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Effect of Anakinra and Infliximab on Oxidative Stress and Caspase Activation in PTZ-Induced Acute Seizure in Rats

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ABSTRACT

We aimed to examine the effects of Anakinra (AKR) and Infliximab (IFB) on increased oxidative stress (OS) and pro-apoptotic pathway parameters after an epileptic attack. The rats were divided into four groups; control, pentylenetetrazol (PTZ), PTZ+Anakinra, and PTZ+Infliximab groups. AKR and IFB treatment was done 30 min. after PTZ administration, and rats were sacrificed after 24 h. The acute epilepsy model we created with PTZ in the rats and seizure symptoms were evaluated using the Racine classification and the delay time to the first myoclonic jerk. We also examined the levels of total antioxidant status (TAS), total oxidative stress (TOS), neurotrophin brain-derived neurotrophic factor (BDNF), caspase 3 and caspase 9 in the cortex and hippocampus. Post-treatment of AKR and IFB decreased PTZ-induced OS in the cortex and hippocampus while TAS levels also increased. In addition, it was observed that AKR and IFB decreased the levels of BDNF, caspase 3 and caspase 9 compared to the PTZ group. These findings showed that AKR and IFB might be used as important therapeutic agents to inhibit OS and apoptosis mechanisms that may occur after PTZ-induced epileptic attacks.

Keywords: Apoptosis, Epilepsy, Anakinra, Infliximab, Oxidative Stress

Introduction

Epilepsy is one of the most common neurological diseases today (1). During an epileptic seizure, many regions of the brain may be affected, and damage may occur. However, there are still no drugs that ultimately prevent the onset or progression of the disease (2,3). Therefore, it is difficult to predict exactly when an attack will occur in an epileptic patient. Still, many studies are being conducted to understand the mechanisms of damage after seizure and to minimise the damage. (4,5). In addition, recent studies have shown that neuroinflammation plays an active role in forming epileptic seizures and epileptogenesis (6,7). A different study showed that inflammation in the brain increased neuronal hyperexcitability and seizures. Pro-inflammatory cytokines play an essential role in epileptic seizures and epileptogenesis, although the functions of many inflammatory mediators have not been resolved yet (8). Karan et al. examined the cytokine expression changes caused by epileptic activity in rats. They determined that epileptic activity caused pro-inflammatory responses, especially in both the ventral and dorsal parts of the hippocampus (9). A different study emphasized that there was an increase in oxidative stress (OS) parameters in parallel with the increase in Interleukin (IL)-1β, IL-6 and IL-17 levels in brain tissue samples of pentylenetetrazole (PTZ) application rats (10). Sitges et al. determined that rats' IL-1β and TNF-α mRNA expression increased after epileptic seizures. In the same study, they found that decreasing the levels of IL-1β and TNF-α mRNA expression in the rat brain after the treatment also effectively prevented rats' seizures (11). There are studies indicating that neurotrophin brain-derived neurotrophic factor (BDNF) supports the survival and growth of hippocampal and cortical neurons and also has a vital role in epileptogenesis (12).

Anakinra (AKR) is an IL-1 receptor antagonist that reduces autoinflammatory-induced damage

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(13). IL-1 is a prototypical proinflammatory cytokine involved in various autoinflammatory disorders (14). A study on febrile infection-related epilepsy syndrome stated that early intervention with AKR can help prevent the encephalopathy of seizures (14). In addition, Parian et al. found that AKR treatment decreased mitochondrial oxidative stress by activating superoxide dismutase-2 against increased production of reactive oxidative species and activation of NLRP3 (15). Another study noted that AKR treatment reduced neuronal loss in hippocampal areas in a lithium-pilocarpine-induced epilepsy model (16).

Infliximab (IFB) is an antagonist for TNF-α, a pro-inflammatory cytokine (17). Sahin et al. found that IFB treatment may suppress inflammation and OS in chronically stressed rats (18). Furthermore, in a study investigating the effect of IFB on OS in ovarian tissue of hyperstimulated rats, it was seen that IFB reduced the OS caused by hyperstimulation in ovarian tissue (19).

In our literature review, we determined that a limited number of studies show the effect of AKR and IFB after epileptic seizures. Therefore, we aimed to investigate how AKR and IFB changed oxidant/antioxidant, caspase, and BDNF levels in the cortex and hippocampus regions of the brain after an acute epileptic seizure model created with PTZ.

