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Original Article

Effects of Artemisinin on Anti-epileptogenic, Antioxidant and Cholinesterase Enzymes in Pentylenetetrazole-induced Kindling Model in Mice

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ABSTRACT

Background: Artemisinin (ART) is a compound synthesized from the plant *Artemisia annua*. This compound has various therapeutic effects and is widely used against malaria. However, ART is known to have modulating effects on GABA (gamma-aminobutyric acid) receptors, which are thought to be responsible for epileptic seizures. This study aimed to evaluate the effects of ART on anti-convulsant, antioxidant, and cholinesterase enzyme activities in Pentylenetetrazole (PTZ)-induced kindling model in mice. **Materials and Methods:** In the experiment, 6 groups were formed, with seven mice in each group. Mice received a total of 11 intraperitoneal injections of PTZ (35 mg/kg). On the last day of the study, a threat dose of PTZ (75 mg/kg) was administered. In addition, behavioral analysis tests (Locomotor activity and rotarod) and biochemical measurements were performed. **Results:** Compared with the PTZ group, ART attenuated the severity of the kindling, decreasing the seizure score. ART and VPA reversed increased oxidative stress. Decreased cholinesterase enzymes in PTZ-induced brain increased with ART treatment. While the PTZ application impaired locomotor activity in mice, the ART application provided improvement in locomotor activity. However, no significant difference was found between the groups in the motor performance of the mice. **Conclusion:** The findings show that ART may have the potential to prevent PTZ-induced oxidative stress, neurochemical changes, behavioral disorders, and seizures.

Keywords: Anti-convulsant, Antioxidant, Cholinesterases, Pentylenetetrazole, Kindling model, Artemisinin.

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INTRODUCTION

Epilepsy is a chronic disease caused by abnormal discharge of neurons that carry out information transfer to the brain. It is one of the most common neurodegenerative diseases in society after Alzheimer's and stroke.[1] This disease affects about 1% of the world's population.[2] Epilepsy is more common in childhood and individuals over the age of sixty, and men are more affected than women. The sudden death rate in epilepsy is very high compared to the general population.[3] Today, although the anti-epileptic drugs used partially eliminate the epileptic seizures and cognitive disorders of the patients, it is known that resistance develops due to the long-term use of these drugs.[4] Therefore, there has been a growing interest in the search for compounds with a high therapeutic index and prophylactic effect, or pharmacologically active substances that prevent neural damage.

Pentylenetetrazole (PTZ) is a compound used as a nervous system stimulant. PTZ triggers the formation of seizures by creating a selective antagonist effect on the GABA receptor.[5] Therefore, it is a widely used model for the evaluation of pharmacological agents thought to have anti-epileptic potential.[6,7]

The oxidant-antioxidant mechanism in the organism is balanced under normal physiological conditions. However, disruption in this mechanism causes an increase in the balance in favor of oxidant and also tissue damage formation. Free oxygen radicals, which contribute to the formation of damage, attack the lipids in the cell and lead to Lipid Peroxidation (LPO). Malondialdehyde (MDA) is the end product of LPO and increases in the case of oxidative stress. This parameter is widely used in the evaluation of LPO.[8,9] Glutathione S-Transferases (GST) are among the

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multifunctional enzymes involved in antioxidant defense and phase II metabolism of cells. GST also plays a role in the metabolism of all chemicals (xenobiotics) including drugs, together with the enzymes found in the blood-brain barrier and also as an antioxidant in cells.[10]

Neurochemical markers Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) are enzymes that perform the hydrolysis of acetylcholine. These enzymes also have functions that provide the restorations of excitations in cholinergic nerve endings, neural synapses, and motor endplates.[11,12] It has been reported that inhibition of cholinesterase with neurotoxic substances such as PTZ may affect the increase of acetylcholine in the brain and the formation of seizures.[11] In addition, cholinergic dysfunction may cause various symptoms such as cognitive disorders in neurodegenerative diseases, memory problems, anxiety, and depression.[13] Cholinesterase also plays a vital role in the cholinergic system by ending the nerve conduction disorders that cause these symptoms in the brain.[11,13]

Artemisinin (ART) is a sesquiterpene lactone derivative isolated from the plant *Artemisia annua*, which grows in the Asian continent. Today, artemisinin is a widely used anti-malarial compound and plays a critical role in malaria treatment worldwide.[14,15] ART antagonizes NMDA receptors.[16] It has a relaxing effect on chronic neuropathic pain.[17] It has also been reported in previous studies that it modulates GABA receptors.[15]

It has been reported that damage to the brain due to oxidative stress may trigger the formation of chronic neurodegenerative diseases such as epilepsy, Alzheimer's, and Parkinson's.[8,18] The main purpose of our study is to investigate a compound that may have antioxidant and antiepileptic properties, which have a protective effect on neurons. Therefore, the effect of ART on anti epileptogenic, motor performance, cholinesterase enzyme activity, and oxidative stress was evaluated in the PTZ-Kindling model.

