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Potential neuroprotective effect of γ-glutamylcysteine ethyl ester on rat brain against kainic acid-induced excitotoxicity

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Abstract
The aim of this study was to investigate the effect of γ-Glutamylcysteine Ethyl Ester (GCEE) on the levels of GSH, caspase-3 activity, DNA damage and the expressions of Bcl-2, Bax and p53 mRNAs in rat hippocampus after status epilepticus (SE) induced by systemic kainic acid (KA). The male rats were divided into four groups as controls, KA (10 mg/kg), GCEE (10 mg/kg) and KA+GCEE. Glutathione (GSH) levels and caspase-3 activity were determined spectrophotometrically and colourimetrically, respectively. DNA damage and Bcl-2, Bax and p53 mRNA expressions were quantified by comet assay and reverse transcription followed by RT-PCR, respectively. KA treatment significantly depleted GSH levels, induced DNA damage, caspase-3 activity and the expressions of p53 and Bax mRNA. GCEE treatment protected GSH levels, decreased DNA damage and the levels of p53 and Bax/Bcl-2 mRNA against KA injection. These results indicate that GCEE treatment at the dose of 10 mg/kg is capable to protect the depleted levels of GSH and shows an anti-apoptotic activity due to the decreased levels of apoptotic biomarkers in the rat hippocampus after SE induced by KA.

Keywords: *Kainic acid, γ-glutamylcysteine ethyl ester (GCEE), p53 mRNA, Bcl-2 mRNA, Bax mRNA, DNA damage, caspase-3 activity*

Introduction

Excitotoxicity resulting from excitatory amino acids such as glutamate is considered to be a contributing factor in pathogenesis of a number of central nervous system (CNS) disorders such as stroke, epileptic seizures, Parkinson's disease, etc. [1–3]. Kainic acid is an excitatory neurotoxic substance. It stimulates a sub-type of the ionotropic receptor of the brain neurotransmitter glutamate and results in transmembrane ion imbalance, causing calcium influx, which, in turn, generates reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$) and the hydroxyl radical (HO•). These reactive oxygen species attack macromolecules within neurons, resulting in membrane lipid peroxidation, structural and functional changes in proteins and DNA strand breaks [4,5]. The administration of KA to rodents can trigger limbic seizures and leads to neuronal death, especially in the hippocampus, amygdala and thalamus both *in vitro* and *in vivo* [6,7]. Bcl-2 family of proteins, transactivating factor p53 and caspase proteases such as caspase-3 are involved in neuronal apoptosis induced by KA [8,9]. KA-induced seizures lead to GSH depletion [10–13]. It has been shown that intracerebroventricular [14,15] or intraperitoneal [16,17] administration of GCEE increase GSH levels in the rat brain. GCEE has been proposed to increase GSH biosynthesis by providing the limiting substrate in GSH biosynthesis, γ-glutamylcysteine. In addition, GCEE shows an antioxidant activity in the brain against peroxynitrite [16], amyloidβ(1-42) protein [17,18], 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) [20] and traumatic injury-induced oxidative stress [21].

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Cellular stressors such as ROS can lead to the activation of the p53 protein. The activated p53 increases the expression of two pro-apoptotic Bcl-2 family proteins, Bax and Bak [22]. Caspases constitute another essential component of the apoptotic process. The release of cytochrome *c* from the mitochondria to the cytosol allows the formation of the apoptosome, which, in turn, activates effector caspases. This important pathway of caspase activation is regulated by the pro- and anti-apoptotic Bcl-2 family proteins [23].

The regulation of molecules activated in apoptosis by antioxidants such as GCEE could offer the possibility to delay neuronal death. However, there is no data available showing its regulator effect on the genes or proteins activated by apoptosis. KA administration greatly affects hippocampal areas due to their high levels of kainate receptors [24]. In this study, we focused on the potential anti-apoptotic effects of GCEE on the rat hippocampus and determined the p53, Bax and Bcl-2 mRNA expressions, caspase-3 enzyme activity and DNA damage following status epilepticus induced by systemic KA injection.

Materials and methods

Animals and treatments

Thirty-two adult male Sprague-Dawley rats weighing 200–250 g were used for the present study. All animals were kept in the same laboratory conditions of temperature (25 ± 2°C) and lighting (14:10 h light: dark cycle) and were given free access standard laboratory chow and tap water. The protocol for the experiments was approved by the appropriate Animal Care Committee of Faculty of Pharmacy at Ege University, Izmir, Turkey. Animals were divided into four groups of eight animals each; controls (saline), KA only (10 mg/kg), GCEE only (10 mg/kg) and KA + GCEE (10 mg/kg GCEE 10 min after kainic acid injection).

KA, GCEE and saline were administered to rats intraperitoneally (i.p). A standard dose of 10 mg/kg body weight i.p injection of kainic acid is used to create seizures and neurodegeneration in specific brain regions. Animals that received KA injection exhibited the clinical characteristics shown earlier [6,25,26]. Following KA injection, rats were observed for 4 h to assess seizure activity. Seizures occurred ~ 45 min after KA injection. Only rats exhibiting full limbic seizures, forelimb clonus with rearing, were included in this study. A single high dose of GCEE (150 mg/kg body weight) has been used for the rats [16,17,21].

In this study the dose of GCEE was based on our dose-response study (data not shown). All animals were decapitated at 4 h after all injections and the hippocampus was dissected on an ice-cold plate. All samples were stored at –80°C until they were used.

Chemicals

Kainic acid [2-carboxy-4-isopropenyl-pyrrolidin-3-acetic acid] was purchased from Ocean Products International (Canada). γ-Glutamylcysteine ethyl ester [(Des-Gly)-Glutathione-monoethyl ester] was obtained from Bachem (Switzerland). GCEE was dissolved in saline. Kainic acid was dissolved in phosphate-buffered saline (PBS) and the pH adjusted to 7.4 with NaOH.