Materials and Methods

Animals: The protocol of this study was carried out with the revised Declaration of Helsinki in 2000. Thirty-two male Wistar Albino rats were used for this study. Before starting the experiment, the weights of the rats were measured separately, and the rats were selected into the groups by the block random technique so that the weighted average of each group (240±10 g) was close to each other. In this sampling method, each population item has the same probability of entering the sample. PTZ, AKR and IFB doses and durations were determined according to the literature (16,20,21).

Drug administration: Anakinra (Kineret, Amgen, USA), infliximab (Remicade, Merck, USA), and PTZ (Sigma-Aldrich, USA) were purchased commercially. Saline was used as a solvent for PTZ, AKR, and IFB. All solutions were prepared daily.

Experimental study: The groups were created as follows (n=8);

Control group: the rats received no treatment.

PTZ group: 30 min. after PTZ (45 mg/kg) application (21), saline was injected intraperitoneally (i.p.) at a dose of 1 mL/kg.

PTZ+Anakinra: 30 min. after PTZ application, anakinra (100 mg/kg) was injected i.p. (16).

PTZ+ Infliximab: 30 min. after PTZ application, infliximab (5 mg/kg) was injected i.p. (20).

Experimental protocols: The seizure severity was scored based on Racine's Convulsion Scale (RCS). Seizure stages are defined by RCS as follows: 0 = no convulsion; 1 = twitching of vibrissae and pinnae; 2 = motor arrest with more pronounced twitching; 3 = myoclonic jerks; 4 = tonic-clonic seizure while the animal remained on its feed; 5 = tonic-clonic seizure with loss of the righting reflex; 6 = lethal seizure (22). The rats were observed after PTZ injection, both for behavioural scoring according to RCS, and for determining the time of the first myoclonic jerk (FMJ), for 30 min. (Figure 1).

Passive Avoidance Test: The working setup has been given in detail in our previous studies (22). During this test, the persistence test phase was performed 1 h after the training sessions (23).

Preparation of cortex and hippocampus tissue homogenates: After the cortex and hippocampus were carefully separated from the brains, they were taken into cold phosphate-buffered saline solution (pH: 7.4) and homogenized with the help of a mechanical homogenizer (Turrax homogenizer, ISOLAB, Germany). Homogenates taken to eppendorfs were centrifuged 4000 *xg* and 10 min. (22). The supernatant was carefully collected to study with ELISA. The Bradford protein assay kit (Merck, Germany) was used to rate the total protein levels (24).

Measurement of TAS, TOS, BDNF, Caspase-3 and Caspase-9: TAS, TOS, BDNF, Caspase-3 and Caspase-9 levels in supernatants of cortex and hippocampus samples after the epileptic attack was determined using ELISA (BT Lab, China). The protocols determined by the company for commercial kits were performed for analysis (Thermo Fisher Scientific, Altrincham, UK) (22).

Statistical Analysis: In this study, a statistical power analysis was performed by taking the effect size of 0.65 (calculated with η^2), alpha=0.05 and power=0.80, and the required minimum total number of samples was determined as 32. Descriptive statistics of results were expressed as mean ± standard deviation (STD). The Shapiro-Wilk test was used to confirm the normality of the distributions of the variables. Levene's test was

used to control the homogeneity of group variances. Following the Kruskal Wallis analysis, first one-way ANOVA and then the Tukey post-hoc comparison test was applied to determine the differences of the variables according to the groups. P value < 0.05 was considered statistically significant. SPSS (version 23.0, SPSS Inc, Chicago) program was used for statistical data analysis.

Results

RCS, FMJ and GTCS% in PTZ-induced seizures in rats: Epileptic attacks were determined using the video recordings after 45 mg/kg i.p. PTZ injection to rats. The means ± SEM of seizure stages (RCS) were 5.00 ± 0.21 in the PTZ (45 mg/kg) group, 4.5 ± 0.22 in the PTZ + Anakinra (100 mg/kg) group, 4.66 ± 0.21 in the PTZ + Infliximab (5 mg/kg) group. There was no statical significance between the groups in respect of RCS.

The means ± SEM of FMJ (sec) were at 55.33 ± 3.65 in the PTZ (45 mg/kg) group, 57.50 ± 4.52 in the PTZ + Anakinra (100 mg/kg) group and 56.83 ± 4.59in the PTZ + Infliximab (5 mg/kg) group. There was also no statical significance in respect of FMJ ($p > 0.05$). As seen in Table 1, the percentage of generalized tonic-clonic seizure stages was %100 for PTZ, PTZ + Anakinra and PTZ + Infliximab groups.