MATERIALS AND METHODS

Animals

Swiss albino male mice (*n*=42) were used in this study. The animals were eight weeks old and weighed 20-25 g. Seven mice in each group were placed in plastic cages. They were kept on a 12 hr light/12 hr dark cycle and had easy access to feed and water. Pre-approval was obtained from the Local Ethics Committee to perform the experiments (2021/01-07).

Design of the experimental protocol

Experimental animals were randomly divided into six groups of seven animals each (*n*=7). The first group was given saline (0.9% NaCl i.p.), and the second group was given saline-dissolved PTZ (35 mg/kg, i.p.). The third group was given the reference drug VPA (100 mg/kg, i.p.). ART in different doses of 30, 60, and 120 mg/kg was administered to the fourth, fifth, and sixth groups. The groups that were administered VPA (Group 3) and ART (Group 4, 5, 6) were injected with PTZ (35 mg/kg, i.p.) 30 min later.

PTZ-Kindling Model

A sub convulsive dose of PTZ (35 mg/kg) was administered to mice for a total of 11 injections every other day to form kindling (Figure 1). Animals were observed for convulsive episodes for 30 min after administration of VPA, ART, and PTZ. The PTZ sub-convulsive dose was completed on the 24th day of the trial. In addition, PTZ (75 mg/kg) dose was administered to the test groups (Groups 2, 3, 4, 5, and 6) on the 26th day of this study. This dose is convulsion (clonic and tonic), status epilepticus, and lethality dose.[7] Kindling was determined using the following scale: Stage 0, No response to PTZ; Stage 1, long-term twitching of the ear and face; Stage 2, Axial convulsive fluctuations throughout the body; Stage 3, Recurrent quick and severe myoclonic reflexes in the body; Stage 4, Generalized clonic seizures and side-lying position in animals; Stage 5, Generalized seizures with status epilepticus and muscle stiffness (Tonic extension); Stage 6, Mortality.[19]

Locomotor and motor coordination activity

Open-Field Test (OFT)

In the OFT test, a 50x40x40 cm, open-topped assembly with a base divided into 16 equal squares and surrounded by 30 cm long black plastic was used. Each mouse was placed in the center of the assembly for 5 min. During this period, the number of square-to-frame transitions and rearing of mice was counted. The floor was cleaned with ethyl alcohol so that the smell of the animals did not affect the behavior of the next animal.[20]

Rota rod performance test

The motor coordination test was carried out with the rotor device placed in a chamber. Mice were placed on the main axis of the rotating device. All animals were tested on the device five times. The first two tests were for educational purposes. The next three tests were done with adapting animals. Mice were tested on the rotating shaft for a maximum of 300 sec. The times the animals fell off the stick were recorded.[21]

Biochemical analysis

Mice were intracardiac blood drawn at the end of the experiment (day 26). It was centrifuged at 3500 x g for 15 min. Phosphate buffer (pH 7.4, 1.8 mL 50 mM) was added to brain tissue (200 mg). Homogenization was achieved with a homogenization device (Ultra Turrax-T25). The obtained homogenate was centrifuged at 10000 rpm for 30 min. Both samples were stored in a deep freezer at -80°C until the day of the study.

MDA measurements were made by taking samples from a previous study.[22] Serum and brain tissue samples prepared at

Table 1: PTZ alone and co-administration PTZ with artemisinin comparison of the latent period (s) and lethal effects of PTZ (75 mg/kg, i.p.) in mice on day 26.

Groups	Latent period (s)	Lethality
PTZ	57 ± 25.33	6/7
PTZ+VPA 100 mg/kg	73 ± 31.19	4/7
PTZ+ART 30 mg/kg	217 ± 71.26	3/7
PTZ+ART 60 mg/kg	379 ± 123	3/7
PTZ+ART 120 mg/kg	452 ± 147.34	2/7

Data are expressed as mean SEM (n=7/ group). Pentylenetetrazol=PTZ; Artemisinin=ART; Valproate=VPA.

different concentrations were loaded into High-Performance Liquid Chromatography (HPLC). These concentrations were read at 527 nm and 551 nm at excitation and emission wavelengths, respectively. The GST enzyme catalyzes the conjugation of glutathione. In GST measurement, enzyme activity can be measured with an absorbance value of 340 nm by reaction of 1-Chloro-2,4-dinitrobenzene (CDNB) substrate. Absorbance values were measured and recorded for 3 min at baseline and every minute.[23]

Cholinesterase enzyme activity values are one of the biomarkers used in the diagnosis of various pathological diseases. AChE catalyzes the hydrolysis of acetylcholine to thiocholine. The rate of formation of thiocholine was determined by the reaction of thiocholine resulting from the formation of 5-thio-2-nitrobenzoic acid and giving a yellow color with 5,5'-dithiobis (2-Nitrobenzoic acid) (DTNB). Butyrylthiocholine Iodide (BChI) and DTNB were used to measure BChE enzyme activity. Both enzymes were measured spectrophotometrically at 412 nm at the start of the reactions.[24,25]

