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ORIGINAL ARTICLE

Polypharmacy with tiagabine, levetiracetam, and perampanel in status epilepticus: Insights from EEG, biochemical, and histopathological studies in rats

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Abstract

Objective: Status epilepticus (SE) is a condition of neurological emergency, which precipitates various functional and morphological changes in the brain. Due to the risk of drug resistance associated with SE, this study aimed to evaluate a multitargeted approach to treat SE by combining clinically used antiseizure drugs.

Methods: In this study, we intraperitoneally administered tiagabine (TGB), levetiracetam (LEV), and perampanel (PER) alone and in combination as a duo and trio therapy after 30 min of SE in electrode-implanted male Sprague–Dawley rats subjected to lithium–pilocarpine-induced convulsive SE. The rats were monitored for SE-associated behavioral and electroencephalographic (EEG) changes. Moreover, at the end of the experiment, rats were sacrificed and brains were excised for biochemical and histopathological evaluation.

Results: The control rats showed behavioral progression to the seizure of Stages 4–5 with 30–40 min of pilocarpine administration along with the appearance of uninterrupted fully blown epileptic spikes on EEG noted up to 2 h. The rats treated with TGB, LEV, and PER alone failed to provide behavioral and ictal attenuation. However, when combinations were tested, there was an improvement in seizure presentation while TGB + PER and LEV + PER also reversed SE-associated electrographic changes. However, the most prominent seizure attenuation was noted in rats receiving trio therapy with TGB, LEV, and PER. Moreover, the trio-treated rats demonstrated marked protection from SE-induced oxidative stress and morphological alterations in different regions of the brains.

Significance: We observed that intraperitoneal administration of TGB, LEV, and PER alone did not significantly alter the ictal activity recorded by EEG but pharmacological manipulation of acutely coadministered drugs caused a reduction of electrographic, biochemical, and histopathological eruptions

providing preclinical evidence of a novel multitargeted combination treatment to ameliorate the acute SE.

Plain Language Summary: This study investigates and compares the efficacy of mono- and polytherapy approach to counter the behavioral, electrographic, and histopathlogical manifestations of status epilepticus. The tiagabine as monotherapy was administered after 30 min of uninterrupted SE, and the outcomes were compared with levetiracetam and perampanel alone as well as their duo and trio combinations. We noted that combining the low doses of tiagabine, levetiracetam, and perampanel notably interrupted the seizure progression through distinct mechanism in rat model of status epilepticus. Thus, we conclude that this novel combination may be a promising multitargeted approach for management of status epilepticus.

KEYWORDS

EEG, pilocarpine, polytherapy, status epilepticus, tiagabine

1 | INTRODUCTION

Generalized convulsive status epilepticus (SE) is a life-threatening medical emergency that if remains untreated leads to an increased risk of mortality in epileptic patients.[1] SE is characterized by continuous seizures lasting over 30 min, or intermittently occurring repeated seizures without recovering consciousness.[2] The repetitive seizures occurring during SE lead to damage to various brain regions, especially limbic structures and the hippocampus. 30–40% of patients fail to respond to traditional antiseizure drugs (ASDs), leaving them at high risk of mortality[3] and management of SE relies on intravenously administered benzodiazepines to potentiate the GABAergic neurotransmission.[4] Among important variables influencing the effectiveness of drugs is the duration of SE as animal studies suggest that prolonged SE (30–50 min) results in reduced efficacy of benzodiazepines[5,6] as prolonged SE results in the internalization of GABA receptors while the concentration of glutamate receptors is increased at synapses.[7] Thus, the pathophysiology of SE and resistance to benzodiazepines after prolonged SE suggest polytherapy with the drugs targeting distinct mechanisms of action. The limited development of newer ASDs requires the researchers to utilize the early therapy comprising the combination of already available ASDs with different mechanisms to manage the SE.[8]

Pilocarpine works as a muscarinic agonist and its administration results in the development of SE presented as generalized convulsions with uninterrupted electrical activity in brain.[9] Pilocarpine-induced SE is a widely used laboratory method and involves the stereotyped development of uninterrupted seizures for hours resulting in neuronal injury in an animal's hippocampus and limbic structures.[10] Being highly isomorphic with human disease, pilocarpine-induced SE is a broadly accepted laboratory model as induction of SE is very rapid in comparison with intraperitoneally administered kainic acid.[11,12]

Tiagabine (TGB) is a GABA-modulating drug that blocks transporter type-1 (GAT-1). Halonen et al. reported that TGB 50 mg/kg/day showed promising outcomes as seizure severity and associated hippocampal damage were reduced in rats with SE.[13] Walton and colleagues reported that administration of TGB in rats with SE worked effectively in managing the seizures at the dose of 8.3 mg/kg.[14] This study aimed to evaluate

Key points

- Treatment with low-dose tiagabine (TGB), levetiracetam (LEV), and perampanel (PER) as monotherapy proved insufficient to provide behavioral and ictal attenuation in experimental model of status epilepticus.
- Combining the low doses of TGB, LEV, and PER prominently caused seizure attenuation and reversal of SE-associated electrographic changes.
- The drug combination markedly protected the rats from SE-induced oxidative stress and morphological alterations in CA1 and DG regions of hippocampus.

the comparison of TGB with LEV and PER alone and in combinations as duo and trio therapy in pilocarpine-induced SE. As literature reports that prolonged SE precipitates pharmcoresistance, this study hypothesized that multitargeting by combining the TGB, LEV, and PER might prove effective in dealing with monotherapy-resistant SE and ameliorate electrographic changes and hippocampal damage.

2 | MATERIALS AND METHODS

2.1 | Animals and their housing

In this study, 6- to 8-week-old male Sprague–Dawley rats weighing 150–200 g were used. The rats were obtained from the animal house situated at Faculty of Pharmacy, Bahauddin Zakariya University, Multan. The rats were locally bred and kept in a well-regulated environment with 25°C temperature, 12-h light/dark cycle and provided with water and standard rodent chow. The experimental procedures were permitted by Ethical Committee (03/PHDL/2018) of the Department of Pharmacology, B.Z. University, Multan and were under instructions from the "Institute of Laboratory Animal Resources" (ILAR), Commission on Life Sciences, National Research Council (NRC, 1996).

2.2 | Drugs and chemicals

Tiagabine (TGB) was procured from BOC Sciences USA and used at a dose of 6 mg/kg after dissolving the drug in normal saline.[15] Levetiracetam of 99% purity was obtained from Hilton Pharma, Pakistan, and used at a dose of 400 mg/kg after dissolving the drug in distilled water.[16] Fycompa® containing PER (Eisai. Co. Ltd.) was suspended in 1% Tween 80 to be used at 5 mg/kg.[17,18] Pilocarpine (Pilo), lithium chloride (LiCl), and methylscopolamine bromide were obtained from Sigma Aldrich, Germany. On the day of the experiment, all drugs were freshly diluted and injected through the intraperitoneal route.