Effects of AKR and IFB on passive avoidance test after PTZ-induced seizure in rats: The passive avoidance test was used to evaluate the effect of AKR and IFB post-treatment after PTZ-induced seizures on memory impairment. There was a statistically significant difference between the PTZ (45 mg/kg) and the control groups in terms of the test trial ($p < 0.001$). Besides, The PTZ + Anakinra (100 mg/kg) and PTZ + Infliximab (5 mg/kg) groups were significantly higher compared to the PTZ group ($p < 0.001$, Figure 2).

Effects of AKR and IFB on TAS and TOS levels after PTZ-induced seizure in rats: As shown in Figure 3, the TAS level was the lowest in the PTZ group compared to the other groups ($p < 0.001$). The TAS level was the lowest in the PTZ group compared to the other groups. It was determined that AKR and IFB, used as post-treatment after PTZ incubation, increased the TAS level compared to the PTZ group ($p< 0.001$) (Figure 3a). When the TOS level between the groups was examined, it was seen that the TOS level was significantly higher in the PTZ group compared to the other groups, while the TOS level was significantly lower in the AKR and IFB groups compared to the PTZ group ($p< 0.001$, Figure 3b).

Effects of AKR and IFB on BDNF levels after PTZ-induced seizure in rats: BDNF levels in the groups were measured by ELISA. The BDNF level was significantly higher in the PTZ group compared to the other groups, while the BDNF level in the AKR and IFB groups was significantly lower than in the PTZ group ($p< 0.001$, Figures 4a and b).

Effects of AKR and IFB on Caspase-3 and Caspase-9 levels after PTZ-induced seizure in rats: Caspase 3 and caspase 9 levels were detected by ELISA in cortex and hippocampus homogenates. The caspase 3 and caspase 9 levels were significantly higher in the PTZ group compared to the other groups, while the caspase 3 and caspase 9 levels were significantly lower in the AKR and IFB groups compared to the PTZ group ($p< 0.001$) (Figures 5a and b).

Discussion

In recent years, it has been understood that oxidative, inflammatory and apoptotic processes play important roles in the etiopathogenesis of neurological diseases, including epilepsy. Therefore, it has directed the attention of researchers to find new agents that have the potential to treat such diseases by modulating the aforementioned processes. In our post-treatment study, the effects of IL-1 antagonist AKR and TNF-α antagonist IFB after epileptic seizures were investigated in a PTZ-induced in vivo epilepsy model. After PTZ treatment, seizure stages, Racine scale, and FMJ onset time of all the groups with PTZ were statistically close, and there was no significant difference (Figure 2). However, when the cortex and hippocampus samples at the end of our study were examined, it was seen that the antagonists decreased the amount of TOS and increased the TAS level compared to the PTZ group after PTZ treatment. In addition, it was observed that the AKR and IFB decreased the levels of BDNF, caspase-3, and caspase-9 compared to the PTZ group.

In epilepsy, the hippocampus plays a central role in the occurrence and spread of seizures. In experimental studies, it was stated that there was a decrease in the number of neurons in the CA1 region of the hippocampus and an increase in the astrocyte population in the CA1 regions in the

Table 1. Effect of AKR and IFB on RCS, FMJ, and GTCS after PTZ-induced Seizures in Rats

Groups	Racine Convulsion Scale (RCS)	The onset of the first myoclonic seizures (FMJ) (sec)	Percentage of generalized tonic-clonic seizures (GTCS%)
Control	None	None	None
PTZ (45 mg/kg)	5.00 ± 0.21	55.33 ± 3.65	100 %
PTZ + Anakinra (100mg/kg)	4.5 ± 0.22	57.50 ± 4.52	100 %
PTZ + Infliximab (5 mg/kg)	4.66 ± 0.21	56.83 ± 4.59	100 %

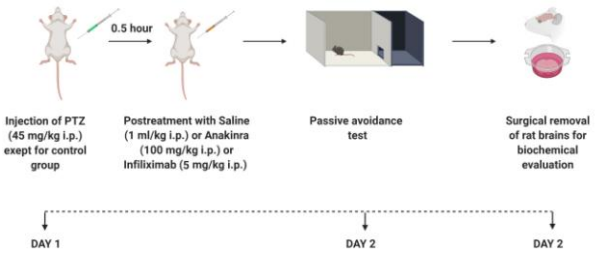

Fig. 1. Experimental protocol of the study (Created by BioRender)

