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EXPERIMENTAL STUDY

Anti-seizure effect of zinc on PTZ-induced epilepsy in rat model

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ABSTRACT
Epilepsy is a widespread and mainly severe neurological condition portrayed by recurring spontaneous seizures caused by the brain's abnormal electrical activity. According to new research, inflammation may be both a result and the cause of epileptic seizures. The highest zinc levels in the brain have been found in the hippocampus which is one of the most studied regions of the brain regarding epilepsy. Zinc may have an anti-inflammatory potential as zinc co-factors affect numerous biochemical and physiological reactions. In this study, we evaluated the effects of intraperitoneal zinc administration on seizure activity in murine PTZ model. Rats received either intraperitoneal (IP) zinc sulfate at two different dosages (50–100 mg/kg) or a placebo followed by pentylenetetrazole (IP), a strong seizure-inducing drug. The spike percentages were considerably lower in the PTZ (35 mg/kg) and 50 or 100 mg/kg zinc-treated groups (A3 and A4) than in the PTZ (35 mg/kg) and saline-treated group (A2; $p < 0.001$). When the PTZ (70 mg/kg) and zinc sulfate-administered groups (B3 and B4) were compared to the PTZ (70 mg/kg) group (B2), the zinc-administered groups had a substantially reduced RCS ($p < 0.001$). The onset time of FMJ was substantially longer in the PTZ (70 mg/kg) and 50 mg/kg or 100 mg/kg zinc sulfate groups (B3 and B4) than in the PTZ (70 mg/kg) and saline (B2; $p < 0.001$). We discovered that MDA levels were considerably lower in the zinc-treated groups. SOD and HSP-70 levels were also increased significantly with zinc sulfate administration. In conclusion, our findings indicate that zinc has the potential to exhibit antiepileptogenic effects by alleviating acute oxidative stress and neuroinflammation in a rat PTZ-induced epilepsy model. Zinc (50 or 100 mg/kg i.p.) successfully decreased the spike percentages and RCS associated with PTZ kindling epilepsy, as well as considerably decreased MDA and increased SOD and HSP-70 levels in rat brain. According to these results, zinc sulfate may be used as an adjuvant therapy in combination with other antiepileptic drugs in the future *(Tab. 3, Fig. 1, Ref. 27)*.
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KEY WORDS: anti-seizure effect of zinc, epilepsy, abnormal electrical activity, antiepileptic drugs, rat model.

Introduction

Epilepsy is a widespread and mainly severe neurological condition portrayed by recurring spontaneous seizures caused by the brain's abnormal electrical activity. Epilepsy affects up to 3 % of people at some point in their lives (1). Epilepsy has a variety of causes, ranging from hereditary and congenital conditions to neuronal abnormalities caused by earlier damage such as infection, hypoxia, and inflammation.

The goal of epilepsy medications is to give an adequate life quality while minimizing the negative effects of antiepileptic medicines and guaranteeing total seizure control. Monotherapy prevents seizures in the large majority of epileptic patients (2). When monotherapy is ineffective, antiepileptic medication combinations can be used. While the expanding variety of antiepileptic medications are available, roughly 30 % of patients continue to have episodes despite proper treatment (3).

Zinc is a runner-up trace element. It is a non-redox active metal that only occurs as a divalent cation. Zinc is necessary for the catalytic action of more than 300 enzymes as well as for stabilization of protein subdomain folding (4). Zinc is also essential for the brain to function properly, also an optimal concentration of zinc is necessary for the limbic system which includes the hippocampus and amygdala (5). Many mental and neurological illnesses such as Parkinson's and Alzheimer's diseases, schizophrenia, attention deficit hyperactivity disorder (ADHD), and epilepsy are associated with zinc dysregulation (6). The highest zinc levels in the brain have been found in the hippocampus which is one of the most studied regions of the brain regarding epilepsy (7, 8).

According to new research, inflammation may be both a result and the cause of epileptic seizures. Seizures *per se* can cause brain inflammation, and recurring seizures can aggravate chronic inflammation. Seizure-related cell death can contribute to inflammation, although it is not prerequired for inflammation to occur (9).

It is suggested that zinc may have an anti-inflammatory potential as zinc co-factors affect numerous biochemical and physiological reactions (10). In this study, we evaluated the effects of intraperitoneal zinc administration on seizure activity in murine PTZ model.