2.3 | Stereotaxic electrode implantation for EEG studies

All rats were cortically implanted with EEG electrodes. For the surgical procedure, every rat was anesthetized with chloral hydrate (400 mg/kg; i.p.).[19] After mounting in a stereotaxic frame (Stoelting Co), the required area on the head was shaved, cleaned with 70% ethanol, and a 2-cm incision was made in skin to expose the skull. To implant the tri-polar EEG electrode, the electrical drill was used to drill the holes at AP +3.5 mm; LL ±2 mm from bregma taking one screw implanted at AP −2.5 mm; L −2 mm as the reference electrode. The electrode was implanted by using the screws and fixed at its position by dental cement. Immediately after surgery, the rats were subcutaneously administered with a bolus of 0.9% normal saline to avoid dehydration. All rats were kept individually and monitored until their recovery from anesthesia and resumption of the normal feeding behavior. Before experimentation, the rats were allowed to recover for at least 1 week after electrode implantation.

2.4 | Animal grouping and EEG acquisition

A total of 54 SD rats were used in this study. The rats were divided into nine groups ($n = 6$) as sham control (Group I), SE control (Group II), TGB alone (6 mg/kg) (Group III), LEV alone (400 mg/kg) (Group IV), PER (5 mg/kg) (Group V), TGB (6 mg/kg) + LEV (400 mg/kg) (Group VI), TGB (6 mg/kg) + PER (5 mg/kg) (Group VII), LEV (400 mg/kg) + PER (5 mg/kg) (Group VIII), and TGB (6 mg/kg) + LEV (400 mg/kg) + PER (5 mg/kg) (Group IX).

All rats were intraperitoneally (i.p.) treated with lithium chloride (127 mg/kg) 18–24 h before administration of pilocarpine.[20,21] On experiment day, rats were connected to the 8-channel bioamplifiers (ADInstruments Ltd.) and an analog–digital converter (PowerLab 8/35, ADInstruments). The EEGs were recorded using a signal sampling rate of 200 Hz and bandpass filtered between 0.1 and 60 Hz[22] through LabChart Pro version 8.1.19. After 30 min of baseline recording, methyl scopolamine bromide (2 mg/kg; i.p.) was administered to prevent the peripherally exerted cholinergic effects of pilocarpine. The rats were injected with pilocarpine (30 mg/kg) and noted for seizure development including behavioral arrest, nodding of head, and wet-dog shakes continuing to rearing, jumping, and unconsciousness according to Racine scale.[23] The whole experimental scheme has been presented in Figure 1.

2.5 | Preparation of tissue homogenates for biochemical studies

Immediately after completion of EEG recording, the rats ($n = 3$) were decapitated through cervical dislocation, and brains were dissected to isolate the cortex and hippocampus. Tissues were homogenized with PBS (pH 7.4) followed by centrifugation of samples for 10 min at

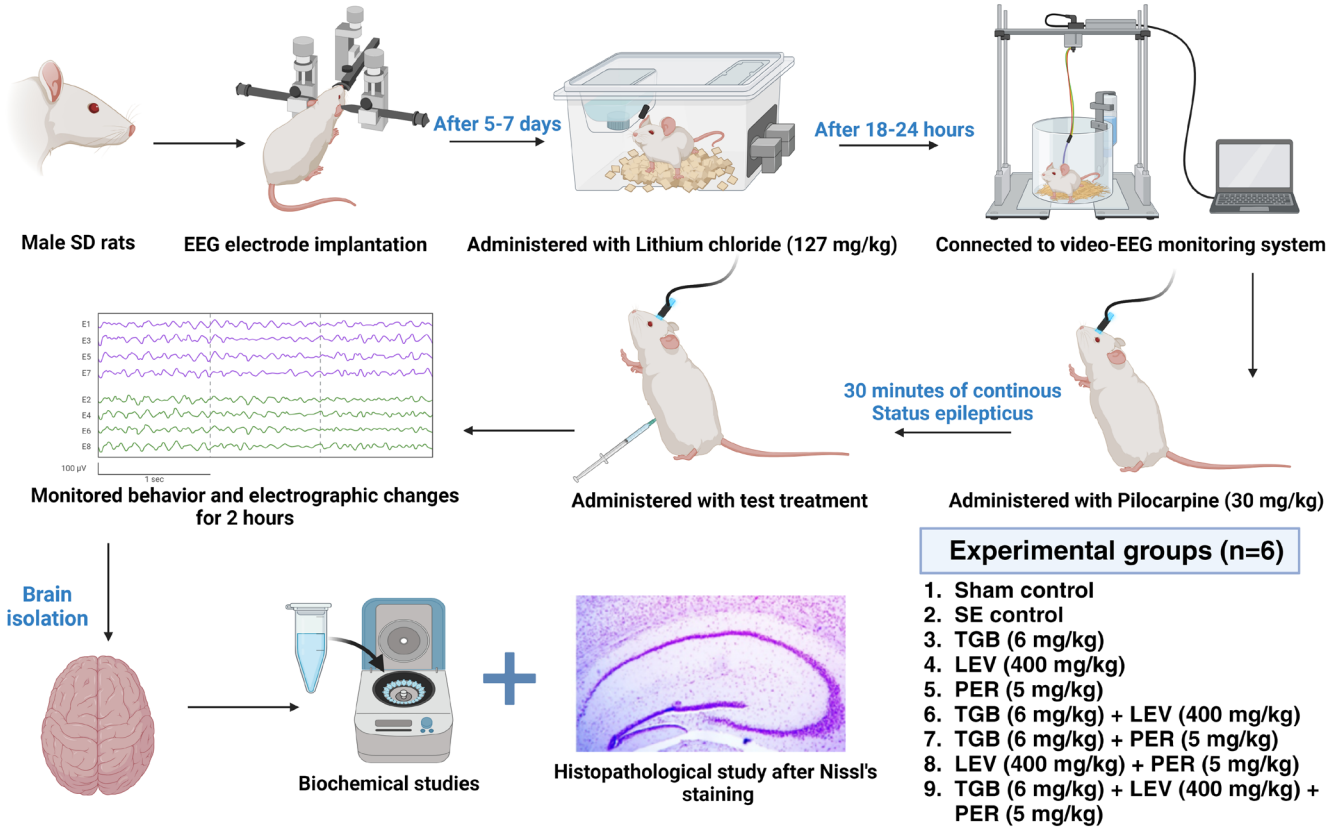

FIGURE 1 The graphical presentation of the whole experimentation scheme. The 54 rats were stereotaxically implanted with cortical electrodes and after the recovery phase were electrographically monitored to evaluate the impact of test treatments after 30 min of SE. The behavioral and electrographic monitoring was carried out for 120 min after administration of test treatments. After 120 min, the brains were dissected out for biochemical and histopathological analysis. The experimental layout has been designed using Biorender.com (HO27EHJ0FN: Dated October 9, 2024).