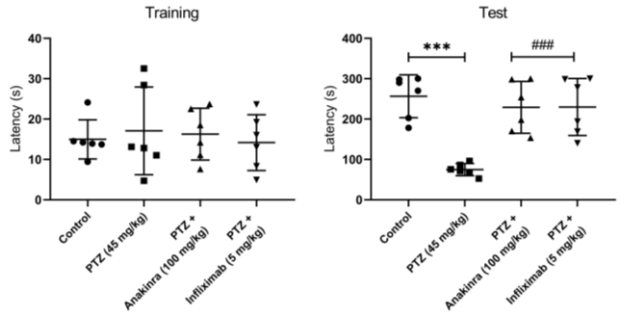

Fig. 2. Effects of AKR and IFB on passive avoidance test after PTZ-induced seizure in rats. (Mean±STD). (***$p< 0.001$ versus control group, $^{###}p< 0.001$ versus PTZ group)

dentate gyrus after PTZ application (25,26). Ekici et al. revealed that 8-OHdG level and caspase activity increased in the hippocampus and cortex after PTZ-induced epileptic seizures (21). Another study reported that OS markers increased significantly in the cerebral cortex in the PTZ-induced epilepsy model (27). The above studies and other similar studies showed that after an epileptic seizure, the damage could occur both in the hippocampus and in the cortex. For these reasons, we investigated the inhibition effect of AKR and IFB on PTZ-induced OS and apoptosis in the hippocampus and cortex. OS and BDNF

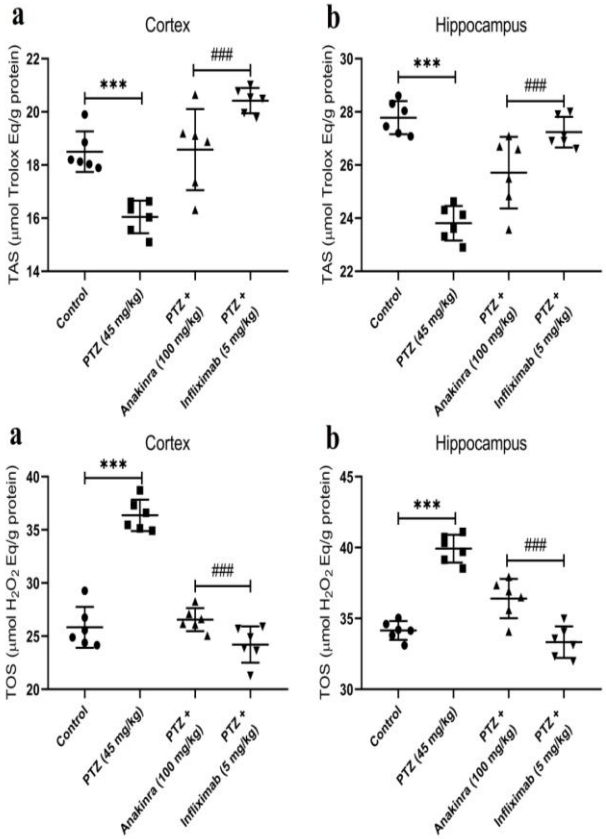

Fig. 3. Effects of AKR and IFB on TAS and TOS levels in the cortex (a) and hippocampus (b) after PTZ-induced seizure in rats. (Mean±STD). (***$p< 0.001$ versus control group, $^{###}p< 0.001$ versus PTZ group)

dysregulation is a critical factors in the development of epilepsy. BDNF is known to have a vital role in epileptogenesis (28). It has been reported in the literature that epileptic conditions increase the level of BDNF expression. In addition, infusion of BDNF into the rat hippocampus has been reported to induce seizures (29). In the mouse model induced by PTZ, Kazmi et al. emphasized that MDA and NO levels were high after PTZ administration, while antioxidant levels were low. In particular, they stated that modulation of OS and BDNF levels also decreased brain tissue damage (30).

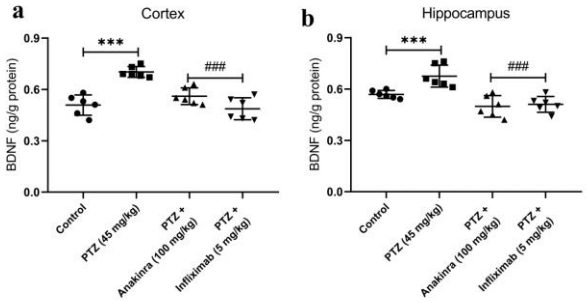

Fig. 4. Effects of AKR and IFB on BDNF levels in the cortex (a) and hippocampus (b) after PTZ-induced seizure in rats. (Mean±STD). (****p< 0.001* versus control group, ###*p< 0.001* versus PTZ group)