Protein measurement, 4900 µL of drabkin, 90 µL of distilled water, and 10 µL of homogenate were added to the prepared tissue homogenates, and after 10 min of incubation, it was read at 595 nm and the results were calculated as µg/mL with the help of a standard curve.[26]

Statistical Analysis

The results obtained from the study were shown as mean ± SD. Seizure severity was analyzed by two-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons; biochemical data were analyzed with one-way ANOVA followed by the Tukey test. $p<0.05$ was set as significant in all tests (GraphPad Prism version 8.0).

RESULTS

PTZ-Kindling subconvulsive dose

Experimental animals were given 11 injections every other day for 24 days. PTZ (35 mg/kg) administered at repeated doses increased seizure scores in mice. It increased seizure scores compared to the control group. Seizure severity reached a score of four on injection days 8, 9, 10, 11. ART pretreatment resulted in a significant reduction in PTZ-induced seizure severity in 7, 8, 9, 10, 11 injections. In addition, VPA was not effective in reducing seizure scores. The effects of increasing doses of ART on PTZ-induced kindling are shown in Figure 2.

PTZ threatening doze

PTZ-threatening dose (75 mg/kg) was administered to the test groups on the 26th day of the experiment. Mice exhibited gradual progressive convulsive episodes such as twitchy hyperactivity, sustained clonic-tonic seizures, and hyperextension of extremities after injection. Seizure scores and survival rates were found to be close to each other between PTZ and VPA groups. It was observed that the animal groups in which ART was administered as a pre-treatment protected against convulsive attacks and mortality (Table 1).

Behavioral analysis

Mice were left in the middle of the assembly for 5 min for the OFT test and frame-to-frame transitions were counted. While there was a decrease in the number of frame transitions in the PTZ group, a significant increase was observed in the medium and high doses of ART ($p<0.05$; Figure 3A). However, there was no statistically significant difference between the groups in the rearing test ($p>0.05$; Figure 3B).

Motor coordination was assessed using the rotarod test. The ART groups performed better than the PTZ group. ART increased the motor activities of the mice. However, there was no statistically significant difference between the groups ($p>0.05$, Figure 3C).

The effects of ART administration on oxidative biomarkers in brain tissue and serum

Tissue and serum MDA levels were increased in the PTZ group compared to the other groups ($p<0.05$). ART dose-dependently decreased MDA levels in the brain tissue compared to the PTZ group (Figure 4). The ART-60 dose had a lower serum MDA level than all test groups (Figure 5). Brain GST levels were decreased in the PTZ group. It increased at medium and high doses of ART. ART increased brain tissue GST levels better than VPA ($p<0.05$, Figure 5). Serum GST levels increased in both PTZ and VPA, ART-60 and ART-120 groups compared to the control group ($p<0.05$). In addition, the GST activities of the ART-30 group were lower than the control group ($p<0.05$, Figure 4).

The effect of repeated ART administration on AChE and BChE

Brain tissue AChE levels were highly inhibited in the PTZ group. It was much higher in the ART-30, 60, and especially in the ART-120 group compared to the other groups ($p<0.05$, Figure 4). VPA group AChE level was higher than both control and PTZ groups ($p<0.05$). Serum samples' AChE levels did not change in the PTZ, ART-30, and ART-120 groups compared to the control group, while the VPA and ART-60 groups increased significantly compared to the control group (Figure 5).

As seen in Figure 4, Brain tissue BChE activities were partially similar to AChE activities. BChE activity of PTZ and VPA groups was inhibited compared to other groups ($p<0.05$). BChE levels of ART-60 and ART-120 groups increased compared to Control and PTZ groups. BChE activities measured in serum samples were different from BChE activities measured in brain tissue ($p<0.05$, Figure 4). PTZ and ART-30 groups had the highest values, while VPA had the lowest. Other BChE levels are detailed in Figure 5.

DISCUSSION

ART is a compound widely used worldwide for the treatment of malaria. Antioxidant, anti-inflammatory, antibacterial, anticancer, and neuroprotective effects have also been reported. In addition, ART has been recommended to be used against Coronavirus Disease (COVID-19) due to its antiviral properties.[27,28] The fact that ART has the above-mentioned pharmacological activities makes it attractive to investigate its effect on different diseases.