12 000 rpm using reduced temperature[24] and supernatant was stored at −40°C.

The prepared tissue homogenates were evaluated for malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) according to previously reported methods[25–27] (provided in supplementary information) and normalized with protein content quantified in tissues by Lowry's method.[28]

2.6 | Histopathological examination

The isolated brains ($n = 3$) were immediately fixed using 4% formalin in PBS for at least 3 days at 4°C. The paraffin blocks were prepared and 10-μm-thick sections were cut between −2.5 and −4.5 mm.[29] The processed sections were stained using 0.1% cresyl violet[30] and studied under the microscope. The histopathological changes were analyzed in cornu ammonis (CA1) and dentate gyrus (DG) regions of the hippocampus through the ImageJ software and quantification of the intact neurons was carried out at 10×.

2.7 | Statistical analysis

GraphPad Prism version 8.0 was used to evaluate the data. The Kruskal–Wallis test was used to evaluate the seizure score. After evaluation of normality of data through Shapiro–Wilk test, one-way ANOVA followed by Dunnett's test was used to evaluate biochemical and histopathological outcomes while two-way repeated measure ANOVA followed by Dunnett's test was used to evaluate electrographic parameters. The $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | Effects of TGB, LEV, and PER alone and in combination on seizure scoring

The seizure score was noted in rats after every 30 min of administration of treatment, and outcomes were compared with SE control. In detail, sham control rats

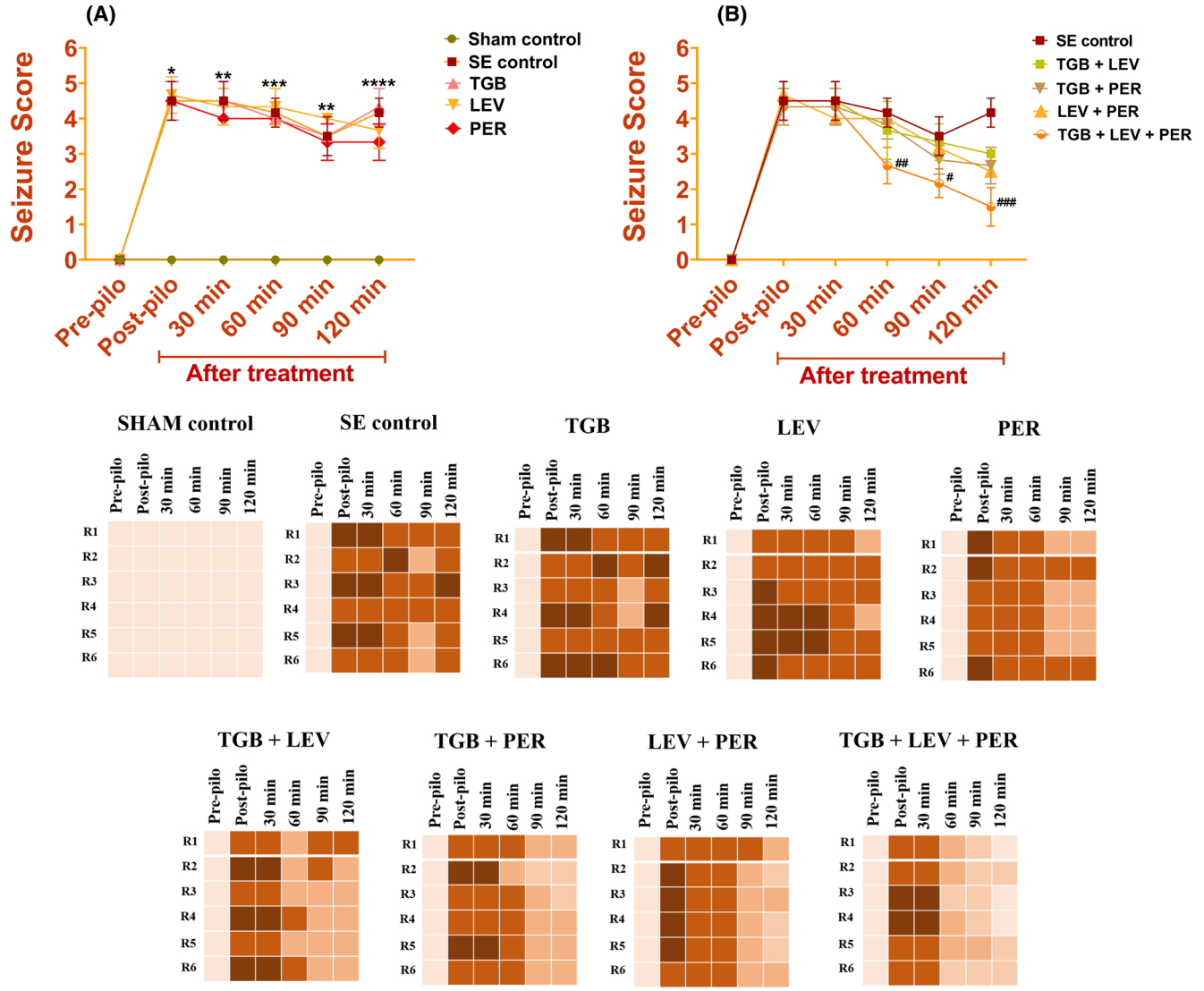

FIGURE 2 After 30 min of continuous SE, rats were administered with TGB, LEV, and PER as alone and combination to evaluate the effect of post-treatment of TGB + LEV, TGB + PER, and LEV + PER duo therapy and TGB + LEV + PER trio therapy on behavioral manifestation of SE. The rats were treated with group-wise designated treatments after 30 min of continuous SE monitored for behavioral changes for up to 2 hrs and outcomes were expressed as (A) seizure score compared among sham control, SE control, TGB, LEV, and PER treated rats and (B) seizure score noted in rats treated with TGB + LEV, TGB + PER, LEV + PER, and TGB + LEV + PER in comparison with SE control. These data have been expressed as mean ± SD ($n = 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ shows comparison between sham and SE control rats while $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ shows change in seizure score in rats receiving TGB + LEV + PER compared with SE control rats. The colors in heatmap shows severity of seizures as: .

receiving normal saline instead of pilocarpine showed normal activity without any seizure-associated behavioral impairments revealing no impact of surgical procedure on seizure development. However, when SE control rats were compared with sham control rats, the intraperitoneal injection of pilocarpine caused continuous convulsions with bilateral forelimb clonus, recurrent rearing, and falling rats on their one side with loss of righting reflex and a seizure score of 4.50 ± 0.22 ($p < 0.05$) was noted in SE control rats. When monitored every 30 min, these behavioral changes remained persistent, and the seizure score was 4.16 ± 0.16 ($p < 0.0001$) after 120 min as shown in Figure 2A.