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Materials and methods

Animals and design of experiment

The methodologies utilized in this study's experiments were authorized by the Animal Ethics Committee. (Scientific University, 202101-a). All methods followed the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. (The United States)

For this study, 48 male Sprague–Dawley rats weighing 200–250 g were utilized (24 for EEG recording and 24 for behavioral investigations). The rats were kept in calm rooms with 22–24 °C ambient temperature and a 12/12-hour light/dark cycle (light from 07.00 to 19.00). They were fed conventional laboratory diet and given unlimited access to tap water.

Experimental procedures

In total, 48 rats were assigned into two groups at random: Group A for EEG recordings and Group B for behavioral evaluation. Rats in Group A were heavily sedated. Then, under stereotaxically controlled settings, a small hole was bored using a drill. The electrodes (0.1 mm diameter polyamide-coated stainless steel wires, electrical resistance 1/10 mm) were implanted on the *dura mater* over the left frontal cortex (2.0 mm lateral to the midline, 1.5 mm anterior to the bregma) and the reference electrode was implanted over the cerebellum (1.5 mm posterior to the lambda), on midline for EEG recording.

The electrodes were then stabilized with dental acrylic (dental acrylic is a compound of several alloys used for dental restoration). Intraperitoneal administration of ketamine (80 mg/kg) and xylazine (4 mg/kg) anesthetized rats severely (i.p.). While 35 mg/kg is adequate for seeing alterations in EEG spikes, it does not regularly elicit evident behavioral changes. A dose of 70 mg/kg provides observable behavioral changes, but EEG measurements have a poor signal-to-noise ratio, making it difficult to distinguish changes in drug concentrations. After 12 days, 24 rats were randomly divided into four groups (n = 6): Group A1, A2, A3, A4

Group A1 was designated as the control group and received no medication. Group A2 received saline intraperitoneally, Group A3 received 50 mg/kg zinc sulfate intraperitoneally. (Zinco, 50 mg, Berko Drug), and Group A4 received 100 mg/kg zinc sulfate intraperitoneally. The medications were administered 30 minutes before the administration of pentylenetetrazol (PTZ; 35 mg/kg, intraperitoneally.). Except for Group A1, all animals received 35 mg/kg PTZ and EEGs were recorded. After 5 minutes of PTZ treatment, EEG recordings were made in a separate container in conscious rats.

All EEG recordings and behavioral evaluation procedures were performed exactly as instructed. In brief, the EEG recordings were performed for 60 minutes, and the signals were magnified 10,000 times and filtered between 1 and 60 Hz. The BIOPAC MP150 Data Acquisition System (Biopac System Inc., Santa Barbara, CA, USA) was used to gather the EEG data, and the spike percentage was calculated. The EEG data were assessed by two clinical neurophysiologists for the spike percentage, which is a reproducible way of detecting epileptiform activity that measures the ratio of 1-second bins with at least one spike-wave, referred to as the "spike-wave percentage."(11). When compared to the baseline values, the initiation and termination of this complex were detected by a larger amplitude (at least two-fold). Within 2-minute intervals, the total period of the spike was analyzed.

The remaining 24 rats (Group B) were also separated into four groups (n = 6): Group B1, Group B2, Group B3, and Group B4. The first group (Group B1) was designated as the control group and was not given any medication. Group B2 received saline intraperitoneally, Group B3 received 50 mg/kg zinc sulfate intraperitoneally, and Group B4 received 100 mg/kg zinc sulfate intraperitoneally. The medicines were given 30 minutes before the PTZ administration (70 mg/kg, intraperitoneally.). To assess the seizures (for just PTZ 70 mg/kg), Racine's convulsion scale (RCS) and onset times of 'first myoclonic jerk' (FMJ) were utilized as follows: 0 = no convulsion; 1 = twitching of vibrissae and pinnae; 2 = motor arrest with more pronounced twitching; 3 = motor arrest with generalized myoclonic jerks (this time was recorded for evaluating FMJ onset time); 4 = tonic-clonic seizure while the animal remained on its feet; 5 = tonic–clonic seizure with loss of the righting reflex; 6 = lethal seizure. As previously described, the rats were monitored for FMJ onset timings (11). The timings of the onset were recorded in seconds. Almost all of the animals with tonic generalized extension perished. The observation time for PTZ-induced seizures was set at a 30-minute duration (11, 12). The rats were put down after this period.

Brain lipid peroxidation measurement via MDA

Using malondialdehyde (MDA) levels, the amount of lipid peroxidation in tissue samples was measured as thiobarbituric acid reactive substances (TBARS). Shortly, trichloroacetic acid and the TBARS solution were added to the tissue, which were then combined and incubated for 60 minutes at 100 °C. The specimen was centrifuged at 3,000 rpm for 20 minutes after being chilled on ice, and the absorbance of the mixture was measured at 535 nm. MDA levels were estimated using tetraethoxypropane from the standard calibration curve and represented as nmol/gr protein.

