# Fresh Green Tea and Gallic Acid Ameliorate Oxidative Stress in Kainic Acid-Induced Status Epilepticus

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**ABSTRACT:** Green tea is one of the most-consumed beverages due to its taste and antioxidative polyphenols. However, the protective effects of green tea and its constituent, gallic acid (GA), against kainic acid (KA)-induced seizure have not been studied. We investigated the effect of fresh green tea leaf (GTL) and GA on KA-induced neuronal injury in vivo and in vitro. The results showed that GTL and GA reduced the maximal seizure classes, predominant behavioral seizure patterns, and lipid peroxidation in male FVB mice with status epilepticus (SE). GTL extract and GA provided effective protection against KA-stressed PC12 cells in a dose-dependent manner. In the protective mechanism study, GTL and GA decreased $Ca^{2+}$ release, ROS, and lipid peroxidation from KA-stressed PC12 cells. Western blot results revealed that mitogen-activated protein kinases (MAPKs), RhoA, and COX-2 expression were increased in PC12 cells under KA stress, and expression of COX-2 and p38 MAPK, but not RhoA, was significantly reduced by GTL and GA. Furthermore, GTL and GA were able to reduce $PGE_2$ production from KA-stressed PC12 cells. Taken together, the results showed that GTL and GA provided neuroprotective effects against excitotoxins and may have a clinical application in epilepsy.

**KEYWORDS:** antioxidant, epilepsy, mitogen-activated protein kinases, ROS, COX-2

## ■ INTRODUCTION

Green tea is one of the most popular and widely consumed beverages in the world. It is well-known that green tea has important physiological properties and healthy benefits due to the presence of chemical compounds, such as polyphenols, amino acids, and vitamins.[1] Phenolic compounds are important in plant defense mechanisms against bacteria and environmental stress. Gallic acid (GA) is found in almost all plants including green tea. GA is known for its anti-inflammatory, antiallergic, antiviral, and antiproliferative activities against cancer cells.[2−5] Studies show that green tea and its bioactive components may decrease the incidence of dementia, Alzheimer's disease, and Parkinson's disease;[6,7] however, its effect on epilepsy has not been reported.

Status epilepticus (SE) is defined as a period of continuous seizure activity and has been implicated as a major predisposing factor for the development of mesial temporal sclerosis and temporal lobe epilepsy.[8] This emergency condition requires a prompt, appropriate treatment to prevent brain damage and eventual death. In animal models, similar pathologic changes have been observed with electrically and chemically induced seizures.[9−11] Animal studies showed that SE caused recurrent spontaneous seizures, that is, epilepsy,[12] and releases of free radicals in experimental models of kainic acid (KA),[13,14] pilocarpine,[15] pentylenetetrazole,[16] and ferric chloride.[17]

KA, a glutamate-related chemical, increases nerve excitability and is widely used to induce limbic epilepsy in animal models.[2] KA causes neuron epilepticus and excitotoxicity with the increased production of reactive oxygen species (ROS) and lipid peroxidation.[18−20] Mitogen-activated protein kinases (MAPKs) and Rho kinases are associated with seizures, inflammation, and apoptosis.[21−23] KA triggers neurons membrane depolarization by the release of calcium ions, which are involved in nerve impulse transmission as the calcium action potential reaches the synapse.[20] The apoptosis of nerve cells can be triggered by release of a large number of calcium ions, due to a large intracellular calcium increase, and activation of the calcium ion dependence of the enzyme, resulting in DNA fragmention of the nerve cells with fracture death.[24]

The antioxidant activity of *Machilus thunbergii* (Lauraceae) extract can reduce the glutamate-induced calcium ion release in the neuron primary cells of the rat cerebral cortex.[25] Another plant extract with an antioxidant effect, *Ginkgo biloba* leaf extract, can also reduce the KA-induced calcium ion concentration of nerve release and provide neuroprotection in the rat.[26] Polyphenols present in champagne wine may induce a neuroprotective effect against oxidative neuronal injury.[27] Because GA is abundant in green tea and has a strong antioxidant activity, it can protect human cells from injury by scavenging free radicals.[28] Therefore, the aim of this study was to investigate the protective mechanism of GA and green tea leaf extract against KA-induced injury in neuronal cells in vivo and in vitro.