A nonsignificant difference in seizure score was noted when TGB, LEV, and PER-treated rats were compared with SE control. The treatment with one drug after 30 min of continuous SE proved ineffective in mitigating the seizure progression as generalized convulsions with intermittent rearing were nonsignificantly different from SE control when noted after every 30 min of treatment for up to 120 min after treatment.

When TGB, LEV, and PER were administered as duo therapy, the presentation of generalized convulsions with intermittent rearing (4.50 ± 0.22) disappeared in rats treated with TGB + LEV, and the seizure score was reduced to 3.66 ± 0.33 after 60 min. Although the difference remained statistically nonsignificant in comparison with SE control, the improvement continued and the seizure score was reduced to 3.33 ± 0.21 after 90 min followed by further reduction to 3.00 ± 0.00 after 120 min. Similarly, TGB + PER rats had seizure score of 4.33 ± 0.21 after 30 min, which showed a further reduction to 3.83 ± 0.16 after 60 min, which reduced to 2.83 ± 0.40 after 90 min, and this score was eventually reduced to 2.66 ± 0.51 after 120 min of treatment. Moreover, LEV + PER treatment resulted in seizure score of 4.00 ± 0.00 after 30 and 60 min of treatment, which reduced to 3.16 ± 0.40 and 2.50 ± 0.54 after 90 and 120 min, respectively.

On administration of TGB as trio therapy with LEV and PER, rats nonsignificantly varied from SE control and presented seizures of advanced stage including bilateral forelimb clonus with rearing after 30 min of administration of TGB + LEV + PER. But, after 60 min of treatment, presentation of seizures was improved and rats were only nodding their heads with mild–moderate facial clonus, that is, 2.66 ± 0.21 ($p < 0.01$) after 60 min, and this score was further reduced to 2.16 ± 0.16 ($p < 0.05$) after 90 min of treatment. After 120 min, most of rats presented seizures with facial movements and twitching of vibrissae, and seizure score was 1.50 ± 0.22 ($p < 0.0001$) as shown in Figure 2B.

3.2 | Effects of TGB, LEV, and PER alone and in combination on EEG

The behavioral assessment of rats to analyze the impact of TGB, LEV, and PER alone and their combinations as duo and trio therapy was combined with EEG monitoring to understand the correlation of electrographic activity with convulsive severity. The electrographic activity recorded in sham control revealed no impact of cortical implantation of EEG electrode as the EEG remained qualitatively the same as basal activity.

In SE control rats, the electrographic changes began to appear on EEG within 30–35 min of pilocarpine administration. Initially, intermittent spiking activity was commenced with simultaneous cholinergic signs such as salivation and piloerection. With time, the intermittently occurring spikes were replaced with repetitive spikes of higher amplitude when rats were having facial movements and whisker trembling. Gradually, the nonstop spiking activity was transformed into paroxysmal discharges of variable amplitude while seizures progressed to bilateral tonic–clonic seizures. The SE control rats remained untreated, and the intermittently happening generalized convulsions with rearing and falling were noted, which were presented as continuous epileptiform discharges on electroencephalogram, which was notably higher in amplitude than baseline.

In rats treated with TGB and LEV alone, the convulsive activity remained consistent, and no significant impact on SE-associated electrographic alteration was obtained except a mild reduction in amplitude of epileptic discharges was observed. However, the mild reduction in amplitude was noted in PER-treated rats. In rats treated with TGB + LEV, high-voltage epileptiform spikes continued but rats treated with TGB + PER and LEV + PER showed mild protection from bilateral generalized convulsions after 90–120 min, which were in accordance with EEG changes as the amplitude of epileptiform discharges was reduced simultaneously. However, treatment of rats with TGB + LEV + PER resulted in the reversal of SE-associated electrographic abnormalities, and these improvements were markedly prominent and had reduced latency as compared to TGB + PER and LEV + PER combinations as presented in Figure 3. Moreover, the recorded EEGs were quantified to evaluate the effects of test treatments on number of spikes, amplitude of spikes and power and outcomes have been provided as Tables S1–S3.

3.3 | Biochemical analysis

The MDA levels varied significantly among all groups [$F_{8,18} = 4.38$; $p = 0.004$] as cortical tissues of SE rats had 66.94 ± 24.78 nmol of MDA/mg of protein, which was a marked elevation ($p < 0.05$) from 21.55 ± 9.08 nmol of MDA/mg of protein noted in sham control. The treatment with TGB did not protect rat brains from elevation in MDA levels. Similarly, LEV and PER alone and duo therapy with TGB + LEV, TGB + PER, and LEV + PER showed a nonsignificant difference in MDA levels in comparison with SE and TGB groups. However, MDA levels were only 25.07 ± 6.86 nmol of MDA/mg of protein in rats treated with TGB + LEV + PER ($p < 0.05$ vs. SE control; Figure 4A).

The groups varied for SOD ($F_{8,18} = 5.20$; $p = 0.0009$), catalase ($F_{8,18} = 5.76$; $p = 0.001$), and GPx ($F_{8,18} = 2.11$; $p = 0.08$) in cortical homogenates. In detail, cortical tissues of sham control rats had 3.63 ± 0.48 units of SOD/mg, 278.41 ± 39.90 μmol of catalase/min/mg of protein and 1394.06 ± 709.22 nmol of GPx/min/mg of protein. The SE rats after continuous SE and no treatment had a marked reduction in antioxidants as SOD was 1.12 ± 0.47 units/mg ($p < 0.001$; Figure 4B), catalase was 92.38 ± 22.81 μmol/min/mg ($p < 0.01$; Figure 4C), and GPx was 373.26 ± 153.62 nmol/min/mg of protein

