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RESEARCH

Neurobehavioral effects of gamma-glutamylcysteine ethyl ester on the rats treated with kainic acid

Kainik asit ile indüklenen sıçan epilepsi modelinde gama-glutamil sisteinil etil ester'in nörodavranışsal etkileri

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Abstract

Purpose: This study aims to determine the effect of gamma-glutamylcysteine ethyl ester (GCEE) on the levels of glutathione (GSH) and brain-derived neurotrophic factor (BDNF) mRNA levels in hippocampal and cortical tissues, and changes in seizure severity, anxiety and learning performance against kainic acid (KA)-induced rat model of status epilepticus.

Materials and Methods: Male Sprague Dawley rats were divided into 4 groups: control (saline), KA only (10mg/kg), GCEE only (10 mg/kg), and KA (10mg/kg) + GCEE (10mg/kg). Saline, KA and GCEE were administered to the rats intraperitoneally (i.p.). Seizure behaviors were assessed according to the Racine Scale. Learning and memory were evaluated with the Morris Water Maze (MWM), and anxiety with the Open Field (OFT) and Elevated Plus Maze (EPM) tests. The levels of GSH and BDNF mRNA in hippocampus and cortex were determined spectrophotometrically, and reverse transcription polymerase chain reaction (RT-PCR) followed real-time polymerase chain reaction (Real-time PCR), respectively.

Results: While GCEE treatments significantly reduced the severity of seizures and improved MWM retrieval performance deficiencies during the probing trial, KA treatment did not significantly change anxious behavior. Conversely, GCEE treatment against KA markedly raised GSH levels in the brain.

Conclusion: These results may reflect GCEE's potential to improve cognitive function by reducing seizure severity, along with its ability to increase GSH levels in the cerebral cortex against KA-induced status epilepticus.

Keywords: Kainic acid, neurobehavior, gamma-glutamylcysteine ethyl ester, glutathione, brain-derived neurotrophic factor

Öz

Amaç: Bu çalışma, gama-glutamil sisteinil etil ester (GCEE) uygulamasının hipokampal ve kortikal dokularda glutatyon (GSH) ve BDNF mRNA düzeyleri üzerindeki etkisini ve kainik asit (KA) ile indüklenen status epileptikus sıçan modelinde nöbet şiddeti, anksiyete ve öğrenme performansındaki değişiklikleri belirlemeyi amaçlamaktadır.

Gereç ve Yöntem: Çalışmada yetişkin erkek Sprague Dawley sıçanlar; kontrol (salin), sadece GCEE (10 mg/kg) , sadece KA (10 mg/kg) ve KA (10 mg/kg) + GCEE (10 mg/kg) olmak üzere dört gruba ayrılmıştır. Salin, KA ve GCEE deney hayvanlarına intraperitoneal (i.p.) olarak uygulanmıştır. Nöbet şiddeti Racine skalasına göre değerlendirilmiştir. Öğrenme ve bellek performansı Morris Su Labirenti (MWM) testi ve anksiyete düzeyleri Açık Alan Testi (OFT) ile Yükseltilmiş Artı Labirent (EPM) testi kullanılarak ölçülmüştür. Korteks ve hipokampus dokularında GSH ve BDNF mRNA ekspresyon düzeyleri sırasıyla spektrofotometrik ve RT-PCR'ı takiben gerçek zamanlı PCR (Real-time PCR) yöntemleriyle belirlenmiştir.

Bulgular: GCEE uygulaması nöbet şiddetini anlamlı düzeyde hafifletirken ve prob denemesi sırasında MWM hatırlama performansındaki bozuklukları önemli ölçüde iyileştirmiştir. KA uygulamasıyla tetiklenen anksiyete davranışında anlamlı bir değişiklik oluşturmadığını göstermiştir. KA'ya karşı GCEE tedavisinin beyinde GSH düzeylerini belirgin biçimde artırdığı saptanmıştır.

Sonuç: Bu sonuçlar, GCEE'nin KA'nın neden olduğu status epileptikusta serebral kortekste GSH düzeylerini artırabilmesinin yanı sıra, nöbet şiddetini azaltarak bilişsel işlevleri iyileştirme potansiyelini yansıtıyor olabilir.

Anahtar kelimeler: Kainik asit, nörodavranış, gama-glutamil sistein etil ester, glutatyon, beyin kaynaklı nörotrofik faktör

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INTRODUCTION

Epilepsy affects over 50 million people worldwide and is a major public health issue due to its chronic nature and associated comorbidities[1]. The repetitive seizures inherent to this condition are linked with neurobiological, cognitive, psychological, and social consequences, predisposing individuals to further seizures. Although epilepsy affects both sexes, its prevalence and incidence are higher among males[2]. Antiepileptic drugs control seizures but do not modify long-term progression. Approximately 30% of patients are refractory to medical treatment, leading to progressive cognitive impairment. In addition, quality of life, morbidity, and mortality are significantly impacted, especially in the presence of intractable seizures[3].

Status epilepticus, considered the most severe form of seizure, accounts for most epilepsy-related deaths. In 2015, the International League Against Epilepsy (ILAE) defined status epilepticus as a condition caused by the failure of mechanisms that terminate seizures or by processes that trigger prolonged seizures. This results in long-term changes such as neuronal damage, death, and alterations in neuronal connections, depending on the type and duration of seizures[4,5].