## ■ MATERIALS AND METHODS

**Reagents.** GA and KA were obtained from Sigma-Aldrich (Steinem, Germany) and Cayman Chemical (Ann Arbor, MI), and 2′,7′-dichlorodihydrofluorescein diacetate ($H_2DCF$-DA) was obtained from Molecular Probes (Eugene, OR).

**Fresh Green Tea Leaves.** Fresh green tea leaves were harvested in July 2010 from tea (*Camellia sinensis*) plants growing in the Hsinchu, Taiwan. On the day that the leaves were collected, they were rinsed thoroughly with tap water to remove external contamination. Then, they were dried for 24 h at 50 °C in a hot-air oven, ground to a fine powder with the aid of a stainless steel mill, stored, and finally dried to constant weight in a vacuum desiccator. With regard to the extraction procedure, triplicate 1 g samples of green tea leaf powder from each site were mixed with 20 mL of RO water, vortexed vigorously for 5 min, and then centrifuged at 2000g for 10 min. The tea extracts were sterilized by filtration through a 0.25 $\mu$m Millipore membrane filter.

**Determination of Bioactive Contents.** The quantity of catechins, GA, and caffeine in the extracts of fresh green tea leaves was determined using the method described by Singleton et al.[29] Briefly, the extracts of tea leaves were serially diluted with methanol, and 0.1 mL aliquots of these solutions were transferred to 10 mL volumetric flasks containing 0.5 mL of undiluted Folin−Ciocalteu reagent. One minute after mixing, 1.5 mL of 20% (w/v) $Na_2CO_3$ was added, and the total volume was increased to 10 mL with double-distilled water. After incubation at room temperature for 60 min, the absorbance was determined at 760 nm and compared to a GA standard calibration curve prepared at the same time to provide a measure of the GA equivalents in each extract.

**High-Performance Liquid Chromatography (HPLC) Analysis of GA, EGCG, and EGC Composition.** Polyphenol contents of fresh green tea were characterized by HPLC using a Shimadzu SCL-LC 10A HPLC fitted with a SIL 10AD autosampler. Chromatography was performed with an ODS HYPERSIL (Thermo Scientific) reverse phase column (25 cm × 0.46 cm i.d., 5 $\mu$) and a UV−vis detector (Shimazu Systems Co., Foster City, CA). The mobile phase of catechins assay contained 1% acetic acid (solvent A) and acetonitrile (solvent B), with a linear gradient from A/B (92:8) to A/B (73:27) over a period of 40 min with a flow rate of 1 mL/min. The detector was set at 280 nm.

**Oxidative Stress in Mice.** Adult male FVB mice, body weight 30−35 g, were used for the study. SE was induced by KA [10 mg/mL in phosphate-buffered saline (PBS), 10 mg/kg, sc]. GTL powder and GA were separately diluted in 10 and 1 mg/mL of normal saline, respectively. The animals were fed with GTL (10 mg/kg) and GA by gavage for 3 days before the KA experiment. The control groups were fed with an equal volume of vehicle (normal saline). The procedures were conducted in accordance with the Taichung Veterans General Hospital Animal Care and Use Committee, Taichung, Taiwan (IACUC Approval No. LA-99741), and all possible steps were taken to minimize animals' suffering at each stage of experiments. Diazepam at a lethal dosage, 60 mg/kg ip, was given to stop seizures 2 h after KA injection, and the animal was sacrificed by decapitation under $CO_2$ asphyxia. The whole brains were removed and immediately frozen in liquid nitrogen and stored at −70 °C until use.

Malondialdehyde (MDA) as a part of thiobarbituric acid reacting substances (TBARS) was used as an indicator of lipid peroxidation. To estimate oxidative stress, the amount of TBARS in the brain from each group was measured. Manual homogenization of brains was carried out at 4 °C with a cold buffer. The protein concentration of the homogenate was determined by BCA protein assay using bovine serum albumin as a standard. For the TBARS assay,[30] the sample (0.2 mL) was mixed with the same volume of 20% (w/v) trichloroacetic acid (TCA) and 1% (w/v) thiobarbituric acid in 0.3% (w/v) NaOH. The mixture was heated in the water bath at 95 °C for 40 min, cooled to room temperature, and centrifuged at 5000 rpm for 5 min at 4 °C. The fluorescence of the supernatant was determined by a spectrophotometer with excitation at 544 nm and emission at 590 nm.