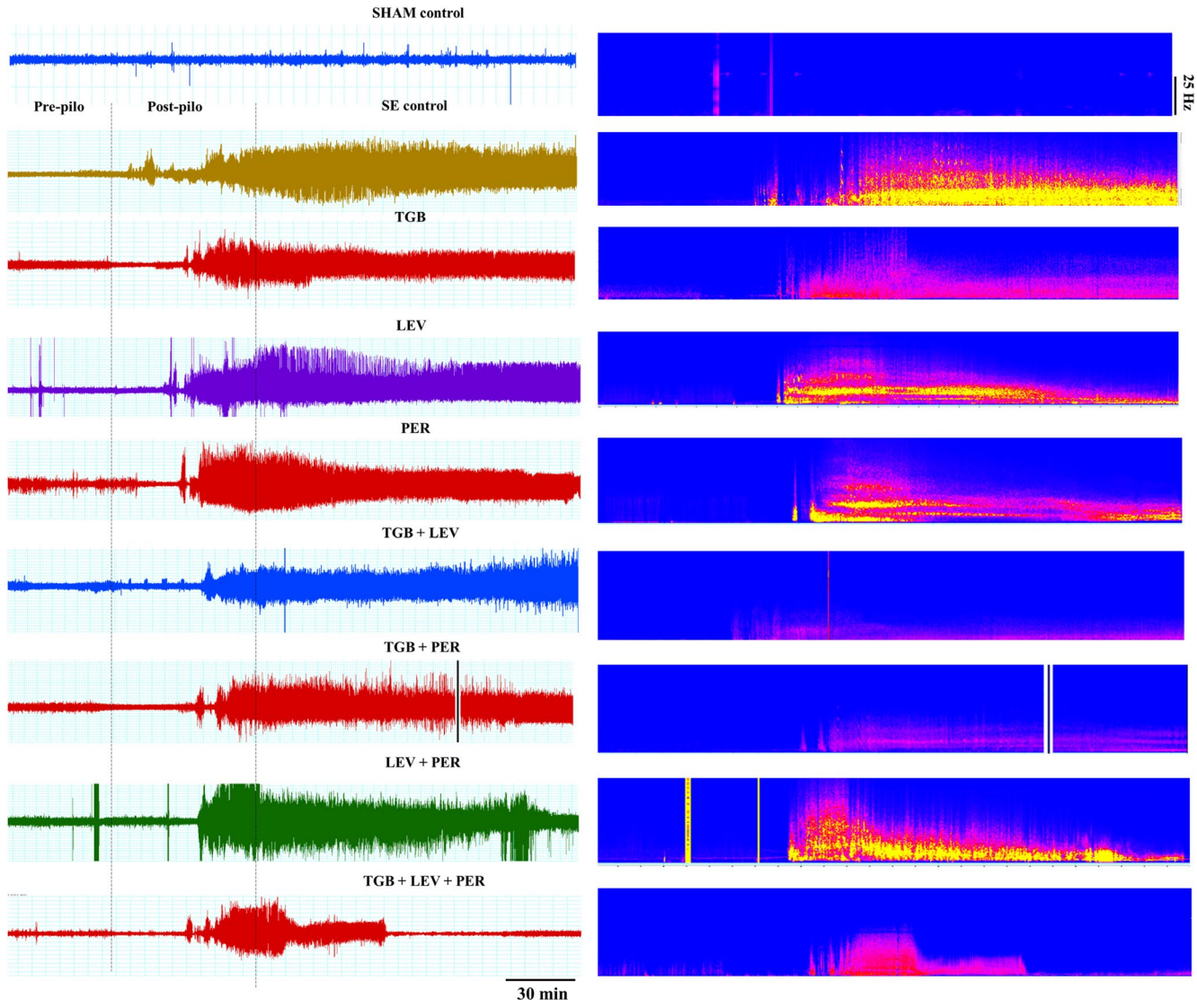

FIGURE 3 The electrode-implanted rats were mounted with EEG acquisition system to evaluate the impact of test treatments on SE-associated electrographic changes. After inducing the SE through administration of pilocarpine, the rats remained in continuous state of seizures for 30 min after which the SE was interrupted by administration of test treatments and EEG was recorded for 2 h. The laboratory chart-generated representative compressed electrogram with corresponding power spectrum has been presented revealing the impact of TGB, LEV, PER alone, and TGB + LEV, TGB + PER, LEV + PER, and TGB + LEV + PER combinations on SE-associated ictal events and epileptiform discharges.

($p < 0.05$; Figure 4D) revealing the continuous seizures caused depletion of antioxidant enzymes in cortical regions of brains. The post-SE treatment with TGB, LEV, and PER alone and TGB + LEV, TGB + PER, and LEV + PER combinations nonsignificantly protected the rats from SE-associated decline in antioxidant enzymes except catalase was $229.90 \pm 22.06\,\mu$mol/min/mg of protein ($p < 0.05$) in LEV + PER group. However, treatment with TGB + LEV + PER provided a defense as the cortex had 3.09 ± 1.46 units of SOD/mg ($p < 0.01$), $281.77 \pm 36.64\,\mu$mol of catalase/min/mg of protein ($p < 0.01$), and 1478.82 ± 228.29 nmol of GPx/min/mg of protein ($p < 0.05$).

The MDA and GPx levels were nonsignificantly different in LEV and PER alone and TGB + LEV, TGB + PER, and LEV + PER groups in comparison with TGB rats. However, SOD and CAT levels were markedly different in rats receiving TGB + LEV + PER ($p < 0.05$), in comparison with TGB alone.

Moreover, the biochemical changes were noted in isolated hippocampal tissues and a notable intergroup difference in MDA levels was noted ($F_{8,18} = 4.87$; $p = 0.002$). The MDA levels were elevated to 87.23 ± 7.50 nmol of MDA/mg of protein in hippocampus of SE control, which was a noteworthy difference ($p < 0.05$) in comparison with 34.37 ± 10.41 nmol of MDA/mg of protein