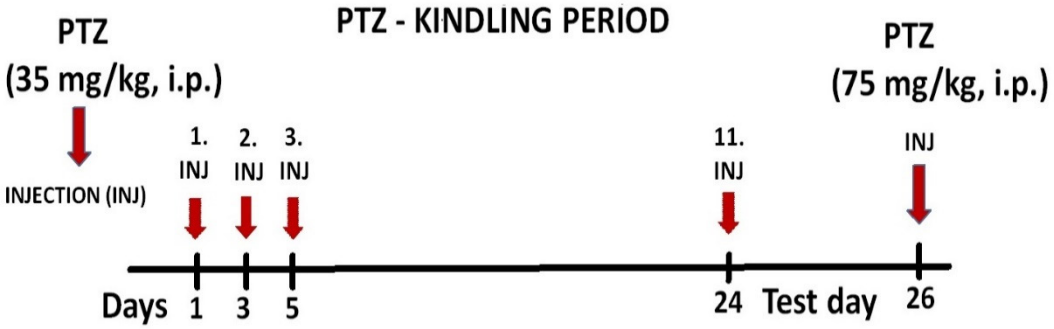

Figure 1: Schematic administration procedure of experimental kindling model. 35 mg/kg dose of PTZ was administered (i.p.) to groups on days 1,3, 5, 8, 10, 12, 15, 17, 19, 22, and 24 (Kindling period). Then 75 mg/kg threatening dose of PTZ was administered (i.p.) to groups on day 26 (Test day). Pentylenetetrazol=PTZ, Injection=INJ, i.p.=intraperitoneal.

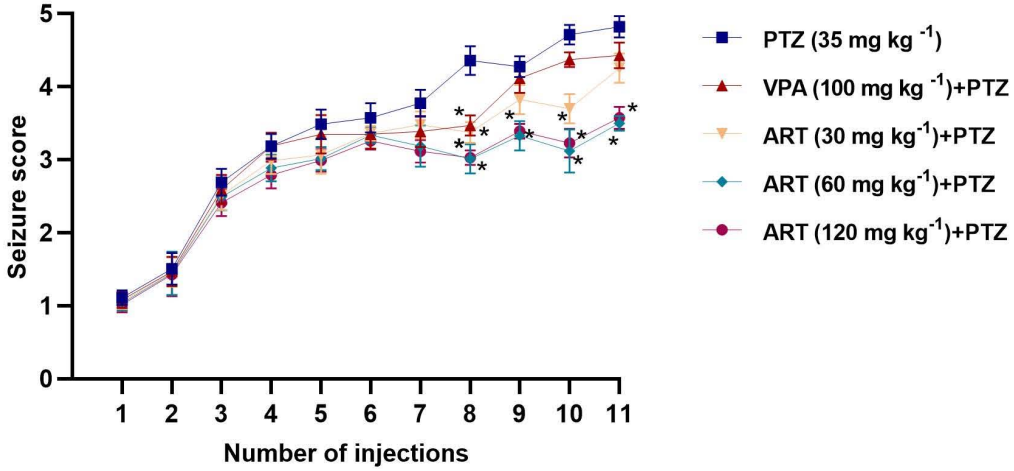

Figure 2: Effect of artemisinin on the PTZ-induced convulsion in the mice. Data are expressed as mean±SD (n=7/group). The convulsive intensity score was specified utilizing the Racine scale (stages 0-5). Two-way ANOVA was used for comparison between the groups, followed by Bonferroni's *post hoc* test for multiple comparisons. *Significant difference when compared to pentylenetetrazole group ($p < 0.05$); Pentylenetetrazol=PTZ; Artemisinin=ART, Valproate=VPA.