GSH assay

The cellular GSH levels were determined according to Drake et al. [16] by a modified procedure of the DTNB-GSH reductase assay. Results were given as nmol/mg protein.

Caspase-3 enzymatic activity assay

The determination of caspase-3 enzyme activity was carried out according to Bachis et al. [27]. Tissue samples were lysed on ice-cold solution of PBS and Triton X-100 0.2% for 10 min. The extract were centrifuged at 10 000 x g for 5 min and the supernatant were collected. Caspase-3 activity was measured using the caspase-3 colourimetric assay protease kit (Chemicon, Temecula, CA) following the instructions of the manufacturers. One-hundred micrograms of protein of each sample was incubated with 200 μM caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp (DEVD)-p-nitroanilide at 37°C for 2 h. Samples were analysed at 405 nm in a microtiter plate reader (Molecular Devices, CA, USA). Data were expressed as percentage increase or decrease in caspase-3 activity compared with the control group.

Determination of DNA damage (single cell gel electrophoresis)

The single cell gel/comet assay was essentially performed according to the recommendations of Tice et al. [28]. Samples (25–30 mg) of hippocampus were minced, suspended and chilled with a homogenizing buffer (pH 7.5) containing 0.075 M NaCl and 0.024 M Na_2EDTA and then homogenized. Homogenates were centrifuged at 700 × g for 10 min at 0°C and the precipitates were re-suspended in chilled homogenizing buffer. The cell suspension was mixed with 500 μl of LMP agarose in PBS at 37°C and 140 μl of this mixture was applied onto a frosted glass microscope slide pre-coated with a layer of 1% NMP agarose (150 μl). After application of a third layer of LMP agarose, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% N-sodium lauroyl sarcosinate, 1% Triton-X-100, 10% DMSO) for 1 h at 4°C. The slides were then placed in an electrophoresis tank, allowing the DNA to unwind for 15 min in the alkaline solution (300 mM NaOH

and 1 mM Na2EDTA). Electrophoresis was then performed at 300 mA for 25 min in the same alkaline solution at room temperature. The slides were neutralized with 0.4 M tris-HCl buffer (pH 7.4) and were stained using the silver staining method described by Nadin et al. [29]. The dried microscope slides were covered with a coverglass prior to analysing under an Olympus light microscope. The microscope was connected to a charge-coupled device camera and a personal computer based analysis system (CASP-Comet Analysis Software Program, 1.2.2 Version) to determine the extent of DNA damage after electrophoretic migration of DNA fragments in the agarose gel. Results were expressed as a percentage of DNA in the tail (tail%) or in tail moment [Tail DNA% × Tail Length (percentage of DNA in the tail) × (tail length)] or tail length (length of the comet tail measured from right border of head area to end of tail in pixels). Three samples per rat were assayed implicating 50 cells per sample. The means ± standard error means (SEM) of the three samples were calculated for each rat.

Total RNA isolation and reverse transcription polymerase chain reaction

Total RNA was extracted from the hippocampus using Trizol reagent (Gibco BRL Life Technologies, Grand Island, NY) followed by phenol chloroform extraction and isopropanol precipitation [30]. Prior to the reverse transcription reaction, potentially contaminating residual genomic DNA was eliminated with DNAse I (MBI Fermentas, Slovenia). One microgram of total RNA was used for the first strand cDNA synthesis by MuMMLV reverse transcriptase (MBI Fermentas, Slovenia).

Real-time polymerase chain reaction for p53, Bax and Bcl-2 mRNA

Bcl-2, Bax and GAPDH primers were derived from our previous publication [31]. p53 primers were newly designed using Primer3 software [32]. The forward primers for Bcl-2 (L14680), Bax (NM_017059), p53 (NM_030989) and GAPDH (AF_106860) genes were 5′-TCTGTGGATGACTGAGTACCTGAAC-3′, 5′- TGCAGAGGATGATTGCTGAC-3′, 5′-GTC TACGTCCCGCCATAAAA-3′ and 5′-AAGGT CATCCCAGAGCTGAA-3′, respectively. The reverse primers for Bcl-2, Bax, p53 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-AGAGACAGCCAGGAGAAATCAAAC-3′, 5’- GATCAGCTCGGGCACTTTAG -3′, 5′- AGGC AGTGAAGGGACTAGCA -3′ and 5′- ATGTAG GCCATGAGGTCCAC -3′, respectively. Conditions for PCRs were optimized in a gradient cycler (Techne 512, UK) with regard to primers and various annealing temperatures. Optimized settings were transferred to real-time PCR protocols on a Stratagene Mx3000P real-time detection system (Stratagene, CA, USA). Amplification of 1 µl RT mixture (cDNA diluted 1:5) was carried out using 1 µl 15 pmol forward and reverse primers, 12.5 µl Brilliant SYBR® Green Q PCR 2X Master Mix (Stratagene, USA) and 9.5 µl nuclease-free water in a total volume of 25 µl. Cycling parameters were: 3 min at 94°C, 45 s at 94°C followed by 40 cycles of 30 s at 60°C and 50 s at 72°C. An additional cycle for melting curve analyses was 1 min at 95°C and 30 s at 55°C. Both cDNA synthesis and PCR amplifications included negative control reactions, which were set up by excluding RNA and DNA templates, respectively. The expected PCR products for Bcl-2, Bax, p53 and GAPDH were 129, 173, 174 and 338 bp, respectively. The melting temperatures (Tm) for Bcl-2, Bax, p53 and GAPDH were 82, 84, 82 and 83°C, respectively.