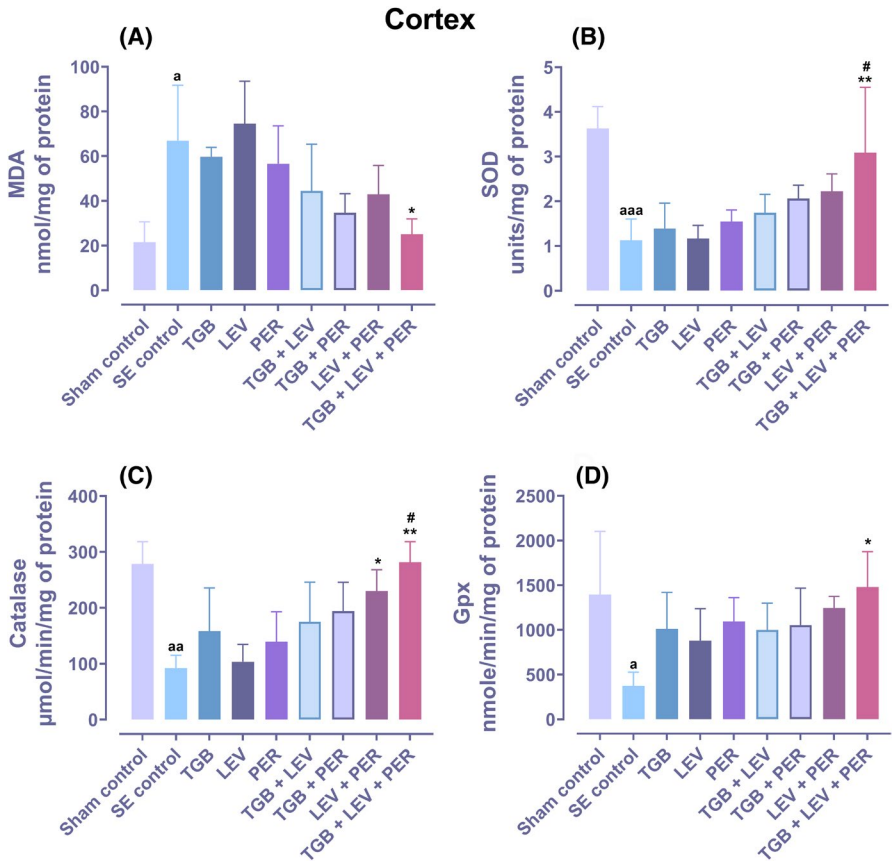

FIGURE 4 Immediately after completion of EEG monitoring for 2 h, the rats were disconnected from the EEG setup and euthanized by cervical dislocation to remove the brains and isolate the cortices. The cortical homogenates were analyzed for (A) malondialdehyde (MDA), (B) superoxide dismutase (SOD), (C) catalase, and (D) glutathione peroxidase (GPx). These data have been expressed as mean ± SD ($n = 3$). $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$ show a comparison between sham and SE controls, $^{*}p < 0.05$, $^{**}p < 0.01$, show the comparison of differently treated rats with SE control while $^{\#}p < 0.05$, shows the change in biochemical parameters in rats receiving different treatments in comparison with TGB group.

noted in sham control. The mono and duo therapy proved ineffective in controlling the seizure-associated lipid peroxidation while treatment with TGB + LEV + PER caused a reduction in MDA levels to were reduced to 25.59 ± 8.97 nmol of MDA/mg of protein ($p < 0.01$ vs. SE control and $p < 0.05$). Moreover, when MDA levels in differently treated rats were compared with TGB, no significant difference was noted in LEV and PER mono and duo therapy, but the difference was noticeable in rats treated with TGB + LEV + PER ($p < 0.05$ vs. TGB; Figure 5A).

The hippocampal levels of SOD ($F_{8,18} = 3.31$; $p = 0.01$), catalase ($F_{8,18} = 5.17$; $p = 0.001$), and GPx ($F_{8,18} = 2.90$; $p = 0.02$) were notably different among groups. The hippocampus of sham control rats had 2.74 ± 1.35 units of SOD/mg, 267.67 ± 17.74 µmol of catalase/min/mg of protein and 1061.94 ± 139.80 nmol of GPx/min/mg of protein, which were notably reduced in rats subjected to continuous SE. The uninterrupted SE reduction in SOD to 0.79 ± 0.51 units/mg ($p < 0.05$; Figure 5B), catalase to 88.62 ± 11.28 µmol/min/mg of protein ($p < 0.05$; Figure 5C) and GPx to 394.25 ± 141.18 nmol/min/mg of protein ($p < 0.05$; Figure 5D). The TGB, LEV, and PER alone and TGB + LEV, TGB + PER, and LEV + PER combinations proved ineffective in protecting the hippocampi of rats from SE-associated waning in antioxidant defenses except catalase levels were protected to 197.10 ± 18.71 ($p < 0.05$) and 199.67 ± 13.21 ($p < 0.05$) by TGB + PER and LEV + PER as compared to SE control, respectively. However, TGB + LEV + PER caused marked protection from reduced levels of antioxidant levels as 3.05 ± 1.11 units of SOD/mg ($p < 0.05$), 222.79 ± 66.45 µmol of catalase/min/mg of protein ($p < 0.01$) and 1128.82 ± 493.26 nmol of GPx/min/mg of protein ($p < 0.01$). The levels of all noted enzymes were nonsignificantly different in rats receiving mono, duo and trio treatments in comparison with TGB rats.

3.4 | Histopathological studies

When hippocampi of isolated brains were evaluated through Nissl staining, the sham rats possessed neurons with well-defined and intact cellular structures comprising Nissl bodies and quantitative analysis showed 342 ± 206 neurons in the CA1 region of hippocampus. The continuous SE caused visible histopathological changes in neurons as pyknotic nuclei, inflammatory cellular infiltration, and neuronal death were observed (Figure 6) while cell count was reduced to 80 ± 29 ($p < 0.05$). The rats treated with TGB, LEV, and PER alone and in duo combinations showed nonsignificant differences in neuronal

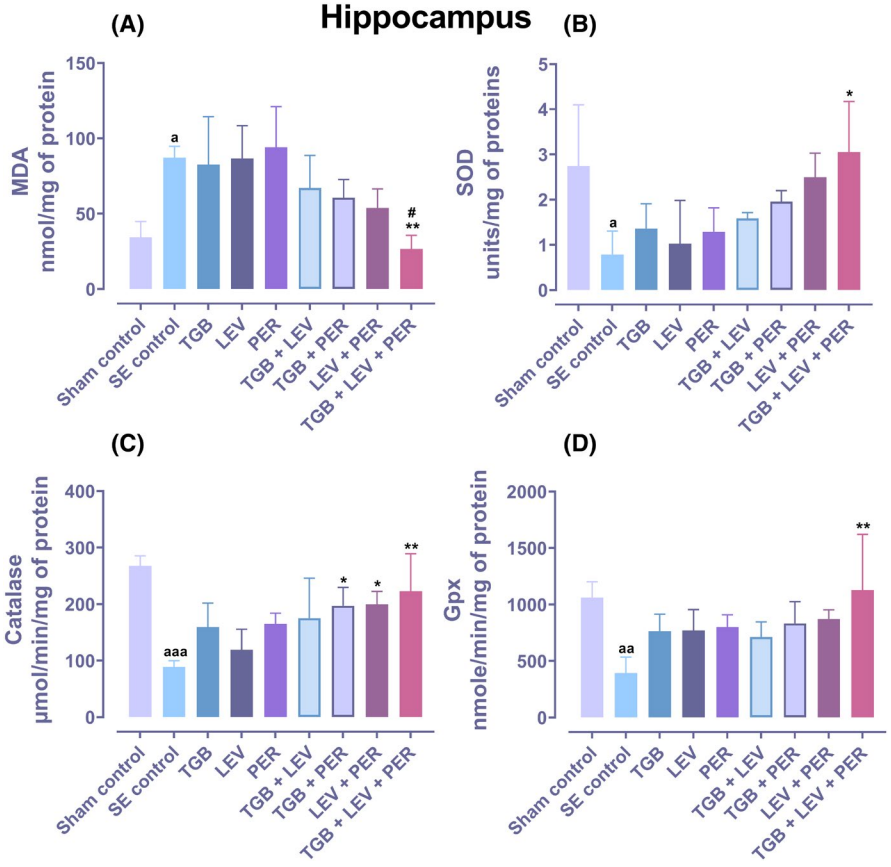

FIGURE 5 The hippocampal homogenates were analyzed for (A) malondialdehyde (MDA), (B) superoxide dismutase (SOD), (C) catalase, and (D) glutathione peroxidase (GPx). The data has been expressed as mean ± SD ($n = 3$). $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$ show a comparison between sham and SE controls, $^{*}p < 0.05$, $^{**}p < 0.01$, show the comparison of differently treated rats with SE control while $^{\#}p < 0.05$, shows the change in biochemical parameters in rats receiving different treatments in comparison with TGB group.

counts from SE control. However, the protection from SE-associated histological abnormalities and neuronal count was 320 ± 107 ($p < 0.05$) in the CA1 region of rats in which SE was interrupted by administration of TGB + LEV + PER (Figure 6A). The histopathological changes in CA1 regions of rats receiving different treatments were nonsignificantly different from TGB rats.