Brain protein level measurement

The total protein content in brain tissues was evaluated using the Bradford technique with bovine serum albumin as a reference.

Total SOD activity levels detection

Total superoxide dismutase activity was measured with the method of Sun et al. As a superoxide generator, this approach uses the inhibition of NBT reduction by the xanthine oxidase system. One unit of superoxide dismutase was defined as the quantity of enzyme that inhibited the NBT reduction rate by 50%. The activity of superoxide dismutase was measured in units per milligram protein (U/mg protein).

Detection of HSP-70 in brain tissue

After sacrification, the tissues were quickly taken out and stored for biochemical analysis at −20 °C. All of the brain tissues were homogenized using a glass homogenizer in 5 volumes of

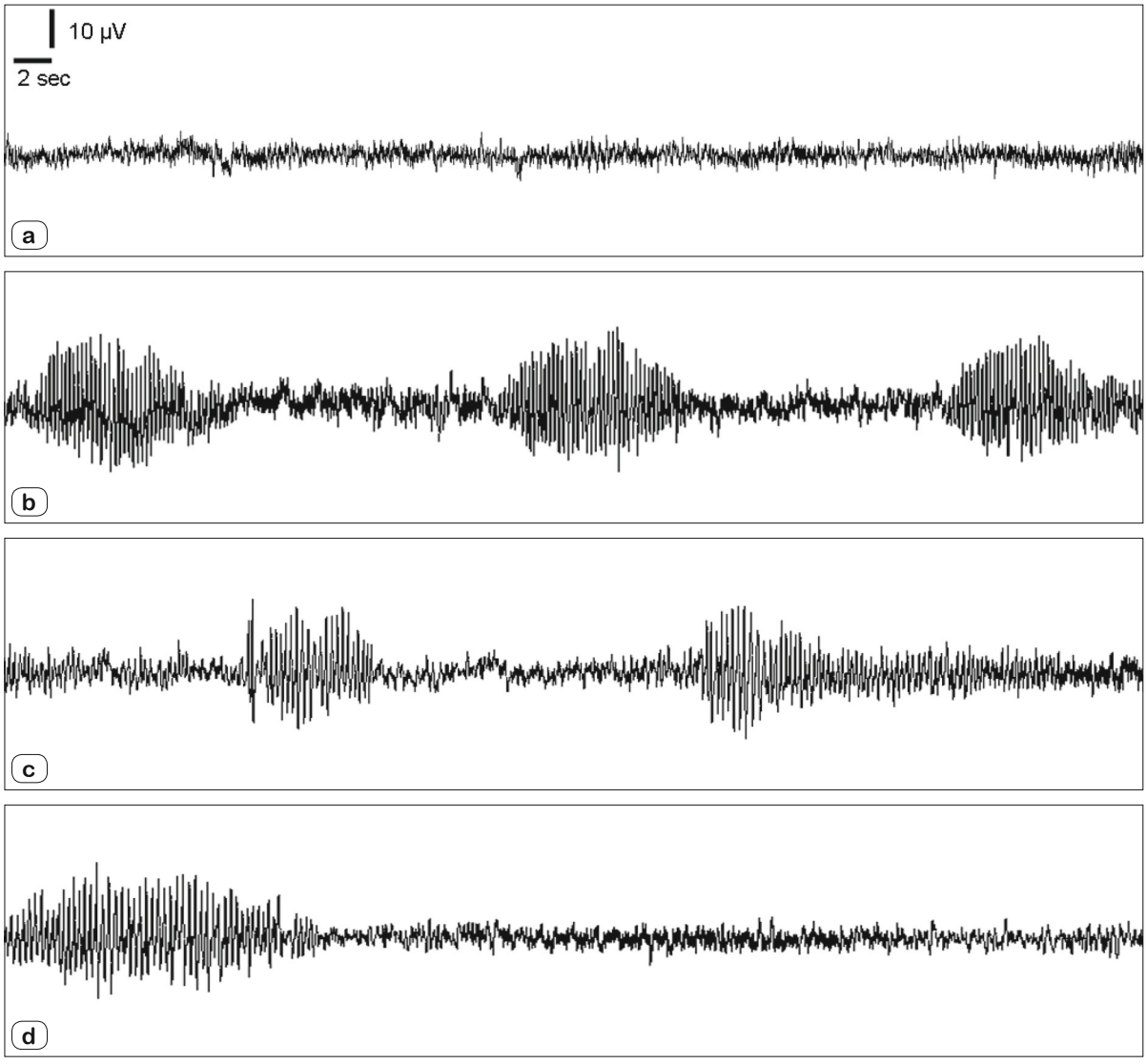

Fig. 1. EEG recordings of groups; (a): A1, (b): A2 (c): A3 (d): A4.