Neuronal hyperactivity and excitotoxicity in brain regions are marked by increased levels of free radicals[6]. The brain, with its vulnerable antioxidant defense mechanisms, is susceptible to neurodegeneration due to the long-term increase in reactive oxygen species (ROS) generated during epileptic seizures[7].

Recurrent seizure activity may trigger neurodegenerative alterations that contribute to the development of diverse neurobehavioral comorbidities, including cognitive impairment, anxiety, depression, schizophrenia, and autism spectrum disorder[8].

Glutathione (GSH) is an endogenous antioxidant molecule. GSH has the capacity to directly inhibit the production and/or activation of ROS in all cells. In neurodegenerative processes, GSH levels decrease and ROS increases[9]. In this context, approaches aimed at increasing cellular GSH levels in neurodegenerative conditions are important. However, these approaches may not be successful in crossing the blood-brain barrier. Gamma-glutamylcysteine ethyl ester (GCEE) is a synthetic substance that provides feedback support in overcoming a rate-limiting step in cellular GSH synthesis, ensuring the continuity of GSH synthesis. Furthermore, due to its ethyl ester structure, GCEE is an antioxidant capable of crossing the blood–brain barrier[10,11].

The protective effects of GCEE against oxidative stress have been demonstrated in bronchial asthma and cataract models in mice, as well as in vitro studies against the AIDS virus[12–14]. GCEE mitigated the loss of mitochondrial function in neuronal cell cultures, prevented H_2O_2-induced oxidative stress in a microvascular endothelial cell line by increasing GSH levels, decreased cell death, and contributed to the recovery of endothelial cells and microvasculature in a mouse model of brain trauma[15–17].

GCEE has been reported to increase GSH levels and consequently exert protective effects against oxidative stress and neurotoxicity in mice with adriamycin-induced cognitive deficits[18]. The protection of GCEE against oxidative stress and cell death due to its antioxidant capability has also been studied in neurodegenerative disease models. For instance, GCEE protects both synaptosomes and mitochondria against peroxynitrite-induced oxidative damage by raising GSH in primary neuron culture in Alzheimer's disease, which is associated with increased oxidative stress and decreased GSH levels[19]. Also, potential effect of GCEE was investigated both in vivo and in vitro to prevent the early GSH loss seen in Parkinson's disease and to provide a protection against the death of dopaminergic neurons[20].

Excitotoxicity is one of the fundamental neurodegenerative processes, including oxidative stress, neuroinflammation, and apoptosis, that arise from the over-induction of glutamate receptors by agents such as glutamic acid and kainic acid (KA). Excitotoxicity is also one of the key molecular mechanisms involved in status epilepticus[21,22].

GCEE provides a protection against KA-induced excitotoxicity in rats by increasing GSH levels in both cortical and hippocampal tissues, decreasing ROS formation in synaptosomes, and reducing apoptotic molecules such as caspase-3 enzyme activity, and Bax and p53 mRNAs[23,24]. The ameliorative effects of GCEE on learning in traumatic brain injury model in mice were tested using the Morris Water Tank (MWM) experiments[25].

Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the adult brain and functions as both a primary neurotrophic signal and a neuromodulator. It serves essential roles in neuronal development, maintenance, transmission, and plasticity, thereby influencing aging, cognition, and behavior. BDNF essentially affects all aspects of neuronal cellular function through tropomyosin receptor kinase B (TrkB) receptor signaling, the disruptions of which vary between brain regions across different pathologies leading to diversified consequences on cognition and behavior[26].

As summarized above, the neuroprotective effects of GCEE at the molecular level in excitotoxicity-induced experimental models have been extensively studied. However, it is observed that the neurobehavioral effects of GCEE in excitotoxicity, which has the capacity to cross the blood-brain barrier due to its molecular structure, have not yet been sufficiently investigated. In this context, it is considered important for neurotherapeutic approaches to examine GCEE together with its effects on neurobehavioral patterns that may be comorbid for epilepsy, such as learning, memory, and anxiety, and on BDNF expression, which is stated to play a central role in these neurobehavioral patterns, as well as its direct relationship with GSH levels, in excitotoxicity induced by KA, a robust status epilepticus model.

MATERIALS AND METHODS

In this study, animal experiments were conducted at the Ege University Laboratory Animal Application and Research Center (EGEHAYMER) and the Department of Physiology, Faculty of Medicine. Molecular analyses were performed at the Department of Biochemistry, Faculty of Pharmacy, Ege University, Izmir, Türkiye. The Ege University Animal Experiments Local Ethics Committee (EGEHADYEK) approved all animal procedures on April 28, 2021 (Decision No.: 2021-028) in accordance with institutional and national guidelines.

Animals

Forty adult male Sprague–Dawley rats, 60 days old and weighing 200–250 g, were used in the present study. The rats were housed under controlled conditions with standard humidity and a temperature of 25°C, on a 12- hour light/12- hour dark cycle. They were provided with water and food ad libitum throughout the experiment. To avoid hormonal fluctuations, only male subjects were included in the study.