The granular cell layer of the DG region demonstrated 259 ± 52 neurons in sham control while a severe reduction in neuronal density to 100 ± 63 ($p < 0.01$) was noted in SE control rats. TGB, LEV, and PER alone and TGB + LEV did not work effectively in retaining the neuronal integrity while treatment with TGB + PER and LEV + PER protected the rats from neuronal damage significantly as cell count was 199 ± 27 ($p < 0.05$) and 210 ± 60 ($p < 0.05$), respectively. A further prominent protection in the DG region was noted with TGB + LEV + PER treatment as cells were remarkably preserved cellular arrangements and neuronal integrity in the granular cell layer of the DG region with the cell counts of 241 ± 19 ($p < 0.01$) as shown in Figure 6B. When outcomes were compared with TGB rats, TGB + PER and LEV + PER duo therapy caused marked protection from SE-induced damage with $p < 0.01$ and $p < 0.001$, respectively. This protection was more noticeable in rats treated with TGB + LEV + PER ($p < 0.0001$), when compared to TGB alone.

4 | DISCUSSION

Lithium–pilocarpine-induced SE is a broadly employed laboratory model to reiterate the effect of antiseizure drugs on behavioral, electrographic changes, and histopathological alterations comparable to the observations noted in patients with SE. The pathophysiological changes happening in SE recommend that treatment with combinations of antiseizure drugs is one of the key determinants associated with beneficial outcomes.[8] Despite this, there is still limited evidence on the efficacy of early polytherapy due to the scarcity of clinical studies, although it is remarkable that many preclinical studies support its implementation. In the present study, we hypothesized to use a duo and trio combination of TGB with LEV and PER and compared the outcomes noted with monotherapy with TGB.

After 30 min of continuous SE, TGB alone at a dose of 6 mg/kg did not prove sufficient to attenuate behavioral manifestations of SE and rats continued to have myoclonic jerking with recurrent presence of rearing and jumping. When SE was interrupted by administration of duo therapy with TGB + LEV and TGB + PER, the seizure with stages 4–5 vanished after 120 min of treatment but there was repeated head nodding and body jerking that did not disappear even after 2 h. of drug administration. However, on coadministration of TGB, LEV, and

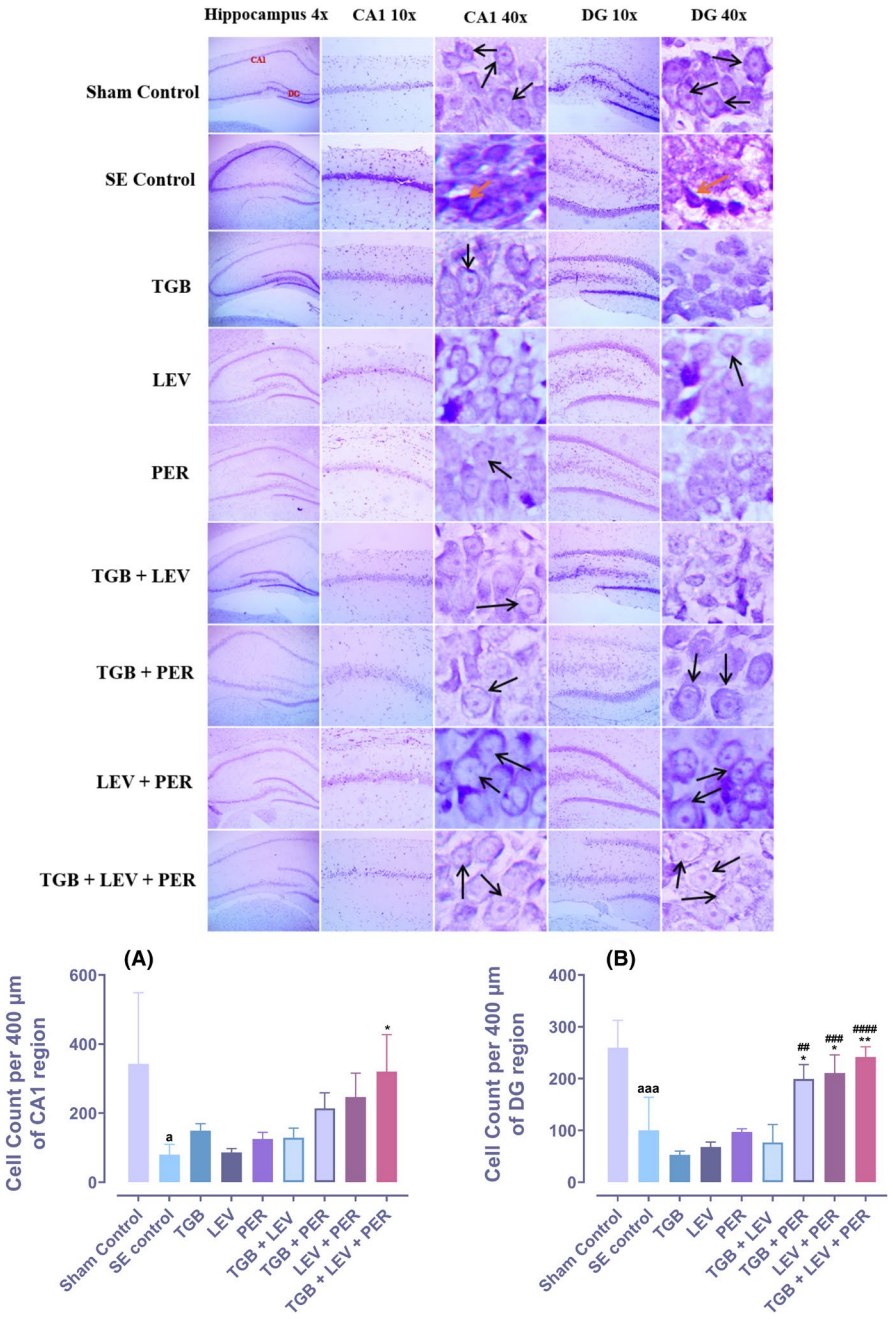

FIGURE 6 To evaluate the impact of TGB, LEV, and PER alone and in combination on continuous SE-induced histopathological changes, rats ($n = 3$) were decapitated and whole brains were isolated and stained with Nissl stain. The cells were intact with well-defined cellular structures in CA1 and DG regions of sham control rats while prolonged SE resulted in increased signs of necrosis, cellular penetrations and apoptosis in hippocampus. To quantify the cellular damage (marked with red arrows), the healthy and intact neurons (marked with black arrows) were counted in (A) CA1 (magnified at 10×) and (B) DG regions (magnified at 10×). These data have been expressed as mean ± SD ($n = 3$). $^{a}p < 0.05$, $^{aaa}p < 0.001$ show comparison between sham and SE controls, $^{*}p < 0.05$, $^{**}p < 0.01$, show comparison of differently treated rats with SE control while $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, shows histopathological changes in rats receiving different treatments in comparison with TGB group.