GAPDH gene was used as an endogenous control for normalization. The relative expressions of target genes were quantified according to ABI Prism 7700 Sequence Detection System User Bulletin No. 2 (Applied Biosystems, Foster City, CA) and Livak and Schmittengen [33].

Determination of protein levels

Protein concentrations were measured using bovine-serum albumin as a standard by the method of Lowry et al. [34].

Statistical analyses

Statistical analysis were performed by one-way analysis of variance (ANOVA) with a post-hoc Fisher's protected least significance difference (PLSD) test. A p-value of <0.05 was accepted as statistically significant.

Results

Animal behaviours

Systemic administration of KA was observed to produce the well described sequential behavioural changes. Rats exhibited immobility and rigid postures that were replaced by 'staring spells' after ~ 45 min, followed by repetitive head nodding 'wet dog shakes' and subsequent rearing and falling. Eventually, the rats developed generalized tonic-clonic seizures with continous convulsions lasting for several hours. Administration of GCEE did not reduce the KA-induced seizure activity compared with animals that only received KA injection.

The effects of KA and GCEE treatments on the levels of GSH in hippocampus were presented in Figure 1. According to our results KA treatment significantly reduced the levels of GSH when compared with

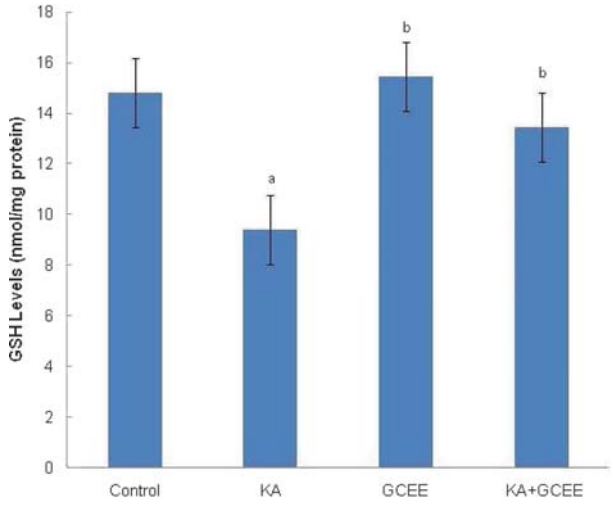

Figure 1. Effect of KA and GCEE treatments on the levels of GSH in the rat hippocampus. The results are given as nmol/mg protein. Data are mean ± SEM. $n=8$ for controls and $n=6$ for KA, GCEE and KA+GCEE groups. (a) $p < 0.05$ vs controls; (b) $p < 0.05$ vs KA.

controls ($p < 0.05$). In addition, GCEE or GCEE+KA treatments significantly protected GSH levels in the hippocampus when compared with the KA-treated group ($p < 0.05$).

To test the ability of GCEE to inhibit caspase-3 enzymatic activity, we assayed the pooled samples of controls, KA, GCEE and KA+GCEE -treated animals using caspase-3 colourimetric protease assay kit. Caspase-3 activity was significantly increased in KA-treated animals compared with controls ($p < 0.05$ and $p < 0.05$, respectively). GCEE treatment significantly decreased the levels of caspase-3 activity when compared with KA-treated animals ($p < 0.05$) (Figure 2).

We used single cell gel electrophoresis to detect DNA damage at the level of individual cells. Figure 3

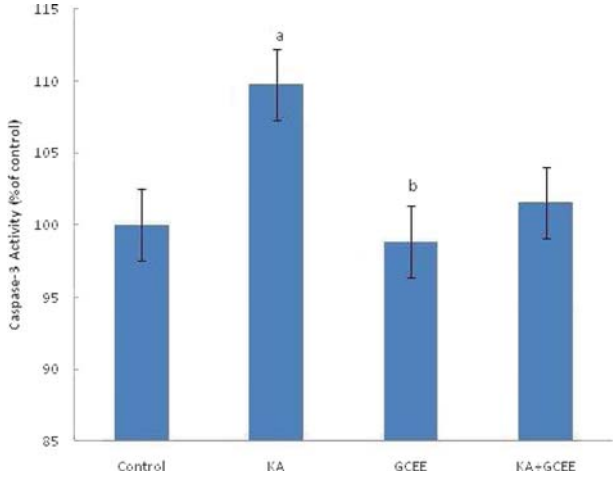

Figure 2. Effect of KA or GCEE treatments on the activity of caspase-3 in the rat hippocampus. Caspase-3 activity was determined in the pooled samples of controls, KA, GCEE and KA+GCEE-treated rats. $n=4$ per group. Each value is the mean ± SD of three independent experiments. (a) $p < 0.05$ vs controls; (b) $p < 0.05$ vs KA.

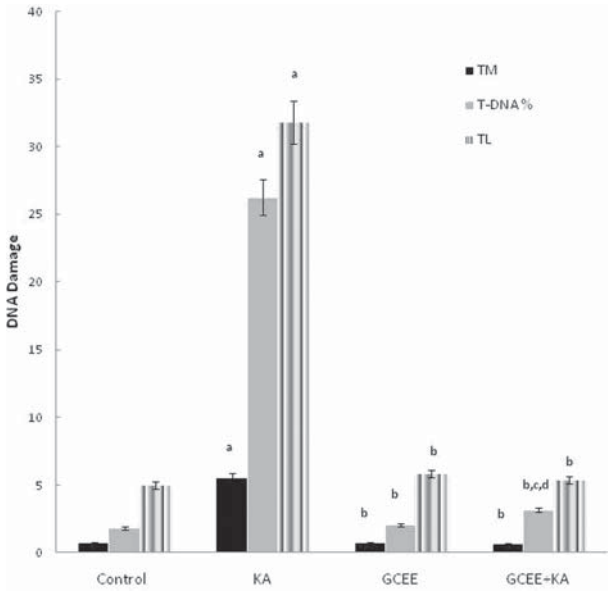

Figure 3. Effect of KA and GCEE treatments on hippocampal DNA damage (comet-Tail-DNA% and TM scores). Data are mean ± SEM of three independent experiments. $n=5$ per group. TM: Tail moment; TL: Tail lenght. (a) $p < 0.001$ vs control; (b) $p < 0.001$ vs KA; (C) $p < 0.05$ vs GCEE; (D) $p < 0.05$ vs control.