Experimental design

Animals were randomly divided into four groups of ten animals each; 1) Control (saline), 2) KA only (10 mg/kg), 3) GCEE only (10 mg/kg) and 4) KA + GCEE (10 mg/kg KA+ 10mg/kg GCEE). KA and GCEE were administered to the animals as a single dose on the same day. In the KA + GCEE group, GCEE injection was given within 10 minutes following KA injection.

KA [2-carboxy-4-isopropenyl-pyrrolidin-3-acetic acid] and GCEE [(Des-Gly)-Glutathione-monoethyl ester] were purchased from Cayman (USA), and Bachem (Switzerland), respectively. KA and GCEE were dissolved in saline. KA, GCEE and saline were administered to rats intraperitoneally (i.p.). A standard dose of 10 mg/kg body weight i.p injection of KA was used to create seizures and neurodegeneration in specific brain regions[27,28].

Rats were monitored for three hours following KA injection in order to measure seizure activity. Following the administration of the KA, a number of distinct behavioral changes were observed. After 45 to 60 minutes, the rats' immobility and rigidity gave way to "staring spells," which were followed by repeated head nodding and wet dog shakes, and lastly by rearing and falling. Rats finally developed a generalized tonic-clonic seizure with continuous convulsions. Only rats having complete limbic seizures and forelimb clonus during rearing, were included in this study. The stages of seizures were scored using the Racine scale[29] (Table 1). Continuous stage IV–V convulsive seizures that continue at least five minutes operationally are referred to as status epilepticus[30].

Table 1. Racine scale used to evaluate seizure severity

Stage 0	Normal behavior
Stage 1	Spontaneous facial movements
Stage 2	Clonus in the face and head
Stage 3	Clonus in the forelimbs
Stage 4	Rearing behavior
Stage 5	Rearing followed by falling backward

Behavioral experiments were started ten days later in all experimental animals that received saline, only KA, only GCEE, and KA + GCEE. Behavioral experiments included the Morris Water Maze (MWM), Open Field Test (OFT), and Elevated Plus

Maze (EPM) tests. After completing all behavioral experiments, the animals were decapitated. The hippocampus and cortex brain regions were quickly removed. Brain tissues were stored at -80°C until they were used for GSH and BDNF analysis.

Behavioral tests

Morris Water maze

The Morris Water Maze (MWM) was used to test spatial learning in rats. The circular tank (130 cm diameter, 75 cm height) was filled with yellow-dyed water at 22°C and divided into four virtual quadrants (North, South, East, West) marked in black. Visual-spatial cues were placed in the experimental setting. The 15×15 cm platform was visible (2 cm above water) on day one and hidden (2 cm below surface) from days 2–6. Rats swam from four different starting points each day. On the seventh day (probe trial), the platform was removed, and each rat's latency to reach the previous platform location was recorded to evaluate retrieval performance. Behavior was recorded and analyzed using Toxtract software[31-33].

Open field test

The Open Field Test (OFT) was used to determine locomotor activity, exploration and anxiety-related behaviors in status epilepticus model rats. The apparatus was an 80×80 cm arena with 45 cm high walls. Each animal was placed in the center and observed for 5 min with video recording under controlled lighting and quiet conditions. The number of entries into the center area, the time spent in the center, and the total distance traveled were measured. Locomotion was analyzed using Toxtract software[32,33].

Elevated plus maze test

The Elevated Plus Maze test was used to evaluate anxiety-related behaviors in status epilepticus model rats. The apparatus has two open and two closed arms (50×10 cm), elevated 50 cm above the floor with a central square (10×10 cm). Open arms have 1 cm edges; closed arms have 40 cm high walls. Each animal was placed facing an open arm and observed for 5 min under controlled lighting and quiet conditions, with video recording. The latency to first entry into the open arm, the number of entries into the open and closed arms, and the time spent in each, were measured.

Molecular and biochemical evaluation

Homogenization of brain tissue samples and determination of protein levels

Protein quantity determination in brain tissues was performed using the BCA Protein Assay Kit (Biovision, USA) according to the manufacturer's instructions. The kit contains reagent A (bicinchoninic acid disodium), reagent B (copper sulfate), and BCA standard solution (2 mg/ml). Phosphate buffer (20 mM, pH=7.4), KCl solution (140 mM), standard solutions, and working solution were prepared for the experiments.

Hippocampus and cortex tissues were weighed and homogenized on ice at +4°C with 20 mM PBS (pH 7.4) containing 140 mM KCl in a volume four times the tissue weight. The homogenates were centrifuged at 16,500xg at +4°C for 30 minutes, and the supernatants were separated. Blanks and samples were prepared according to the kit's instruction manual, incubated at 37°C for 30 minutes, and their absorbances were measured at 562 nm. Experiments were repeated three times for each sample. The protein content of the tissue samples was determined using a standard curve prepared with various protein concentrations.

Determination of GSH levels

The levels of GSH in cortex and hippocampus were determined by a modified procedure of the DTNB (5,5'-dithiobis-(2-nitrobenzoic acid))-GSH reductase assay[34]. The DTNB-GSH assay (Ellman's method) is a sensitive colorimetric method used to quantify reduced GSH in biological samples. It relies on DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) reacting with GSH to produce a yellow product (TNB), which is measured spectrophotometrically at 412 nm.