In addition, it has been emphasized in different studies that BDNF binds to TrkB receptors and activates downstream protein kinases, leading to the increased presynaptic release of mediators due to the phosphorylation of substrates or altering the functions of postsynaptic receptors as $GABA_A$ receptors (31,32). Hao et al. found that PTZ induction significantly increased the expression of BDNF and TrkB. In their study, they determined that Garcinol treatment potentially inhibited the expression of BDNF and TrkB, ultimately down-regulating BDNF signalling, which could help suppress glutamate-mediated stimulation (31). Chmielewska et al. argued that BDNF has an important role in neurobiological mechanisms in their epilepsy model study. They also suggested that BDNF plays a role in the early phase of epileptogenesis but not in the resident, synchronized neuronal activity associated with epileptic seizures (33,34). In our study, we determined that BDNF levels were increased in the PTZ group in the hippocampus and cortex during epilepsy attacks and were decreased in the groups that used AKR and IFB after PTZ treatment (Figure 4).

Alzoubi et al. suggested that the most important role in susceptibility to seizures was increased by OS (35). Filiz et al. found that the concomitant use of vitamin B12 and lamotrigine decreased the increased oxidative stress in rats caused by PTZ. They also found that maintaining the oxidant/antioxidant balance positively regulates the proinflammatory level (36). In our study, we found that PTZ reduces the amount of TOS and increases the level of TAS, which supports the literature.

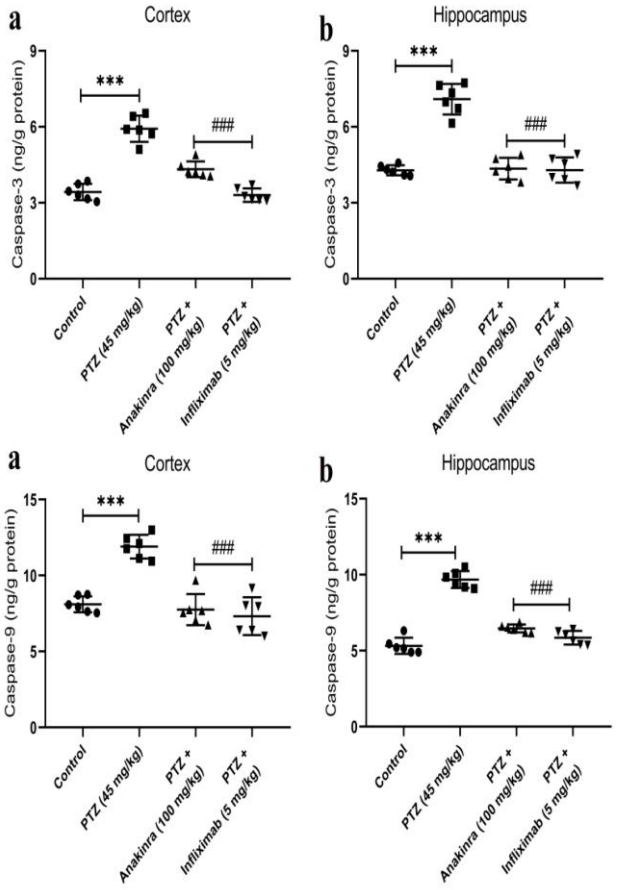

Fig. 5. Effects of AKR and IFB on caspase-3 and caspase-9 levels in the cortex (a) and hippocampus (b) after PTZ-induced seizure in rats. (Mean±STD). (****p< 0.001* versus control group, ###*p< 0.001* versus PTZ group)

However, we observed that the use of AKR and IFB after the PTZ increased the TAS level and decreased the TOS level in the rats (Figure 3).

Lotfy et al. noted a decrease in caspase 3 activation as an important finding against convulsions caused by the PTZ application (37). In another study, the neuroprotective effect of ferulic acid in the epileptic attack model induced by PTZ was investigated. In this study, it was noted that parameters such as MDA and caspase 3/9 were high in the group with only PTZ, while antioxidant parameters were low. Ferulic acid treatment 20 min. before PTZ application was found to inhibit the levels of these parameters. As a result, this study suggested that ferulic acid may be a neuroprotective agent for neurodegeneration in the epileptic model (38). In our study, after PTZ application, we observed that AKR and IFB decreased caspase-3 and caspase-9 levels in cortex and hippocampus samples compared with the PTZ group (Figure 5).

In this study, we observed that AKR and IFB inhibit OS and apoptotic pathways after PTZ-induced seizures. In addition, further studies investigating other parameters and signaling pathways at the molecular level are needed to elucidate the protective effects of AKR and IFB fully.

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Ethics Committee Approval: The Animal Ethics Committee of Cumhuriyet University approved the study protocol (decision no: 65202830-050.04.04-577. date: 12.07.2021).

Conflict of Interest: The authors declare that they have no conflict of interest

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