phosphate-buffered saline for tissue analysis (pH, 7.4) and centrifuged at 5,000 g for 15 min. After collecting the supernatant, the total protein content in the brain samples was measured using Bradford's technique(13). Using ELISA kits, the brain levels of HSP-70 in the tissue supernatants were determined. According to the manufacturer's instructions, all samples from each animal were measured twice. The absorbances were determined using MultiscanGo, Thermo Fisher Scientific Laboratory Equipment, NH, US brand microplate reader.

Statistical analysis

The data were presented as a mean with a standard error of the mean (SEM). SPSS version 15.0 for Windows was used to conduct the data analysis. The Shapiro-Wilk test is used to see if the values in a population have a normal distribution. The Kruskal-Wallis test was used to assess the Racine convulsion scores, and one-way ANOVA analysis of variance was used to assess the first myoclonic jerk (FMJ) time. To distinguish between the experimental groups, the *post hoc* Bonferonni test and the Mann-Whitney U test were used. Statistical significance was defined as a value of $p < 0.05$.

Results

Spike percentage analysis of the groups

The spike percentages were considerably lower in the PTZ (35 mg/kg) and 50 or 100 mg/kg zinc-treated groups (A3 and A4) than in the PTZ (35 mg/kg) and saline-treated group (A2; $p < 0.001$) (Fig. 1, Tab. 1). The mean spike % scores were lower in the PTZ (70 mg/kg) and 100 mg/kg zinc-treated groups (A4) than in the PTZ (70 mg/kg) and 50 mg/kg zinc-treated group (A3; $p = .005$).

Racine convulsion stage and FMJ onset time analysis of the groups

When the PTZ (70 mg/kg) and zinc sulfate-administered groups (B3 and B4) were compared to the PTZ (70 mg/kg) group (B2), the zinc-administered groups had a substantially reduced RCS ($p < 0.001$) (Tab. 2).

The onset time of FMJ was substantially longer in the PTZ (70 mg/kg) groups and 50 mg/kg or 100 mg/kg zinc sulfate groups (B3 and B4) than in the PTZ (70 mg/kg) and saline group (B2; $p < 0.001$) (Tab. 2).

Evaluation of groups in terms of MDA levels

We discovered that malondialdehyde (MDA) levels were considerably lower in the zinc-treated groups. In comparison to the group B2, which had a mean MDA of 105.7 nmol/g, the mean MDA level in group B3 with the reduced zinc dosage fell to the value of 54.9 nmol/g ($p < 0.0001$) (Tab. 3). The mean MDA level in group B4 with the increased zinc dosage dropped to the value of 43.7 nmol/g ($p < 0.0001$) (Tab. 3).

Brain SOD levels were higher in B2 group than in B1 ($p < 0.01$) (Tab. 3) When zinc treatment was applied SOD levels increased significantly;` group B2 was compared to B3 and B4 groups ($p < 0.01$) (Tab. 3).

Also, HSP-70 levels increased in a dose-dependent manner with zinc; group B2 was compared to B3 and B4 groups ($p < 0.01$, $p < 0.001$) (Tab. 3).

Tab. 1. Spike percentage values.

Drugs group	Spike percentage
A1-Control	0
A2-PTZ (35 mg/kg) and saline	70.1±5.8
A3-PTZ (35 mg/kg) and 50 mg/kg zinc sulfate	35.2±4.3 *
A4-PTZ (35 mg/kg) and 100 mg/kg zinc sulfate	24.1±7.2 *

* $p < 0.001$, A3 and A4 groups compared to A2.

Tab. 2. RCS and FMJ onset time statistics.

Drugs group	Convulsion stage (Racine)	FMJ onset time (sec)
B1-Control	0	0
B2-PTZ (70 mg/kg) and saline	5.3±0.2	58.6±10.1
B3-PTZ (70 mg/kg) and 50 mg/kg zinc sulfate	2.8±0.4*	146.3±21.8*
B4-PTZ (70 mg/kg) and 100 mg/kg zinc sulfate	1.6±0.9*	261.2±35.04*

* $p < 0.001$, B3 and B4 compared to B2.