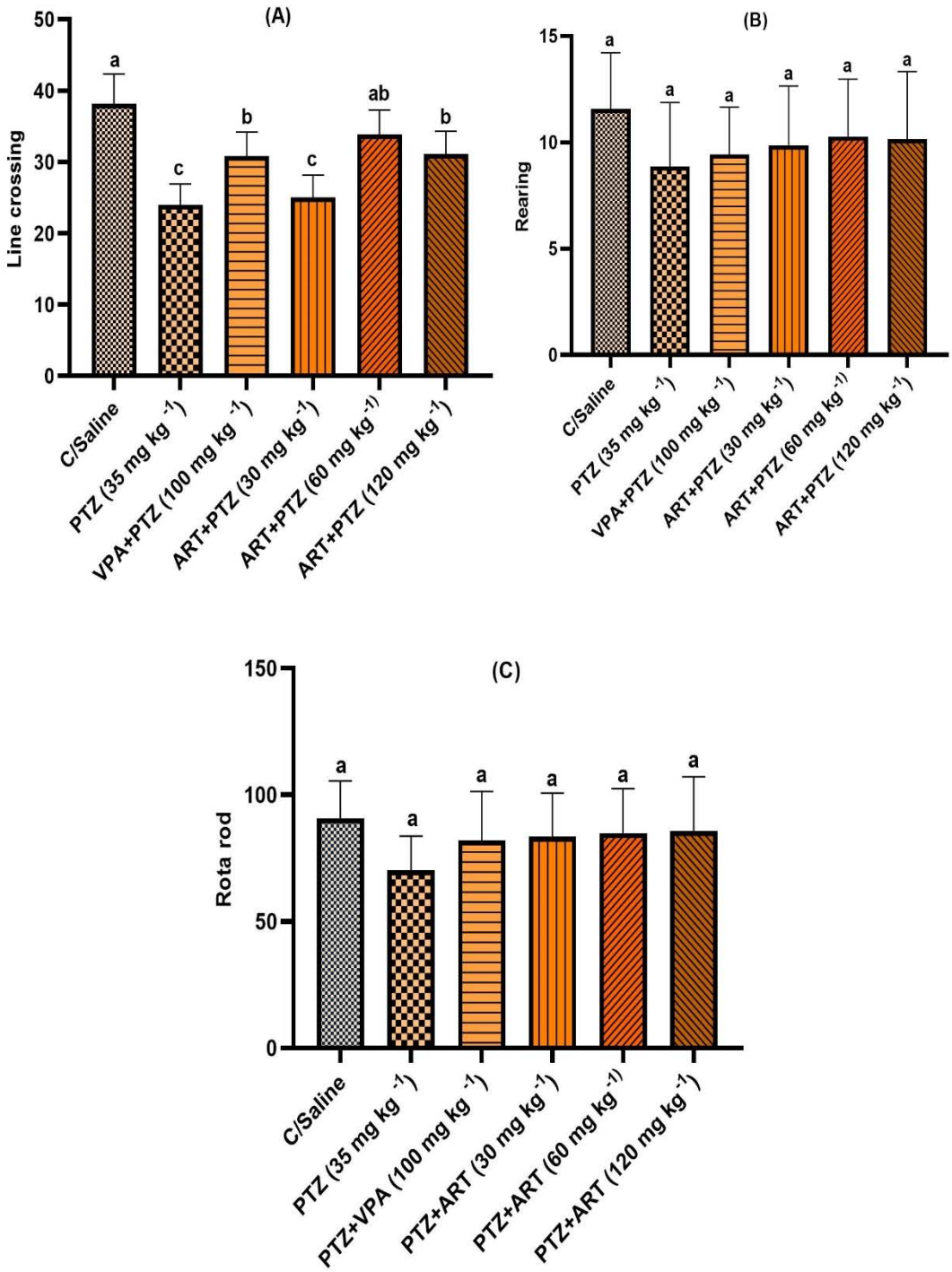

Figure 3: The locomotor activities of the mice were assessed by counting the animals' square transitions for 5 min. The motor activities of mice were evaluated by the Rotarod test. Pentylenetetrazole=PTZ; Artemisinin=ART; VPA=Valproate; C=Control. [a,b,c]*p*: values with different letters are significant when compared with each other ($p<0.05$).

Therefore, the effects of ART on an anticonvulsant, cholinergic enzyme activity, and oxidative stress in PTZ-induced mice were investigated in this study. In the Kindling model, PTZ (35 mg/kg) causes generalized tonic-clonic seizures in animals.[29] ART reduced the score of tonic-clonic seizures in a dose-dependent manner, suggesting that it may have anticonvulsant properties against Kindling-induced temporal lobe epilepsy.[30] In addition, it was determined that ART pretreatment at a lethal dose of PTZ (75 mg/kg) reduced the frequency of seizures and mortality. The anti-seizure activity of ART (60 and 120 mg/kg) was compared to the reference control VPA (100 mg/kg), and ART was found to be better than VPA. PTZ can trigger seizure formation by causing inhibition of GABA receptors and a decrease in GABAergic functions in repeated dosing applications.[29,31] GABA plays an important role in the pathophysiology of epilepsy. In the previous study, it was reported that ART inhibited neuropathic pain by modulating the GABA receptor.[15] Indeed, Lin *et al.* (2018) found that ART inhibits and protects against the glutamate-induced death of neural HT-22 cells in mouse brains.[32] It has also been shown that ART can protect against neurodegenerative disorders