shows the effect of GCEE treatment on DNA damage caused by SE. Comet assay parameters including T-DNA%, TL and TM levels were significantly increased in KA-treated group when compared with controls ($p < 0.001$). The decreased levels of T-DNA%, TL and TM were determined in GCEE or GCEE+KA-treated animals when compared to KA-treated animals ($p < 0.001$ and $p < 0.001$, respectively). Figure 4 shows the typical comets were determined in hippocampus of control, KA, GCEE and KA+GCEE -treated animals.

The effect of KA and GCEE treatments on the relative expression levels of Bcl-2, Bax and p53 mRNAs in the rat hippocampus were presented in Figure 5. Accordingly, KA treatment significantly up-regulated the expressions of Bcl-2, Bax and p53 mRNAs when compared with the GCEE-treated group ($p < 0.01$). Meanwhile, KA+GCEE treatment significantly down-regulated the expression levels of Bax and p53 mRNA when compared with the KA-treated group ($p < 0.05$ and $p < 0.01$, respectively). Figure 5 shows the relation between the expression levels of p53 and Bax/Bcl-2 mRNA in the rat hippocampus. The ratio of Bax to Bcl-2 mRNA and the expression levels of p53 were significantly decreased in GCEE-treated group when compared with KA ($p < 0.01$) and KA+GCEE ($p < 0.05$).

Discussion

The major findings of this investigation provide novel information regarding potential neuroprotective effects of GCEE on the rat hippocampus after SE induced

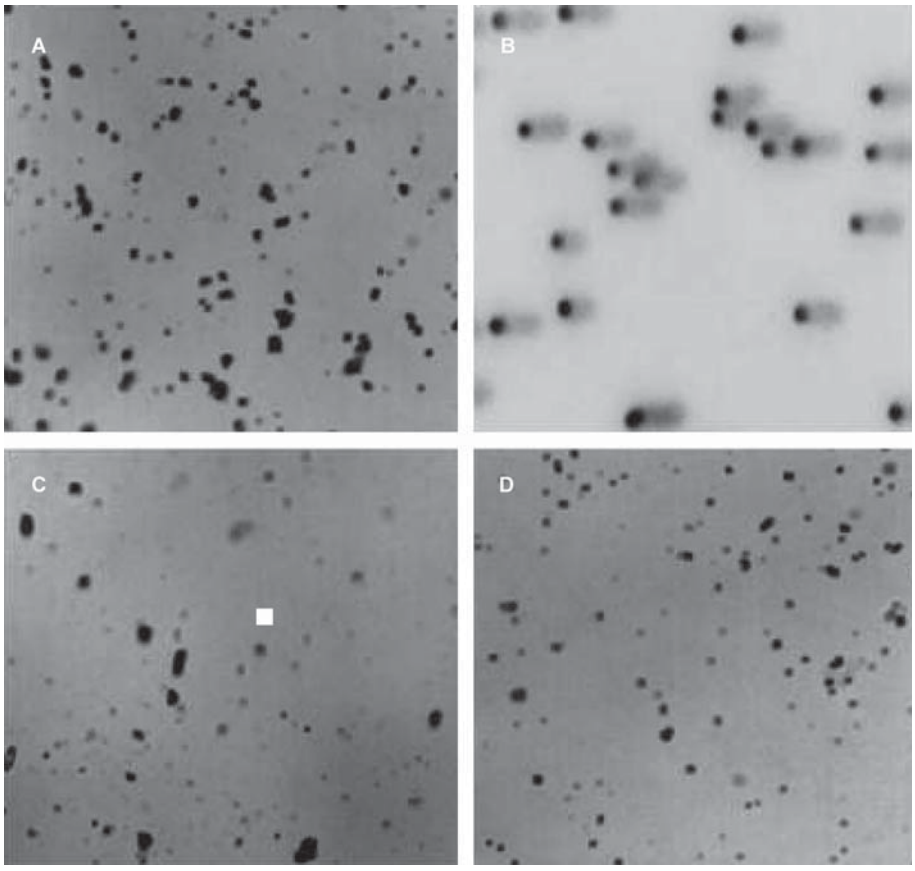

Figure 4. Typical comets from control, KA, GCEE and KA+GCEE-treated groups. Silver staining microscope objective ×20. (A) Control; (B) Kainic acid; (C) GCEE; (D) Kainic acid+GCEE.

by systemic KA injection. First, KA-induced seizures depleted GSH levels, which coupled with an increase in DNA damage. Secondly, KA-induced seizures upregulated the expressions of p53 and Bax mRNA and increased caspase-3 activity. Thirdly, GCEE treatment protected the cellular levels of GSH and decreased DNA damage following SE-induced by KA. Fourthly, GCEE treatment down-regulated the expressions of p53 and Bax expression, which positively correlated with the decreased levels of caspase-3 enzymatic activity. Finally, these results not only confirmed the apoptotic pathway induced by KA treatment, but also showed for the first time the regulatory effect of GCEE on the genes or molecules activated by apoptosis.