**Mortality and Behavior.** Mice were fed with and without GTL extract or GA for 3 days before the SE experiment. The control groups were treated with the vehicle normal saline. SE was induced with KA (10 mg/kg, sc). Each behavioral seizure was recorded and classified using a modified version of Racine's[31] classification: 0, exploring; 1, immobility 2, rigid posture; 3, head nodding; 4, bilateral forelimb clonus and falling; 5, continued clonus and falling; and 6, generalized tonus. Three behavioral patterns of SE were recognized: I, initial (classes 1−2); M, middle (class 3); and C, critical (classes 4−6).

Diazepam, 25 mg/kg ip, was given to stop the seizures at 5 h after induction of SE, and the 10 h mortality rate was recorded.

**TUNEL Staining.** Adult male FVB mice were observed, and the severity of KA stress-induced SE was recorded. After recovery for 24 h, mice were injected with a lethal dose of pentobarbital (120 mg/kg, ip), and brain tissue sections were perfused with 4% paraformaldehyde for fixation. Coronal paraffin sections were prepared with hematoxylin and eosin (H&E) staining for detection of cell damage and TUNEL staining for apoptosis study. After fixation for 1 h, mice brain sections were placed in a freshly prepared permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) and then washed with cold PBS. Then, a label solution of the TUNEL stain mixture (Roche, Mannheim, Germany) was added at 37 °C in the dark for 1 h. The apoptosis of neuronal cells was observed by fluorescence microscopy with excitation at 450−500 nm and detection wavelength at 515−565 nm.

**Cell Culture.** Rat pheochromacytoma (PC12) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 5% horse serum, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin at 37 °C in a humidified incubator under 5% $CO_2$. Confluent cultures were passaged by trypsinization. For experiments, cells were washed twice with warm DMEM (without phenol red) and then treated in serum-free medium. In all experiments, cells were treated with GTL extract or GA with KA stress for the indicated times.

**Preparation of Cell Extracts.** The test medium was removed from culture dishes, and cells were washed twice with ice-cold PBS, scraped off with a rubber policeman, and centrifuged at 200g for 10 min at 4 °C. The cell pellets were resuspended in an appropriate volume ($4 \times 10^7$ cells/mL) of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10 $\mu$g/mL aprotinin, and 5 $\mu$g/mL pepstain A. The suspension was then sonicated. The protein concentration of samples was determined by Bradford assay (Bio-Rad, Hemel, Hempstead, United Kingdom), and samples were equilibrated to 2 mg/mL with lysis buffer.

**Western Blotting.** Protein samples containing 50 $\mu$g of protein were separated on 12% sodium dodecyl sulfate polyacrylamide gels and transferred to Immobile polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated for 1 h with 5% dry skim milk in TBST buffer (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, and 0.1% Tween-20) to block nonspecific binding and then incubated with rabbit anti-COX-2, Rho A (1:1000; Cayman chemical; Cell Signaling, Danvers, MA), and antiphospho-MAPKs. Subsequently, membranes were incubated with secondary antibody streptavidin-horseradish peroxidase conjugated affinity goat antirabbit IgG (Jackson, West Grove, PA).

**ROS Generation.** Intracellular accumulation of ROS was determined with $H_2DCF$-DA. This nonfluorescent compound accumulates within cells upon deacetylation. $H_2DCF$ then reacts with ROS to form fluorescent dichlorofluorescein (DCF). PC12 cells were plated in 96-well plates and grown for 24 h before the addition of DMEM plus 10 $\mu$M $H_2DCF$-DA, incubation for 60 min at 37 °C, and treatment with 150 $\mu$M KA for 60 or 120 min. Cells were then washed twice with room temperature Hank's balanced salt solution (HBSS without phenol red). Cellular fluorescence was monitored on a Fluoroskan Ascent fluorometer (Labsystems Oy, Helsinki, Finland) using an excitation wavelength of 485 nm and emission wavelength of 538 nm.

