of seizures/epilepsy, it has shown that the selective COX-1 inhibitor slowed the development of epilepsy in electrical amygdala kindling in mouse model [26]. Moreover, aspirin, which inhibit the activity of both COX-1 and COX-2, reduced seizures in the absence epilepsy model, in the zebrafish seizure model, and in the pilocarpine-induced epilepsy model [27,28]. However, there is no study in acute and chronic models induced by PTZ in rodents. In our study, we assert that there is increase in COX-1 levels in chronic PTZ models in cortex and hippocampus compared to control and acute PTZ model (Figure 3 A-B; p<0.001). However, the acute
PTZ-induced seizures did not change COX-1 levels in both cortex and hippocampus compared to control and acute PTZ model (Figure 3 A-B, \(p>0.05\)). To the best of our knowledge, it was the first study is about COX-1 expression after two different models of PTZ in the English language. It has been suggested that COX-1 may be related closely to epileptogenesis and may be a new target for epileptogenesis.

Because COX-2 is considered the inducible expressed isoform responsible for propagating the inflammatory response, several studies have been predominantly exploring the COX-2 isoform as the most suitable target for pharmacological intervention in epilepsy studies [29,30]. However, the role of COX-2 inhibition on epileptogenesis and/or seizure suppression remains controversial. Treatment with nimesulide, a COX-2 selective inhibitor, prior to electrical kindling, had antiepileptogenic effects in rodents [31,32]. On the other hand, proconvulsant effects of COX-2 inhibitor have also been shown in kainic acid-induced seizure model [33,34]. In addition to these studies, we found that there is increase in COX-2 levels chronic PTZ models in the cortex and hippocampus (Figure 3 C-D; \(p<0.001\)), also in hippocampal region of acute PTZ model (Figure 3 D; \(p<0.001\)) compared to the control. Nonetheless, the acute PTZ-induced seizures did not alter COX-2 levels in the cortex region compared to the control (Figure 2 C; \(p>0.05\)).

**Figure 3.** Inflammatory enzymes in acute and chronic PTZ models in the cortex and hippocampus. ***\(p<0.001\) compared to control. ###\(p<0.05\) compared to acute PTZ model.
4. Conclusion

In conclusion, based on the literature background and our study inflammation system is relevant epileptogenesis rather than acute epileptic occurring. Regulating inflammatory reactions in the brain and targeting inflammatory mediators may be effective therapeutic strategies to prevent or stop epileptogenesis process in the nervous system. Therefore, controlling inflammation may reduce the risk of developing epilepsy. Nevertheless, further researches are needed to clarify the role of inflammation in the pathogenesis of epilepsy.

Acknowledgment

The authors thank the institution for using the facilities of the Sivas Cumhuriyet University.

Conflicts of interest

Sample sentences if there is no conflict of interest: The authors state that they do have conflict of interests

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