Systemic administration of KA produces free radicals [35] and induces exitotoxicity in neurons and glial cells [36]. KA depletes hippocampal GSH levels [10,25,37–39]. Consistently, our results confirmed that SE induced by KA causes a depletion in the levels of GSH in the rat hippocampus. GSH is a major thiol redox modulator in the cells and acts both as a nucleophilic scavenger of numerous compounds and as a cofactor for glutathione peroxidase and glutathione-S-transferase enzymes [3]. The depleted levels of GSH following SE induced by KA might be explained due to the major roles of GSH in cellular protection and the possible inhibiton of glutathione reductase by oxygen radicals produced by KA. In addition, the decreased levels of cysteine may be responsible for the depleted GSH levels following seizures induced by KA since cysteine is a rate limiting precursor of

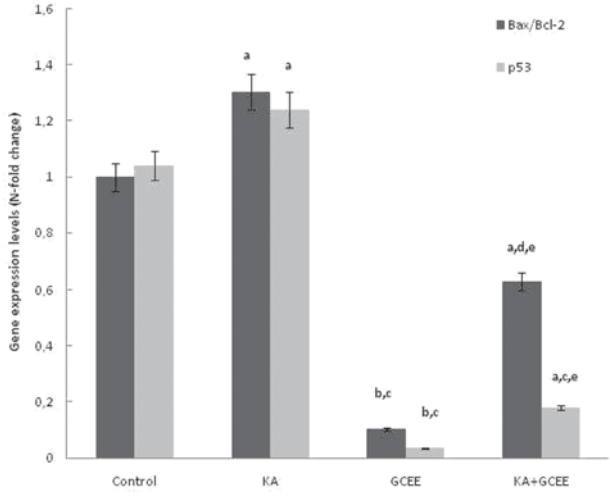

Figure 5. Effect of KA or GCEE treatments on the gene expressions of p53 and Bax/Bcl-2 in hippocampus. Data are mean ± SD of three independent experiments. $n=6$ for controls, and $n=3$ for KA, GCEE and KA+GCEE groups. (a) $p < 0.05$ vs control; (b) $p < 0.01$ vs control; (c) $p < 0.01$ vs KA; (d) $p < 0.05$ vs KA; (e) $p < 0.05$ vs GCEE.

GSH synthesis [13]. It has been shown that GCEE is capable of replacing the depleted levels of GSH in cells or mitochondria after oxidative insults [18–21]. Additionally, GCEE shows an antioxidant activity which is similar to GSH [17] and preserves total antioxidant status in the brain [40]. Our results showed that there was a significant change in GSH levels after GCEE+KA treatment. However, GCEE only treatment did not provide a significant increase in GSH levels when compared with controls. This finding might be related to the relatively low dose of GCEE which we used in this study.

KA-induced apoptosis is dependent on the induction of p53, Bax and caspase-3 [9,41,42]. KA-induced seizures damage both nuclear and mitochodrial DNA [39,43–45] and impair mitochondrial base excision repair capacity in the rat hippocampus [46]. DNA damage activates p53 and this activation influences viability in multiple neuronal sub-types and brain regions after an extitotoxic insult [47,48]. p53 activation induces DNA repair proteins such as XRCC1 and APE/Ref-1 following KA-induced seizures in the rat brain [49]. In addition, death associated protein (DAP) kinase has a significant role in neurological diseases and is involved in the p53 pathway during seizure-induced neuronal death [50]. Consistently, our results confirmed that KA-induced seizures increase DNA damage in the rat hippocampus. These results also indicated that there was a positive relation between the increased levels of DNA damage and the induction of p53 mRNA expression following KA-induced seizures. It has been reported that DNA fragmentation varies directly as a linear function of the duration of focally or KA-induced SE between 30 and 120 min, suggesting this marker is highly responsive to neuroprotective intervention [51]. In our study, GCEE treatment decreased DNA damage. This result was consistent with previous studies showing the protective role of GCEE on DNA fragmentation induced by several oxidative agents [16–18,52].

p53 mediates apoptosis in response to death stimuli by transcriptional activation of pro-apoptotic genes and by transcription-independent mechanisms. In the transcription-independent mechanism p53 induces permeabilization of the outer mitochondrial membrane by forming an inhibitory complex with a protective Bcl-2, resulting in cytochrome *c* release [53] It has been reported that p53 knock-out animals had a protection against KA-induced cell death and seizures [47] and p53 deficient neurons resisted to excitotoxicity in stroke [41]. GCEE treatment down-regulated the expression of p53 mRNA following SE induced by KA. Therefore, we can suggest that p53 may be one of the molecular targets of GCEE besides DNA damage in the context of a neuroprotective intervention.

Bcl-2 is known to localize to multiple sites within cells (mitochondria, endoplasmic reticulum and nuclear envelope). Bcl-2 has been shown to regulate permeability transition via mitochondrial mega-channel and the prevention of mega-channel opening by Bcl-2 blocks the release of cytochrome *c* and apoptosis initiating factor (AIF) at the level of mitochondria, Bcl-2 appears to be involved in transport of transcription factors such as p53, NFκB, calcium and GSH sequestration via an unknown mechanism at the nuclear level [54]. The upregulation of Bcl-2 in the substantia nigra [55] and hippocampus [31] of KA-injected rats were observed, implicating a significant role of Bcl-2 protein in preventing KA-induced neurodegeneration. Bcl-2 increases the GSH pool and decreases GSH depletion and cellular damage caused by lipid peroxidation [56]. Consistenly, KA treatment caused an increase in Bax and Bcl-2 expression levels, indicating that KA might initiate cell death by varying the balance between Bax and Bcl-2. Our results suggest that the tested dose of GCEE may interfere with the mitochondrial apoptotic pathway triggered by KA via modulation of Bax, Bcl-2 and p53.