Cortex and hippocampus tissues were weighed and homogenized on ice in 20 mM PBS (pH 7.4) containing 140 mM KCl in a volume four times the tissue weight at +4°C. Homogenates were centrifuged at 16,500xg for 30 minutes at +4°C, and the supernatants were separated. 5% TCA was added in a volume four times the supernatant and allowed to stand for 5 minutes, then centrifuged at 1000xg for 15 minutes. The pellet was discarded, and the supernatant was collected. 50 µl of supernatant and 5 µl of DTNB were added to 200 µl of TE (Tris–EDTA) buffer and mixed. For the blank, 50 µl of ultrapure water and 5 µl of DTNB were added to 200 µl of TE buffer and mixed. All samples were

incubated in the dark for 5 minutes, after which their absorbance was measured at 412 nm. Experiments were repeated three times for each sample. For GSH analysis, a standard curve was prepared using stock and various concentrations of GSH working standards (2.5-50 µg/ml GSH) diluted with Tris buffer.

GSH levels of the tissue samples were determined using a standard curve prepared with various GSH concentrations. Results were given in µg/mg protein.

Total RNA isolation and reverse transcription polymerase chain reaction

Total RNA was extracted from the hippocampus and cortex tissues using Trizol reagent (Invitrogen, USA) followed by phenol–chloroform extraction and isopropanol precipitation[35].

One microgram of total RNA was used for the first strand cDNA synthesis by MuMMLV reverse transcriptase using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific).

Real-time PCR for BDNF and GAPDH mRNA

GAPDH primers were derived from previous publication[28]. BDNF primers were newly designed using Primer3 software (Rozen S, Skaletsky HJ. Primers on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–386). The forward primers for BDNF (Gene bank no: NM 001270638.1) and GAPDH (Gene bank no: AF106860) genes were 5'-GAAGGCTGCAGGGGCATAGAC-3' and 5'-AAGGTCATCCCAGAGCTGAA-3', respectively. The reverse primers for BDNF and GAPDH genes were 5'-TACACAGGAAGTGTCTATCCTTAT-3', and 5'-ATGTAGGCCATGAGGTCCAC-3', respectively. Conditions for PCRs were optimized in a gradient cycler (Techne 512, UK) with regard to primers and various annealing temperatures.

Real-time PCR was performed using the Stratagene Mx3000P real-time detection system (Stratagene, CA, USA). Each reaction included 1 µl of diluted cDNA, 1 µl of forward and primers (15 pmol), 10 µl of qPCR Master Mix (Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Stratagene, USA)) and 7 µl of nuclease-free water. Cycling parameters were 95°C for 3 minutes, followed by 40 cycles of 95°C for 20 seconds and 60°C for 20 seconds. For melting curve analyses, an additional cycle of 1 minute at 95°C, 30 seconds at 55°C and 30 seconds at 95°C was added. The expected PCR products were 135 bp and 338 bp for BDNF and GAPDH, respectively. The melting temperatures were 83°C and 86°C for BDNF and GAPDH, respectively. The comparative $^{\Delta\Delta}$CT method was used to quantify relative gene expression levels[36]. GAPDH was used as an internal reference gene to normalize BDNF mRNA expression.

Statistical analysis

SPSS version 24.0 was used for statistical analysis. Data are presented as mean ± standard error of the mean (SEM). Normality and homogeneity were tested via Shapiro-Wilk and Levene's tests. A p value of less than 0.05 was considered statistically significant.

Behavioral test results, including seizure scores, MWM, OFT, and EPM, were analyzed using Duncan's and Tukey's post hoc multiple comparisons tests following one-way analysis of variance (ANOVA) to determine intergroup differences. One-way ANOVA was considered appropriate because the study involved comparison of more than two independent experimental groups (Control, KA, GCEE, and KA + GCEE). Tukey's test was used for multiple pairwise comparisons, whereas Duncan's test was additionally applied to increase sensitivity in detecting subtle but biologically meaningful differences, particularly in behavioral parameters.

Biochemical and molecular parameters, including GSH levels and BDNF mRNA expression levels in the cortex and hippocampus, were also analyzed using one-way ANOVA, followed by Tukey's post hoc test for pairwise group comparisons.

RESULTS

The animals were observed for three hours following the treatments to evaluate seizure behavior. Seizure severity was assessed in six time intervals (0–30, 30–60, 60–90, 90–120, 120–150, and 150–180 min) according to the Racine Scale. No significant differences were observed during the first 30 minutes ($p > 0.05$). During the 60–90 min interval, seizure severity in the KA group was significantly higher than in the control, GCEE, and KA + GCEE groups ($p < 0.05$). Similarly, during the 90–120 min and 120–150 min intervals, the KA group continued to exhibit significantly higher seizure severity compared with the control, GCEE, and KA + GCEE groups ($p < 0.05$). During the 150–180 min interval, seizure severity remained significantly higher in the KA

group than in the control and GCEE groups, whereas the KA + GCEE group showed lower seizure severity compared with the KA group ($p < 0.05$) (Fig. 1).