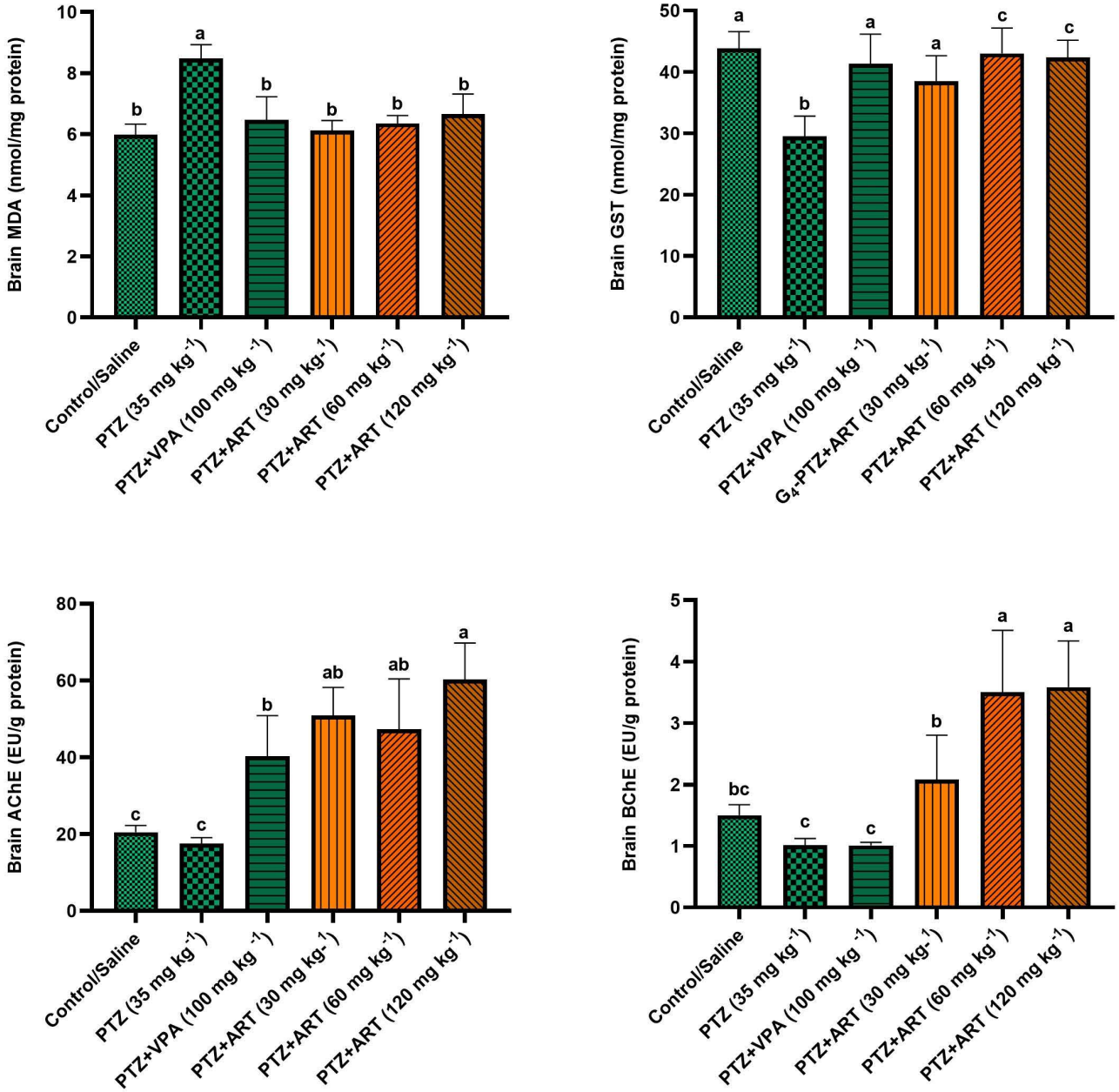

Figure 4: Comparison of the values of parameters measured in brain homogenates. PTZ: pentylenetetrazol, VPA: Valproate, ART: artemisinin, MDA: malondialdehyde (nmol/10 mg protein), AChE: acetylcholinesterase (EU/g protein), BChE: butyrylcholinesterase (EU/10 g protein), GST: glutathione S-transferases, [a,b,c]p: values with different letters are significant when compared with each other ($p<0.05$).

by suppressing neuronal apoptosis.[33] In addition, ART can cross the blood-brain barrier due to its lipophilic nature and may provide advantages in the treatment of neurodegenerative disorders.[32] It supports that ART may have anticonvulsant potential in PTZ-induced seizures with the above-mentioned therapeutic mechanisms of action.

PTZ administration may cause limitation of motor functions and an increase in motor dysfunction depending on the frequency