Indeed, Bax/Bcl-2 ratio was decreased in the GCEE+KA group when compared to KA treatment only. This finding may implicate the potential role of GCEE treatment in the reciprocal regulation of Bcl-2 family proteins because the ratio of Bax to Bcl-2 is an important determinant of neuronal survival [57]. Chinta et al [20] showed the potential neuroprotective effects of GCEE on GSH depletion, cell viability and loss in N27 cells against MPTP-induced oxidative stress, suggesting GCEE has an ability to either act directly itself as an antioxidant or indirectly via increased scavenger synthesis of GSH in these cell systems. Normally, neurons destined to develop apoptosis reportedly show up-regulation and nuclear translocation of Bax protein parallel to Bcl-2 down-regulation within the same neurons [58,59]. According to our results the levels of Bax/Bcl-2 and p53 mRNA were decreased in GCEE-treated animals. This observation may suggest more cells in the hippocampus survive apoptotic cell death due to an antioxidant support provided by GCEE.

Caspase-3 activation is an important indicator of apoptosis since different upstream pathways leading to apoptosis depend on caspase-3 induction for final apoptotic execution.

It has been reported that immunreactivity and activity of caspase-3 are determined in mice hippocampus 3 h after KA treatment [60]. Consistently, we observed that caspase-3 activity increased in the KA-treated animals. Caspase-3 is involved in cell death of hippocampal CA1, CA3 and hilar neurons [61] and nitric oxide synthase II contributes to apoptotic cell death in the hippocampal CA3 sub-field via a cytochrome *c*/caspase-3 signalling cascade [62] following induction of experimental temporal lobe status epilepticus in the rat. In addition, the increased levels of ceramide, a mediator of apoptosis, were correlated with the increased immunostaining of Bax protein and the TUNEL

activity of caspase-3-activated fragment (caspase-3a) in KA-induced SE [63]. It is also worth noting that Bcl-2, Bcl-XL and caspase-3 protein levels are elevated in temporal lobe epileptic human brains [64].

It is known that mitochondria dependent apoptotic stimulation initiates intracellular signalling that mediates caspase activation. Drake et al. [17] reported that mitochondria pre-treated *in vitro* with GCEE were protected against oxidative damage induced by peroxynitrite due to the decreased levels of mitochondrial swelling, mitochondrial membrane potential, 3-nitrotyrosine formation, protein carbonyl formation and cytochrome *c*-release. In this study we observed that GCEE treatment decreased the levels of caspase-3 activity. We also determined the similar inhibitor effect of GCEE on the levels of caspase-3 activity against KA treatment, but these results were unsignificant. Recently it has been shown that co-administration of GCEE protects mitochondrial complex 1 activity against buthionine sulphoximine (BSO) treatment in dopaminergic cell cultures [20]. Therefore, our data may implicate the potential protective effect of GCEE on mitochodrial integrity in the rat hippocampus because caspase activation is causally connected to the release of mitochondrial cytochrome *c* as a result of the collapse of the mitochondrial membrane potential and permeability transition caused by oxidative stress [65].

Finally, it can be suggested that GCEE treatment may have beneficial effects on status epilepticus through an anti-apoptotic activity due to the reduced levels of DNA damage and the decreased levels of Bax, p53 expressions and caspase-3 activity which are known as major determinants of apoptosis. Since glutamate excitotoxicity has been implicated in the progression of many neurodegenerative diseases, agents such as GCEE may help to delay neuronal death induced during these pathologies.