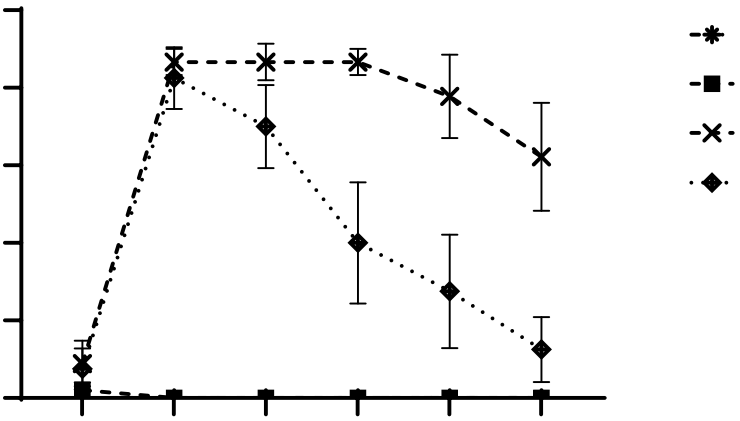

Fig. 1. Seizure severity evaluated according to the Racine scale at different time intervals.

Data are presented as mean ± SEM. (a) $p < 0.05$ vs. Control; (b) $p < 0.05$ vs. GCEE; (c) $p < 0.05$ vs. KA; (d) $p < 0.05$ vs. KA + GCEE.

As shown in Fig. 2A and Fig. 2B, the distance traveled to reach the platform and the escape latency progressively decreased during the training days in all experimental groups ($p < 0.05$). However, no statistically significant differences were observed among the control, GCEE, KA, and KA + GCEE groups during the training trials ($p > 0.05$).

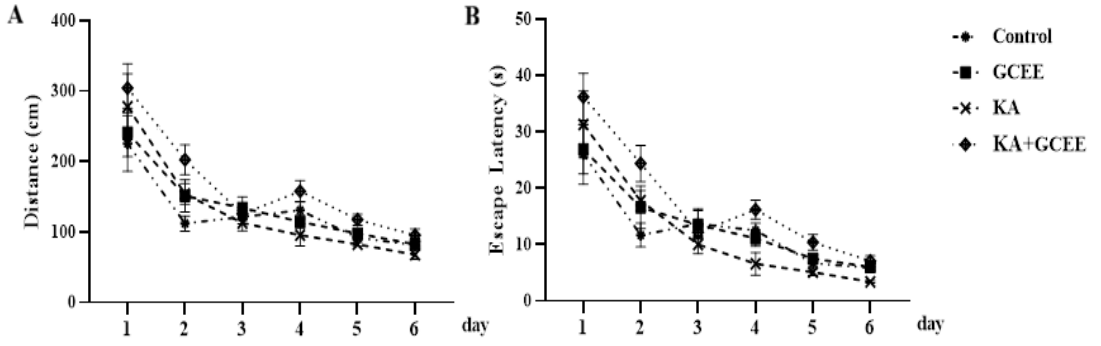

Fig. 2. Effects of treatments on spatial learning performance in the Morris water maze (MWM).

Data are presented as mean ± SEM. (A) Distance traveled to reach the platform during training days. (B) Escape latency during MWM training trials. There were no statistically significant differences among the Control, GCEE, KA, and KA + GCEE groups ($p > 0.05$).

Significant differences in spatial learning were found between the groups in the latency to first reach the quadrant on the 7th day ($p < 0.05$), as shown in Fig. 3. KA negatively affected retrieval performance during the probe trial and this impairment was significantly improved in the KA + GCEE group.

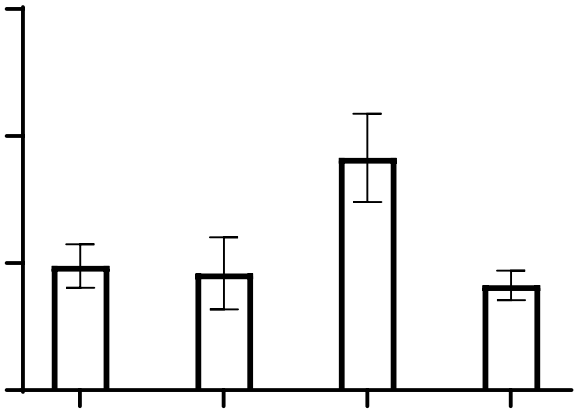

Fig. 3. Effects of treatments on spatial memory performance in the Morris water maze probe trial.

Data are presented as mean ± SEM. Time to first reach the platform on day 7 in the MWM. (a) $p < 0.05$ vs. control.

The OFT results showed that there were no statistically significant differences among the control, GCEE, KA, and KA + GCEE groups in the number of entries into the inner area ($p > 0.05$) (Fig. 4A), the time spent in the center area ($p > 0.05$) (Fig. 4B), or the total traveled distance ($p > 0.05$) (Fig. 4C).