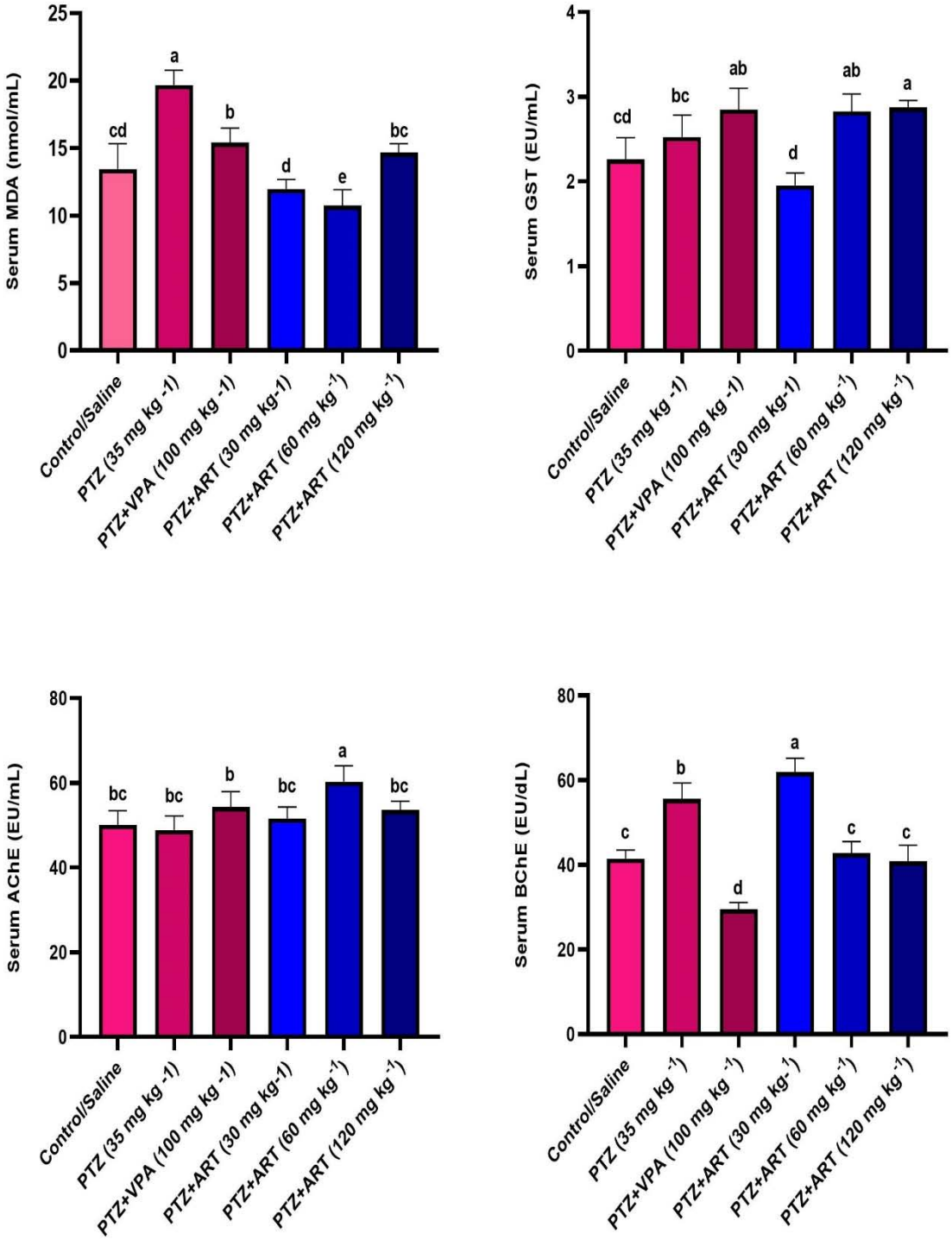

Figure 5: Comparison of the values of parameters measured in serum samples. PTZ: pentylenetetrazol, VPA: Valproate, ART: artemisinin, MDA: malondialdehyde (nmol/10 mg protein), AChE: acetylcholinesterase (EU/g protein), BChE: butyrylcholinesterase (EU/10 g protein), GST: glutathione S-transferases, $^{a,b,c}p$: values with different letters are significant when compared with each other ($p<0.05$).

of seizures.[34] Therefore, OFT and rotarod tests were applied to measure neuromuscular impairment. In OFT, mice were evaluated by Square-to-frame transition and rearing tests. The findings confirmed that PTZ may have caused changes in the basal ganglia, thus causing decreases in locomotor activity.[35] Cognitive behavior of the mice improved when the ART groups were compared with PTZ in a dose-dependent manner. On the other hand, there was no significant change in the motor performances (Rotarod test) of the experimental groups. The cognitive behavior of mice decreased the ability to explore the environment and the number of Square-to-frame transitions in mice exposed to PTZ. ART pretreatment increased the number of frames passes and the motivation to explore the environment. Previous studies have reported that ART prevents isoflurane-induced neuronal apoptosis and improves cognitive behavioral disorders. In the study, it was suggested that pretreatment of ART would contribute to the development of histone acetylation, which is one of the epigenetic mechanisms in the brain, cognitive level, memory, and nervous system development.[36] It has also been shown that ART can restore the learning and spatial memory capacity that develops due to neuronal cell destruction.[37] In addition, ART can cross the blood-brain barrier due to its lipophilic structure and may provide advantages in the treatment of neurodegenerative disorders.[32] This study shows that ART against the neurotoxicity of PTZ can improve the behavior of mice with similar mechanisms of action as in the studies mentioned above.