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References

[1] Choi DW. Ionic dependence of glutamate neurotoxicity. J Neurosci 1987;7:369–379.
[2] Beal MF. Mechanism of excitotoxicity in neurologic disease. FASEB J 1992;6:3338–3344.
[3] Halliwell B. Reactive oxygen species and the central nervous system. J Neurochem 1992;59:1609–1623.
[4] Lesli ML, Hassinger LC, Valentine SL, Baer JD, Coyle JT. L-Type calcium channels reduce ROS generation in cerebellar granule cells following kainate exposure. Synapse 2002;43: 30–41.
[5] Wang Q, Yu S, Simonyi A, Sun GY, Sun AY. Kainic acid-mediated excitotoxicity as a model for neurodegeneration. Mol Neurobiol 2005;31:3–15.
[6] Sperk G, Lassman H, Baran H, Seitelberger F, Hornykiewicz O. Kainic acid-induced seizures: dose-realtionship of behavioural neurochemical and histopathological changes. Brain Res 1985; 338:289–295.
[7] Coyle JT, Puttfarcken P. Oxidative stress glutamate and neurodegenerative disorders. Science 1993;262:689–695.
[8] Savitz SI, Rosenbaum DM. Gene expression after cerebral ischemia. Neuroscientist 1999;5:238–253.
[9] Djebaili M, Lerner-Natoli M, Pascale M, Baille V, Bocakaert J, Rondouin G. Molecular events involved in neuronal death induced in the mouse hippocampus by in-vivo injection of kainic acid. Mol Brain Res 2001;93:190–198.
[10] Gluck MR, Jayatilleke E, Shaw S, Rowan AJ, Haroutunian V. CNS oxidative stress associated with the kainic acid rodent model of experimental epilepsy. Epilepsy Res 2000;39:63–71.
[11] Ong WY, Hu CY, Hjelle OP, Ottersen OP, Halliwell B. Changes in glutathione in the hippocampus of rats injected with kainate: depletion in neurons and upregulation in glia. Exp Brain Res 2000;132:510–516.
[12] Candelario-Jalil E, Ajamieh HH, Sam S, Martínez G, León Fernández OS. Nimesulide limits kainate-induced oxidative damage in the rat hippocampus. Eur J Pharmacol 2000;390:295–298.
[13] Liang PL, Patel M. Seizure-induced changes in mitochondrial redox status. Free Radic Biol Med 2006;40:316–322.
[14] Pileblad E, Magnusson T. Increase in rat brain glutathione following intracerebroventricular administration of γ-glutamylcysteine. Biochem Pharmacol 1992;44:895–903.
[15] Pocernich CB, Cardin AL, Racine CL, Lauderback CM, Butterfield DA. Glutathione elevation and its protective role in acrolein-induced protein damage in synaptosomal membrane: relevance to brain lipid peroxidation in neurodegenerative disease. Neurochem Int 2001;39:141–149.
[16] Drake J, Kanski J, Varadarajan S, Tsoras M, Butterfield DA. Elevation of brain glutathione by γ-glutamylcysteine ethyl ester protects aginst peroxynitrite-induced oxidative stress. J Neurosci Res 2002;68:776–784.
[17] Drake J, Sultana R, Aksenova M, Calabrese V, Butterfield DA. Elevation of mitochondrial glutathione by gamma-glutamylcysteine ethyl ester protects mitochondria against peroxynitrite-induced oxidative stress. J Neurosci Res 2003;74: 917–927.
[18] Boyd-Kimball D, Sultana R, Abdul HM, Butterfield DA. Gamma-glutamylcysteine ethyl ester-induced up-regulation of glutathione protects neurons against Abeta(1-42)-mediated oxidative stress and neurotoxicity: implications for Alzheimer's disease. J Neurosci Res 2005;79:700–706.
[19] Boyd-Kimball D, Sultana R, Poon HF, Mohmmad-Abdul H, Lynn BC, Klein JB, Butterfield DA. Gamma-glutamylcysteine ethyl ester protection of proteins from Abeta(1-42)-mediated oxidative stress in neuronal cell culture: a proteomics approach. J Neurosci Res 2005;79:707–713.
[20] Chinta SJ, Rajagopalan S, Butterfield DA, Andersen JK. *In vitro* and *in vivo* neuroprotection by gamma-glutamylcysteine ethyl ester against MPTP: relevance to the role of glutathione in Parkinson's disease. Neurosci Lett 2006;10:137–141.
[21] Lai Y, Hickey RW, Chen Y, Bayir H, Sullivan M, Cu CT, Kocharek PM, Dixon CE, Jenkins LW, Graham SH, Watkins SC, Clark RS. Autophagy is increased after traumatic brain injury in mice and is partially inhibited by the antioxidant gamma-glutamylcyteine ethyl ester. J Cereb Blood Flow Metab 2008;28:540–550.
[22] Bargonetti J, Manfredi JJ. Multiple roles of the tumor suppressorp53. Curr Opin Oncol 2002;14:86–91.
[23] Liou AKF, Clark RS, Henshall D, Yin X, Chen J. To die or not to die for neurons in ischemia traumatic brain injury and

epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. Prog Neurobiol 2003;69:103–142.
[24] Chittajallu R, Braithwaite SP, Clarke VRJ, Henley JM. Kainate receptors: subunits, synaptic localization and function. Trends Pharmacol Sci 1999;20:26–35.
[25] Floreani M, Skaper SD, Facci L, Lipartiti M, Giusti P. Melatonin maintains glutathione homeostasis in kainic acid-exposed rat brain tissues. FASEB J 1999;11:1309–1315.
[26] Chung S, Han S. Melatonin attenuates kainic acid-induced hippocampal neurodegeneration and oxidative stress through microglial inhibition. J Pineal Res 2003;34:95–102.
[27] Bachis A, Colangelo AM, Vicini S, Doe PP, De Bernardi MA, Brooker G, Mocchetti I. Interleukin-10 prevents glutamate-mediated cerebellar granuler cell death by blocking caspase-3-like activity, J Neurosci 2001;21:3104–3112.
[28] Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guide for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 2000;35:206–221.
[29] Nadin SB, Vargas-Roig M, Ciocca R. A silver staining method for single-cell gel assay. J Histochem Cytochem 2001;49:1183–1186.
[30] Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 1993;15:532–537.
[31] Yalcin A, Kanit L, Sozmen EY. Altered gene expressions in the rat hippocampus after kainic acid with or without melatonin pre-treatment. Neurosci Lett 2004;359:65–68.
[32] Rozen S, Skaletsky HJ. Primers on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–386.
[33] Livak KJ, Schmittengen TD. Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. Methods 2001;25:402–408.
[34] Lowry OH, Rosenbrough NJ, Farr AL, Randal RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951;193:265–275.
[35] Sun AY, Cheng Y, Butterfield F. The biochemical mechanisms of excitotoxicity of kainic acid: Free radical formation. Mol Chem Neuropathol 1992;17:51–63.
[36] Sun AY, Cheng Y, Sun GY. Kainic acid-induced excitotoxicity in neurons and glial cells. Prog Brain Res 1992;94:271–280.
[37] Gilberti EA, Trombetta LD. The relationship between stress protein induction and the oxidative defense system in the rat hippocampus following kainic acid administration. Toxicol Lett 2000;27:17–26.
[38] Candelerio-Jalil E, Al-Dalain SM, Castillo R, Martinez G, Fernandez OS. Selective vulnerability to kainate-induced oxidative damage in different rat brain regions. J Appl Toxicol 2001;21:403–407.
[39] Patel M, Li QY. Age dependence of seizure-induced oxidative stress. Neuroscience 2003;118:431–437.
[40] Clark RS, Bayir H, Chu CT, Alber SM, Kochanek PM, Watkins SC. Autophagy is increased after traumatic brain injury in mice and is partially inhibited by the antioxidant gamma-glutamylcysteinyl ethyl ester. J Cereb Blood Flow Metab 2008;4:88–90.
[41] Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS. Bax involvement in p53-mediated neuronal cell death. J Neurosci 1998;18:1363–1373.
[42] Djebaili M, De Bock F, Baille V, Bockaert J, Rondouin G. Implication of p53 and caspase-3 in kainic acid but not in N-methyl-D-aspartic acid-induced apoptosis in organotypic hippocampal mouse cultures. Neurosci Lett 2002;327:1–4.
[43] Masliah E, Mallory M, Alford M, Tanaka S, Hansen LA. Caspase dependent DNA fragmentation might be associated with excitotoxicity in Alzheimer disease. J Neuropathol Exp Neurol 1998;57:1041–1052.
[44] Lan J, Henshall DC, Simon RP, Chen J. Formation of base modification of 8-hydroxyl-2'-deoxyguanosine and DNA fragmentation following seizures induced systemic kainic acid in the rat. J Neurochem 2000;74:302–309.
[45] Neema M, Navarro-Quiroga I, Chechlacz M, Gilliams-Francis K, Liu J, Lamonica K, Lin SL, Naegele JR. DNA damage and nonhomolougos end joining in excitotoxicity: neuroprotective role of DNA-PKcs in kainic acid-induced seizures. Hippocampus 2005;15:1057–1071.
[46] Jarrett SG, Liang LP, Hellier JL, Staley KJ, Patel M. Mitochondrial DNA damage and impaired base excision repair during epileptogenesis. Neurobiol Dis 2008;30:130–138.
[47] Sakhi S, Bruce A, Sun N, Tocco G, Baudry M, Schreiber SS. Induction of tumor suppressor p53 and DNA damage fragmentation in organotypic hippocampal cultures following excitotoxin treatment. Exp Neurol 1997;145:81–88.
[48] Morrison RS, Wenzel HJ, Kinoshita Y, Robbins CA, Donehower LA, Schwartzkroin PA. Loss of the p53 tumor suppressor gene products neurons from kainate-induced cell death. J Neurosci 1996;16:1337–1345.
[49] Quach N, Chan T, Au Lu T, Schreiber SS, Tan Z. Induction of DNA repair proteins Ref-1 and XRCC1 in adult rat brain following kainic acid-induced seizures. Brain Res 2005;1042:236–240.
[50] Araki T, Shinoda S, Schindler CK, Quan-Lan J, Meller R, Taki W, Simon RP, Henshall DC. Expression, interaction, and proteolysis of death-associated protein kinase and p53 within vulnerable and resistant hippocampal subfields following seizures. Hippocampus 2004;14:326–336.
[51] Kondratyev A, Gale K. Temporal and spatial patterns of DNA fragmentation following focally or systemically evoked status epilepticus in rats. Neurosci Lett 2001;310:13–16.
[52] Joshi G, Hardas S, Sultana R, St Clair DK, Vore M, Butterfield DA. Glutathione elevation by gamma-glutamyl cysteine ethyl ester as a potential therapeutic strategy for preventing oxidative stress in brain mediated by *in vivo* administration of adriamycin: implication for chemobrain. J Neurosci Res 2007;85:497–503.
[53] Endo H, Saito A, Chan PH. Mitochondrial translocation of p53 underlines the selective death of hippocampal CA1 neurons after global cerebral ischemia. Biochem Soc Trans 2006;34:1283–1286.
[54] Voehringer DW. Bcl-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. Free Radic Biol Med 1999;27:945–950.
[55] Chuang JI, Chen ST, Chang YH, Jen LS. Alteration of Bcl-2 expression in the nigrostriatal system after kainate injection with or without melatonin co-treatment. J Chem Neuroanat 2001;21:215–223.
[56] Voehringer DW, Meyn RE. Redox aspects of Bcl-2 function. Antiox Redox Signal 2000;2:537–550.
[57] Schelman WR, Andres RD, Sipe KJ, Kang E, Weyhenmeyer JA. Glutamate mediates cell death and increases the Bax to Bcl-2 ratio in a differentiated neuronal cell line. Brain Res Mol Brain Res 2004;128:160–169.
[58] Isenmann S, Stoll G, Schroeter M, Krajewski S, Reed JC, Bahr M. Differential regulation of Bax, Bcl-2, and Bcl-X proteins in focal cortical ischemia in the rat. Brain Pathol 1998;8:49–62.
[59] Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski MJ, Reed JC. Upregulation of bax protein levels in neurons following cerebral ischemia. J Neurosci 1995;15:6364–6376.
[60] Park HJ, Kim HJ, Park HJ, Ra J, Zheng LT, Yim SV, Chung JH. Protective effect of topiramate on kainic acid-induced cell death in mice hippocampus. Epilepsia 2008;49:163–167.
[61] Becker AJ, Gillardon F, Blümcke I, Langendörfer D, Beck H, Wiestler OD. Differential regulation apoptosis-related genes in resistant and vulnerable subfields of the rat epileptic hippocampus. Brain Res Mol Brain Res 1999;67:172–176.

[62] Chuang YC, Chen SD, Lin TK, Liou CW, Chang WN, Chan SH, Chang AY. Upregulation of nitric oxide synthase II contributes to apoptotic cell death in the hippocampal CA3 subfield via a cytochrome c/caspase-3 signaling cascade following induction of experimental temporal lobe status epilepticus in the rat. Neuropharmacology 2007;52:1263–1273.
[63] Mikati MA, Zeinieh M, Habib RA, El-Hokayem J, Rahmeh A, El Sabban M, Usta J, Dbaibo G. Changes in sphingomyelinases ceramide, Bax, Bcl-2, and caspase-3 during and after experimental status epilepticus. Epilepsy Res 2008;81: 161–166.
[64] Henshall DC, Clark RS, Adelson PD, Chen M, Watkins SC, Simon RP. Alterations in bcl-2 and caspase gene family protein expression in human temporal lobe epilepsy. Neurology 2000; 55:250–257.
[65] Patel M. Mitochodrial dysfunction and oxidative stress: casuse and consequence of epilectic seizures. Free Radic Biol Med 2004;37:1951–1962.

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