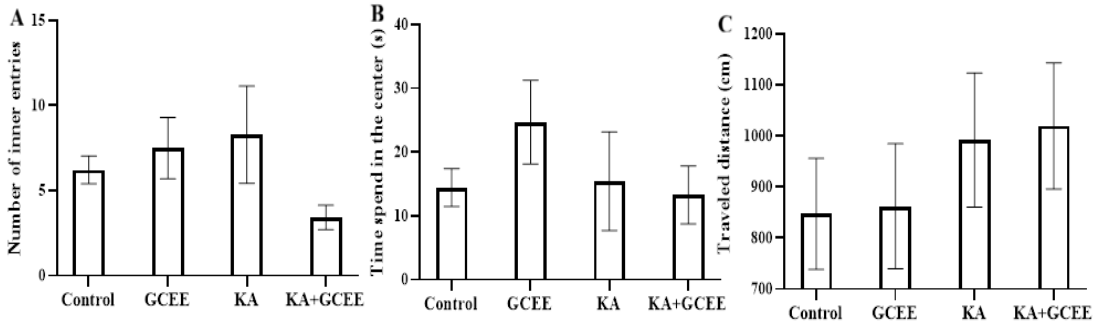

Fig. 4. Effects of treatments on locomotor and exploratory behavior in the open field test (OFT).

Data are presented as mean ± SEM. (A) Number of entries into the inner area. (B) Time spent in the center area. (C) Total traveled distance. There were no statistically significant differences among the Control, GCEE, KA, and KA + GCEE groups ($p > 0.05$).

The EPM results indicated that there were no statistically significant differences among the control, GCEE, KA, and KA + GCEE groups in the number of entries into the open arms ($p > 0.05$) (Fig. 5A), the time spent in the open arms ($p > 0.05$) (Fig. 5B), the number of entries into the closed arms ($p > 0.05$) (Fig. 5C), or the time spent in the closed arms ($p > 0.05$) (Fig. 5D).

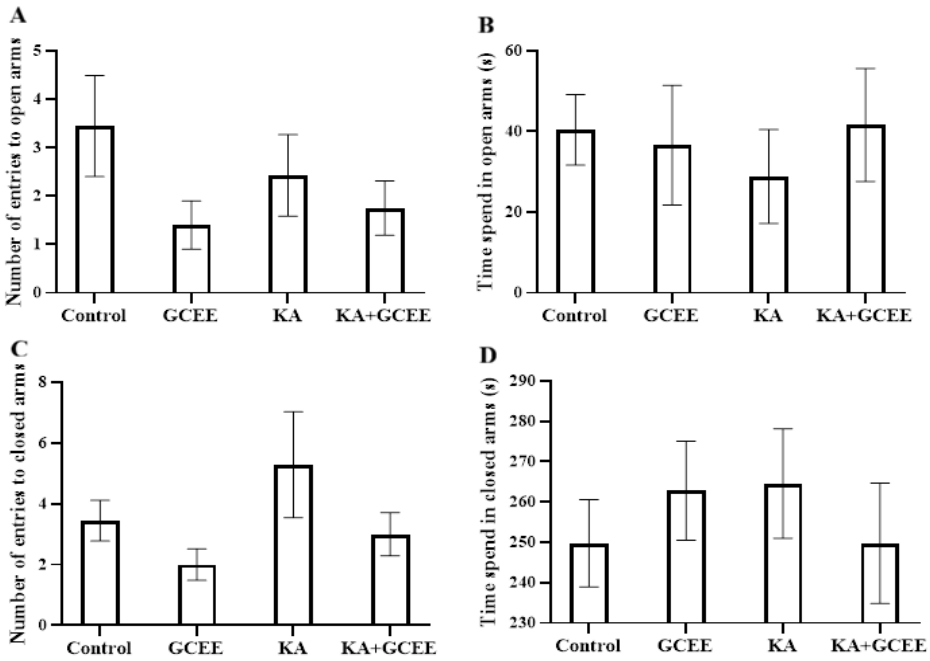

Fig. 5. Effects of treatments on anxiety-like behavior in the elevated plus maze (EPM).

Data are presented as mean ± SEM. (A) Number of entries into the open arms. (B) Time spent in the open arms. (C) Number of entries into the closed arms. (D) Time spent in the closed arms. There were no statistically significant differences among the Control, GCEE, KA, and KA + GCEE groups ($p > 0.05$).

Cortical GSH levels in the GCEE alone and KA alone groups were significantly different when compared to the control group ($p < 0.05$), however, the change in GSH levels detected in the GCEE-only group was not statistically significant when compared to the KA-only group ($p > 0.05$). Cortical GSH levels showed an increasing trend in both the GCEE and KA + GCEE groups, but this trend was not statistically significant when compared to the control group or the KA-only group ($p > 0.05$) (Fig 6A).

Hippocampal GSH levels detected in the GCEE, KA, and KA + GCEE groups were not statistically significant when compared to the control group ($p > 0.05$). Similar to cortical GSH levels, hippocampal GSH levels showed an increasing trend in both the GCEE-only and the KA + GCEE groups, but this trend was not statistically significant when compared to the KA-only group ($p > 0.05$) (Fig. 6B).

According to our results, cortical BDNF mRNA levels were lowest in the KA group compared to other experimental groups. However, the low BDNF mRNA levels detected in the KA-only group were not statistically significant when compared with the control, GCEE-only, and KA + GCEE groups ($p > 0.05$). Furthermore, the KA + GCEE group had the highest BDNF mRNA levels among all experimental groups. However, the high BDNF mRNA levels detected in the KA + GCEE group were not statistically significant when compared with the control, KA-only, and GCEE-only groups ($p > 0.05$). (Fig 7A).