Oxidative stress is known to be caused by the deterioration of the balance between the antioxidant defense system of the organism and the oxidants formed by Reactive Oxygen Species (ROS).[38] The brain needs high amounts of oxygen because it is rich in unsaturated fatty acids and uses glucose as an energy source. Therefore, the brain is an organ that tends to produce more ROS. ROS increases Lipid Peroxidation (LPO) in cells, causing them to be more susceptible to oxidative damage.[8,38] In this study, the effect of ART on oxidative stress was investigated using the PTZ kindling model. This model is widely used to induce oxidative stress in experimental animals.[39,40] Repeated doses of PTZ can increase ROS in brain cells and lead to LPO formation.[8] The results of this study supported the hypothesis that PTZ triggers oxidative stress and increases levels of MDA, the end product of LPO. ART shows that it can reduce MDA levels and suppress oxidative stress in serum and brain tissue at low and moderate doses. In addition, it was determined that there were significant decreases in the GST levels of the same group due to the increase in MDA levels of the PTZ group. GST is an enzymatic antioxidant and detoxifies its harmful products by reacting with free radicals.[41] The decrease in GST activities in the PTZ group indicates that free radicals increase due to oxidative stress and GST may be depleted. In a previous study, it was reported that ART reduced oxidative stress and increased antioxidant biomarkers in rats with diabetes.[42] There are also studies showing that ART has antioxidant potential.[14,43] Therefore, it suggests that the decrease in MDA levels in serum and brain tissues and the increase in GST activity in ART-treated mice may be due to the strong antioxidant property of ART.

AChE and BChE cholinesterase are enzymes that catalyze Acetylcholine (ACh) hydrolysis. These enzymes restore cholinergic nerve pathways. These enzymes restore cholinergic nerve pathways. Both cholinesterase enzymes involved in neurotransmission are used to find the neurotoxic effects of xenobiotics that cause degeneration in the central and peripheral nervous system. In particular, AChE inhibition and accumulation of ACh in the synaptic pathway cause life-threatening symptoms such as epileptic seizures, coma, and death as a result of impaired nerve conduction.[44,45] In this study, it is seen that AChE and BChE levels in the brain tissue of the PTZ group decreased. The AChE and BChE levels of the ART and VPA groups were significantly increased compared to both the control and PTZ groups. Serum AChE levels were similar to those in brain tissue. But surprisingly, serum BChE levels were the opposite of results in brain tissue. Especially in the PTZ group, BChE levels were higher than in the control group. Our results in brain tissue are in agreement with the literature.[46,47] however, no literature has been found to support the increase in serum BChE levels after PTZ application. In a study conducted with stroke patients, it was known that serum BChE levels were increased compared to control.[48] It has also been reported that BChE levels increase in chronic diseases such as diabetes and breast cancer.[49] More detailed and repetitive studies are needed to understand the underlying reasons for the increase in serum BChE levels in chronic diseases.

CONCLUSION

This study showed that PTZ kindling triggers oxidative stress, changes in neurochemical enzymes such as AChE and BChE, and behavioral disorders. However, ART treatment reduced the seizure score and related mortality and learning deficit. During the development of PTZ kindling, ART exhibited a neuroprotective effect by reducing excessive stimulation between neurons with its anticonvulsant effect, and oxidative stress with its antioxidant property. These results suggest that artemisinin may have the potential to prevent seizures caused by excitotoxic substances. ART can be a therapeutic agent for the control of epileptic seizures and the prevention of neurologic disorders. However, it should be kept in mind that further studies are needed to better understand the mechanism of action and side effects of ART on the central nervous system.

ACKNOWLEDGEMENT

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ART: Artemisinin; **VPA:** Valproate; **GABA:** Gamma-aminobutyric acid; **PTZ:** Pentylenetetrazol; **MDA:** Malondialdehyde; **LPO:** Lipid peroxidation; **GST:** Glutathione S-transferase; **AChE:** Acetylcholinesterase; **BChE:** Butyrylcholinesterase; **NMDA:** N-methyl-d-aspartic acid; **HPLC:** High-performance liquid chromatography; **TBA:** Thiobarbituric acid; **DTNB:** 2-Nitrobenzoic acid; **BChI:** Butyrylthiocholine iodide; **CDNB:** 1-Chloro-2,4-dinitrobenzene; **GSH:** Glutathione; **SEM:** Standard error of the mean; **i.p:** Intraperitoneal; **INJ:** İnjection.

SUMMARY

- PTZ administration every other day causes oxidative stress and seizures in mice.
- PTZ administration inhibited AChE and BChE activity in the brain.
- Artemisinin antagonized PTZ-induced seizures in mice and increased AChE and BChE activity.
- Artemisinin prevents cognitive disorders in the PTZ-induced kindling model.
- Artemisinin may be a supplementary drug in the treatment of epilepsy.

Ethics Approval

The study was approved by the Ethical Committee for Animal Experiments of the Van Yuzuncu Yil University Medical Research Centre, Van, Turkey.

AUTHOR CONTRIBUTION

YK: Conceptualization, Project administration, Writing – original draft, Methodology, Resources, Data curation. **OY:** Methodology, Investigation, Resources, Data curation. **ZH:** Methodology, Resources, Data curation. **FT:** Formal analysis, Conceptualization, Investigation.

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