**MTT Reduction Assay for Cell Viability.** Cell viability was measured with blue formazan that was metabolized from colorless 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenases, which are active only in live cells. PC12 cells were preincubated in 24-well plates at a density of $5 \times 10^5$ cells per well for 24 h. Cells with various concentrations of gallate were treated with 150 mM KA for 24 h and grown in 0.5 mg/mL MTT at 37 °C. One hour later, 200 $\mu$L of solubilization solution was added to each well, and absorption values were read at 540 nm on microtiter plate reader (Molecular Devices, Sunnyvale, CA). Data were expressed as the mean percent of viable cells from the control.

**Lactate Dehydrogenase Release Assay.** Cytotoxicity was determined by measuring the release of LDH. PC12 cells with various concentrations of GA were treated with 150 mM KA for 24 h, and the

**Table 1. Polyphenol Composition of Various Teas**

| | levels of polyphenol composition | | | | |
| | ±SEM | | | | |
| sample | GA | EGCG | ECG | unit | ref |
|---|---|---|---|---|---|
| fresh tea leaf (dry) | 51.03 ± 4.02 | 14.83 ± 0.92 | 1.14 ± 0.10 | $\mu$g/mg | present study |
| fresh tea leaf (wet) | 2.91 ± 0.11 | 0.90 ± 0.07 | 1.71 ± 0.14 | $\mu$g/mg | present study |
| green tea | 0.52 ± 0.03 | 13.37 ± 0.47 | 2.91 ± 0.13 | % w/w | Lin et al.[50] |
| black tea | 1.83 ± 0.52 | 0.30 ± 0.10 | ND[a] | % w/w | Lin et al.[50] |
| Pu-erh tea | 1.49 ± 0.04 | 0.15 ± 0.06 | 0.05 ± 0.01 | % w/w | Lin et al.[50] |

[a]ND, not detectable.

supernatant was used to assay LDH activity. The rate of absorbance was read at 490/630 nm on microtiter plate reader. Data were expressed as the mean percent of LDH release from the KA control.

**Calcium Release Assay.** The supernatant from GTL extract or GA-treated PC12 cells under KA stimulation for 24 h was used to assay the release of $Ca^{2+}$. The supernatant (10 $\mu$L) was added to 1 mL of $Ca^{2+}$ reagent (Diagnostic Systems, Holzheim, Germany), mixed well, and allowed to stand for 5 min; then, the resulting 100 $\mu$L of aliquot was transferred to a 96-well plate. The calcium concentration was determined using a microplate reader with an absorbance of 620 nm and quantified with a 10 mg/mL $Ca^{2+}$ standard solution.

**Measurement of Lipid Peroxidation.** Lipid peroxidation was quantified by measuring MDA level of PC12 cells by lipid peroxidation assay kit (Cayman Chemical). This kit works on the principle of condensation of one molecule of either MDA or 4-hydroxyalkenals with two molecules of N-methyl-2-phenylindole to yield a stable chromophore. MDA levels were assayed by measuring the amount expressed in $5 \times 10^5$ cells, and the absorbance at 500 nm was determined using an ELISA reader (spectraMAX 340, Molecular Devices).

**Assay of PGE₂ Concentration and Caspase-3 Activation.** $PGE_2$ release and caspase-3 activity were measured by ELISA assay. PC12 cells ($5 \times 10^5$) were added to 0.5 mL of homogenization buffer (0.1 M phosphate, 1 mM EDTA) and homogenized. The lysate was then centrifuged at 12000$g$ for 15 min at 4 °C. The supernatant was transferred to a new tube, and its total protein content was analyzed using the advanced protein assay. The $PGE_2$ concentration and caspase-3 activity were determined by the $PGE_2$ and caspase-3 ELISA Kits (R&D Systems, Minneapolis, MN). The absorbance at 450 nm was determined using a microplate reader (spectraMAX 340).

**Statistical Analysis.** All data were expressed as the mean ± standard errors of the mean (SEMs). For single variable comparisons, Student's test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's test. $P$ values less than 0.05 were considered significant. The effect on mortality and behavior data was analyzed by Fisher's exact test (small sample sizes), Pearson's $\chi^2$ test (independent groups data, unmatched groups proportion), and Kendall's $\tau$-c (nonparametric correlation) statistical method.