Similarly to the change observed in cortical BDNF mRNA levels, hippocampal BDNF mRNA levels were also lowest in the KA-only group compared to other experimental groups. However, the BDNF mRNA levels detected in the KA-only group were not statistically significant when compared with the control, GCEE, and KA + GCEE groups ($p > 0.05$). Furthermore, the highest BDNF mRNA levels among all experimental groups were found in the KA + GCEE group, and the high BDNF mRNA levels found in this group were not statistically significant when compared with the control, KA-only, and GCEE-only groups ($p > 0.05$). (Fig 7B).

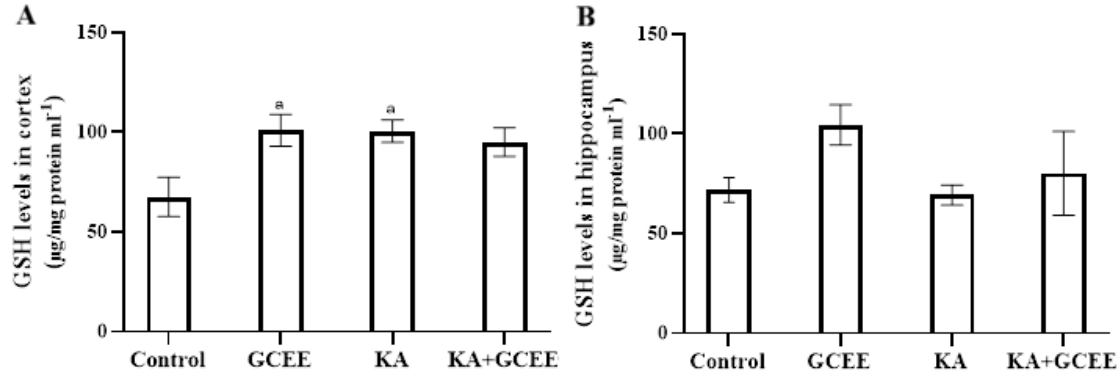

Fig. 6. GSH levels in brain tissues. GSH levels are expressed as % of Control.

Data are presented as mean ± SEM. (A) GSH levels in the cortex. (a) $p < 0.05$ vs. Control. (B) GSH levels in the hippocampus. There were no statistically significant differences among the Control, GCEE, KA, and KA + GCEE groups ($p > 0.05$).

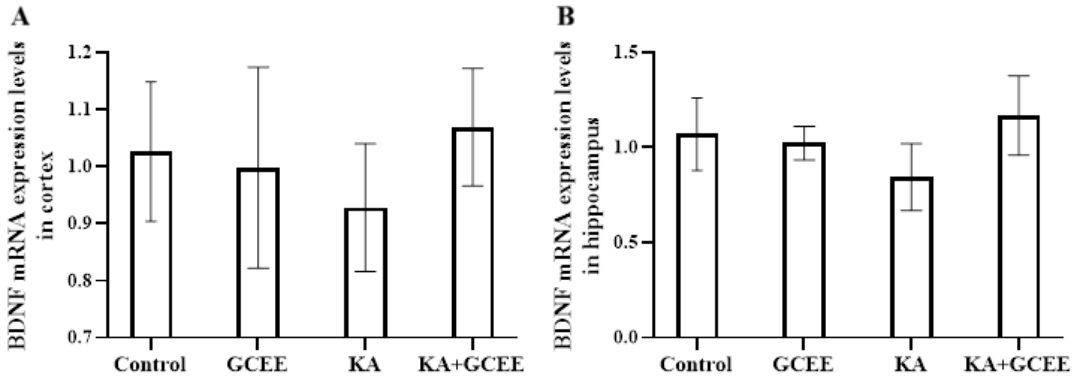

Fig. 7. BDNF mRNA expression levels in brain tissues.

Data are presented as mean ± SEM. (A) BDNF mRNA expression levels in the cortex. (B) BDNF mRNA expression levels in the hippocampus. There were no statistically significant differences among the Control, GCEE, KA, and KA + GCEE groups ($p > 0.05$).

DISCUSSION

Previous studies have determined the effects of a number of antioxidants on seizure severity induced by KA using the Racine Scale. Hyperocidin was found to reduce seizure severity in male rats after KA injection[37], and sitagliptin, used in the treatment of type 2 diabetes, was found to delay the onset of epileptic seizures, reduce their severity, and increase survival rates in male rats in a KA-induced status epilepticus model[38]. Similarly, maslinic acid and asiatic acid have been shown to reduce seizure severity in male mice in a KA-induced epilepsy model through molecular pathways associated with inflammation and oxidative stress[29]. In this study, GCEE was found to reduce seizure severity ~ 45 - 60 minutes after KA administration, and these findings support the previously demonstrated neuroprotective effects of GCEE on KA-induced status epilepticus.

The benefits of many antioxidants on kainic acid-induced learning deficits have been documented in earlier research. Asiatic acid has been shown to protect against oxidative stress, synaptic and mitochondrial function damage in a traumatic brain injury (TMI) model induced by KA, and to reduce learning disabilities and seizure severity in adult male

rats in the MWM test[39]. Because of its anti-inflammatory and antioxidant effects, Huperzine-A has been demonstrated to lessen learning deficits found in the MWM test in a KA-induced epilepsy model[40]. Similar to this, quercetin, which is well-known for its anti-inflammatory and antioxidant effects, has been shown to alter cognitive results from the MWM test in a mouse model of KA-induced status epilepticus by influencing pathways connected to the ferroptosis pathway[41].