Tab. 3. Data were expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA test.

Drugs group	Brain MDA level (nmol/g)	Brain SOD levels (U/mg protein)	HSP-70 (mcg/mg protein)
B1-Control	54.7±6.3	0.021±0.03	12.6±3.3
B2-PTZ (70 mg/kg) and saline	105.7±5.5##	0.045±0.01#	15.2±4.7
B3-PTZ (70 mg/kg) and 50 mg/kg zinc sulfate	54.9±2.6**	0.1±0.05*	25.3±2.8*
B4-PTZ (70 mg/kg) and 100 mg/kg zinc sulfate	43.7±5.1**	0. 17±0.03*	37.9±6.5**

# $p < 0.01$, ## $p < 0.001$ (different from control group), * $p < 0.01$, ** $p < 0.0001$ (different from saline-treated PTZ group)

Discussion

Since epilepsy is a troubling disease that cannot be controlled with appropriate treatment in 1/3 of patients all over the globe (3). In general, drugs are being used to get rid of the symptoms. The pathophysiology behind epileptogenesis needs to be studied furthermore.

According to clinical and experimental findigs, neuroinflammation with increased pro-inflammatory cytokines causes brain hyper-excitability, which is responsible for the seizure onset (9, 14, 15) Studies have shown that IL-1 beta causes hyperexcitability, seizure-evoked excitotoxicity, and in long term, structural and functional changes in glial and neuronal networks (16).

Zinc is the second most abundant trace metal (after iron) and the highest levels of zinc in the brain are in the hippocampus (7), the most relatable studied area in association with epilepsy (8). Zn^{2+} does not play a brain-wide role as a modulator at excitatory synapses. As a result, its effect must be limited to certain neural networks (17).

In this study, our conclusions suggest that intraperitoneal zinc injection benefits the rats significantly in behavioral (Racine convulsion stage and first myoclonic jerk onset time values) and electroencephalograph findings. In a clinical study in India, 38 children with simple febrile seizures were tested and seen to have lower zinc levels in the bloodstream (18). Another study has shown that when pretreated with zinc chelators, the seizure severity has grown in murine PTZ seizure model (19). These findings correlate with our study.

MDA, a result of lipid peroxidation, damages the membrane structure of cells while producing swelling and necrosis. SOD is a vital antioxidant enzyme in the human body. SOD converts superoxide radicals to hydrogen peroxide, which is then degraded further by CAT into water (20). HSP-70 proteins have been demonstrated to protect against Parkinson's disease by controlling α-syn misfolding, oligomerization, and aggregation (21). In our study, we measured MDA, SOD and HSP-70 levels together, so the zinc's neuroprotective effect can be seen as more relatable. Our experimental results showed the rat brain MDA levels to have been lowered statistically with intraperitoneal zinc injection. Brain superoxide dismutase activities and HSP-70 protein levels in rats expanded radically in the groups with zinc treatment. Thus our results also suggest the probability of anti-inflammatory and antioxidant effects of zinc. One study has shown that zinc deficiency is associated with impaired spermatogenesis in the murine model because of increased oxidative stress and apoptosis (22) Another newly published study showed that the MDA measurments in a group with zinc glycinate addition to diet were lower as compared to the control group (23). In another study, malondialdehyde (MDA) levels were lowest in the Zn diet-fed groups of soft-shell turtles. With increasing dietary Zn content, alkaline phosphatase activity (AKP), superoxide

anion (O_2^-), lysozyme activity, and total antioxidant capacity (T-AOC) was enhanced (24). The zinc deficiency may contribute to chronic inflammation in patients (25). Two cohort studies have suggested that zinc intake could lower the type 2 diabetes risk in humans (26, 27). These studies also suggest anti-inflammatory and antioxidant effects of zinc parallel to our results.

In conclusion, our findings indicate that zinc has the potential to exhibit antiepileptogenic effects by alleviating acute oxidative stress and neuroinflammation in a rat PTZ-induced epilepsy model. Zinc (50 and 100 mg/kg i.p.) successfully decreased the spike percentages and RCS associated with PTZ kindling epilepsy, as well as considerably decreased MDA and increased SOD and HSP-70 levels in rat brain. According to these results, zinc sulfate may be used as an adjuvant therapy in combination with other antiepileptic drugs in the future. Nonetheless, further research is required to determine the efficacy of zinc sulfate as an antiepileptic medication or as an adjuvant treatment for epilepsy.

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