PER cocktail, seizures with rearing disappeared after 60 min, which was an earlier response. Further, these rats recovered from full-body myoclonic jerking and returned to seizure with head nodding only after 90 min of treatment. These behavioral changes were following electrographic displays observed in electrode-implanted rats. The EEG recorded from the cortex of rats showed that TGB alone mildly affected the SE-induced electrographic changes while TGB + LEV showed an apparent but statistically nonsignificant reduction in the amplitude of spikes but the ictal bursts reappeared with higher amplitude. However, the TGB + PER worked better in comparison with TGB + LEV. However, after coadministration of TGB, LEV, and PER, a marked reduction in electrographic parameters was noted.

GABA-modulating drugs specifically benzodiazepines (BZDs) enhance GABA-mediated neuro-inhibition and are considered as first-line treatment for SE. However, there is ample evidence suggesting that the effectivity of BZDs is negatively affected by the duration of SE. Goodkin and Kapur[31] reported that 35% of SE patients with SE become unresponsive to BZDs if treatment is not started shortly after the onset of SE. In a previous study on rats, the dose of diazepam stopping seizures after 5 min started to show reduced efficacy after 15 min and became ineffective after continuous seizures of 60 min.[32]

The prolonged seizures cause receptor modulation as endocytosis of GABAergic receptors happens with upregulation of glutamate receptors in the synapse.[33] This might be the reason that TGB alone did not ameliorate behavioral and electrographic alteration occurring after 30 min of SE.

Levetiracetam works as an antiseizure drug by binding to the synaptic vesicle protein SV2A[34] resulting in modulation of neurotransmitter release. Moreover, it has been reported to inhibit neuronal hypersynchronization, *N*-type Ca^{+2} channels, and upregulation of BDNF.[35] In the present study, LEV at a dose of 400 mg/kg when administered with TGB as post-treatment did not prove sufficient to stop SE and associated electrographic, biochemical, and histopathological deteriorations. In a previous study using lithium–pilocarpine model of SE, authors reported that LEV at a dose of 800 mg/kg or more was required to be intravenously administered after 30 min of SE attenuating the transient ictal behavior in rats.[36]

AMPA receptor antagonists work as broad-spectrum antiseizure drugs.[37] PER is a new antiseizure drug that works through its noncompetitive AMPA antagonizing potential. In our study, TGB when coadministered with PER after 30 min of SE, the rats showed improvements in behavioral outcomes and SE-induced electrographic and histopathological alterations were significantly reversed. During SE, increased release of glutamate leads to excessive stimulation of glutamate receptors present postsynaptically, which causes increased intracellular Ca^{2+} influx causing cell death. Efficient inhibition of Ca^{2+} permeability by PER prevents neuronal damage in rodents during seizures.[38] In a study by Wu et al.,[39] PER exerted neuroprotection in 100% of diazepam-resistant rats after intravenous administration of drug at 6 mg/kg, diazepam-resistant SE.

In the present study, the prolonged SE was associated with increased lipid peroxidation and reduced antioxidant enzymes in isolated cortices and hippocampi and these findings were in accordance with Liu et al.[40] who noted increased levels of hippocampal MDA and nitric oxide in the hippocampus within 2 h of SE. Freitas et al.[41] also reported a rise in lipid peroxidation and a reduction in glutathione levels in different areas of the brain after pilocarpine-induced SE. In the rats in which SE was interrupted by administration of the TGB + LEV + PER cocktail, significant protection from SE-associated elevated oxidative stress was noted. The high proportion of polyunsaturated fatty acids present in the brain makes it more vulnerable to peroxidation affects the integrity and permeability of BBB, which further precipitates various other neurological disorders.[42]

The histopathological analysis of excised brains showed a marked reduction in neuronal population and increased neuronal necrosis in CA1 and DG regions of untreated SE control rats, and these findings are supported by previous literature.[43] In present study, TGB alone at a dose of 6 mg/kg did not attenuate seizures and associated morphological changes, and these findings are supported by a previous study involving perforant pathway stimulation model of SE in which TGB reduced seizure severity and loss of pyramidal cells in CAI and CA3 regions of hippocampus when used at the dose of 50–200 mg/kg[13]. However, when we coadministered with PER, the neuroprotection provided by TGB + PER varied by brain region as partial reduction of neuronal loss in DG region was noted. These findings are correlated with a study revealing that PER prevented neuronal loss in MD region more than CA1 area of hippocampus.[39]

5 | CONCLUSION

This study describes the improvement of behavioral and electrographic deteriorations when experimentally induced SE was interrupted by coadministration of TGB with LEV and PER. In addition, the administration of this trio cocktail noticeably attenuated biochemical and histopathological alterations in the brain. However, among duo combinations used in the present study, TGB + PER and LEV + PER were found beneficial than TGB + LEV combination at used doses. However, the current study was carried out using a single dose of TGB, LEV, and PER, which might be insufficient to predict the type of interaction among these drugs thus further preclinical studies must be carried out in the future to provide the clinical benefits of the current novel combination of drugs.

AUTHOR CONTRIBUTIONS

Experiments were performed by Sana Javaid, Abida Parveen, and Zohabia Rehman. Experiments were designed by Faleh Alqahtani, Waseem Ashraf, Syed Muhammad Muneeb Anjum, and Imran Imran. Sana Javaid, Abida Parveen, Waseem Ashraf, and Tanveer Ahmad analyzed the data. Sana Javaid, Abida Parveen, Waseem Ashraf, and Imran Imran wrote the paper, with assistance from Tanveer Ahmad, Faleh Alqahtani, and Zohabia Rehman in editing and revisions. Faleh Alqahtani, Syed Muhammad Muneeb Anjum, and Imran Imran supervised the work.

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CONFLICT OF INTEREST STATEMENT

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

DATA AVAILABILITY STATEMENT

The data presented in this study are available upon request from the corresponding authors.

ETHICS STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. This study was approved by Departmental Ethical Committee (03/PHDL/2018).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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