In a previous study, GCEE was shown to protect against autophagy by increasing antioxidant capacity in a TMI model in mice, increasing learning scores in the MWM test and reducing latency[25]. Our study examines, for the first time, the effects of GCEE on learning and memory performance in the Morris Water Maze (MWM) in a KA-induced status epilepticus model. According to our results, KA treatment affected the initial entry times (delay) of rats onto the platform quadrant on the last day of the MWM test and impaired recall performance during probe testing. However, this impairment was reduced in KA + GCEE- treated group. These results demonstrate that GCEE may have an effect on learning and memory performance.

When comparing KA alone and GCEE alone treatment groups in terms of anxiety-like behavioral changes, a trend towards a decrease in anxiety-like behavior was observed in the GCEE-treated group, although this change was not statistically significant. The effect of GCEE on anxiety-like behavior in KA-induced status epilepticus was investigated for the first time in our study. However, according to our results, no specific effect of GCEE on anxiety-like behavior was observed. This can be explained by the fact that GCEE was administered acutely, in a single, and relatively small dose, the behavioral tests were performed relatively long after the acute administration, and no simultaneous antiepileptic treatment was given.

Considering previous studies examining the relationship between epileptic seizures and BDNF expression, it is noted that changes in BDNF expression levels may not be consistent[42,43]. Although a previous study reported increased BDNF mRNA and protein levels in specific brain regions following KA administration, no detectable levels of BDNF protein were found in cerebrospinal fluid or plasma[44]. Tulke et al.[45] showed that following KA-induced seizures, there was a fluctuating level of BDNF mRNA expression in the hippocampus, along with changes observed in both the granule cell layer and the Cornu Ammon Area 2 pyramidal neurons. In our study, KA-induced status epilepticus was found to reduce BDNF mRNA expression in both the cortex and hippocampus. However, while GCEE administration showed a trend towards increased BDNF mRNA expression in both brain regions compared to KA administration, this increase was not statistically significant. This observation may be related to the dose and duration used for GCEE in our study.

The effects of KA and GCEE administration on GSH levels in particular brain regions have been examined in earlier research[23-28]. KA-induced seizures were found to decrease GSH levels in the hippocampus along with an increase in DNA damage[28]. Furthermore, it was shown that GCEE administration at a dose of 10 mg/kg maintained GSH levels in the cortex and hippocampus against KA after three hours of observation[23]. However, in a pharmacokinetic study[46] GSH levels in the hippocampus and cortex regions were determined using HPLC pharmacokinetics at twelve different time intervals ranging from 0 to 96 hours, using GCEE-loaded microspheres. Acute or chronic administration of GCEE significantly increased GSH levels in the hippocampus and cortex, with the most significant changes observed at 4-6 hours.

The doses of KA and GCEE used in this study were chosen based on previous studies[23,28]. However, we obtained results that may be inconsistent with previous studies regarding changes in GSH levels in both the hippocampus and cortex regions. In our study, GCEE administration alone increased GSH levels in both the hippocampus and cortex compared to the control, but this increase was only significant for the cortex region. Similarly, GCEE administration against KA significantly increased GSH levels only in the cortex region. According to our results, GSH levels showed a significant increase only in the cortex after GCEE administration against KA, but no such increase was observed in the hippocampus. When previous studies examining the effects of GCEE against KA are reviewed, it is observed that, unlike our study, no long-term (20-day) behavioral studies were conducted after KA and GCEE administration, and brain tissue samples were collected 3-4 hours after the KA and GCEE administrations. Therefore, there are differences between our study and previous studies in both the experimental period and the time taken to collect

tissue samples. These differences may have also affected the changes in GSH levels in the cortex and hippocampus regions after KA and GCEE administrations.

The effects of KA and GCEE on neurobehavioral alterations, GSH, and BDNF mRNA levels were the main focus of this study. At this point, it may be useful to point out some limitations of our study. In particular, after KA and KA + GCEE treatments, significant changes were seen in behavioral experiments and in the levels of GSH and BDNF mRNA expression in the hippocampus and cortex regions; however, these changes were not found to be statistically significant because of the high standard deviations of the values. Studies using larger sample size can address this issue as long as they adhere to ethical guidelines. Additionally, employing a range of histological and molecular analysis techniques to examine the effects of GCEE in a KA-induced status epilepticus model will allow for a broader definition of this agent's potential neuroprotective effects. In conclusion, considering the importance of neurobehavioral studies in epilepsy[47], the central role of GSH in cognitive performance[48] and the therapeutic potential of BDNF in neurobehavioral disorders associated with epilepsy[26], it can be said that studies demonstrating the regulatory effects of GCEE on these processes may contribute to the development of new treatment approaches.

To advance understanding of these mechanisms, future studies are recommended to consider developmental stages, sex differences, dosage variations, additional behavioral outcomes, neurophysiological assessments, and combinations with antiepileptic agents.

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