## ■ RESULTS

We investigated the fresh green tea leaves (C. sinensis) harvested in the summer of 2010 from tea plants in Hsinchu, Taiwan. The bioactive components from this dry tea leaf were GA, 51.03 ± 4.02 mg/g; EGCG, 14.83 ± 0.92 mg/g; EGC, 6.86 ± 0.3 mg/g; caffeine, 5.52 ± 0.04 mg/g; and ECG, 1.14 ± 0.10 mg/g (Table 1).

**Effect on Mortality and Behavior.** Treatment of KA-induced SE with GTL or GA did not affect the mortality in FVB mice (Table 2). However, both significantly attenuated the maximal seizure classes and the predominant behavioral seizure patterns in the SE mice as compared with the vehicle (GTL, $p = 0.014$; GA, $p < 0.01$; Table 2).

**Protection from KA Toxicity.** We then evaluated H&E staining of brain sections from KA-stressed FVB mice. We found that KA (10 mg/kg) caused SE and neuronal damage.

**Table 2. Effects of Green Tea Leaf Extract and GA on the Predominant Behavior Patterns/Maximal Seizure Class (MSC) and 10 h Mortality Rate of the Mice with 5 h KA-Induced SE[a]**

| variables | V-10 $n$ (%) | GTL-10 $n$ (%) | $p$ value | GA-1 $n$ (%) | $p$ value |
|---|---|---|---|---|---|
| mortality | 0 (0) | 0 (0) | 0.093[b] | 0 (0) | 0.005[b] |
| behavior pattern/MSC | | | | | |
| I/class 1–2 | 0 (0) | 0 (0) | 0.028[c] | 0 (0) | 0.002[c] |
| M/class 3 | 0 (0) | 4 (33) | 0.014[d] | 7 (58) | 0.000[d] |
| C/class 4–6 | 12 (100) | 8 (67) | | 5 (42) | |

[a]I, initial (class 1-2); M, middle (class 3); and C, critical. GTL-10, green tea leaf extract, 10 mg/kg; GA-1, GA, 1 mg/kg. V-10, vehicle control, with normal saline. [b]Fisher's exact test. [c]Pearson's $\chi^2$ test: all seizure classes taken together. [d]Kendall's $\tau$-c: all seizure classes taken together.

However, after GTL (10 mg/kg) or GA (1 mg/kg) treatment, the damage to cortical neuronal cells was reduced (Figure 1). The TUNEL staining assay showed that GTL or GA significantly reduced KA-induced apoptosis in the hippocampus of the FVB mice as compared to the control (Figure 2). To understand the protective mechanism, KA-induced injury in neuronal PC12 cells was investigated with LDH released and MTT assay. As shown in Figure 3, PC12 cells were protected from the injury by GTL extract (10 $\mu$g/m) and GA (0.1, 1, and 10 $\mu$M). GTL extract and GA reduced LDH released and increased cell viability, which was consistent with the in vivo data.

**KA-Induced Calcium Release.** KA triggers neuronal membrane depolarization by the release of calcium ions.[26] In the present study, KA induced calcium release from PC12 cells in a time-dependent manner (data not shown). GTL extract and GA significantly reduced KA-induced calcium release in PC12 cells (Figure 4).

**ROS and Lipid Peroxidation.** ROS and lipid peroxidation can damage neuronal cells.[18,20] DCF fluorescence of KA-treated cells increased by nearly 1-fold after 120 min as compared with the control cells. Treatment with GTL extract or GA protected cells against KA cytotoxicity by decreasing the KA-induced ROS accumulation. Only a higher dose of GTL extract (10 $\mu$g/mL GTL) or GA (1 and 10 $\mu$M) effectively reduced the ROS production of KA-stressed PC12 cells at 120 min (Figure 5). A marked increase in MDA and 4-hydroxyalkenals levels was observed in KA-exposed cells, as compared with the control cells (Figure 6A). GTL extract and GA protected cells against KA toxicity by lowering MDA levels ($p < 0.01$, as compared to the KA-treated cells). GTL and GA consistently and effectively reduced TBARS levels in the KA-induced SE mice (Figure 6B, $p < 0.01$ as compared to the KA control).
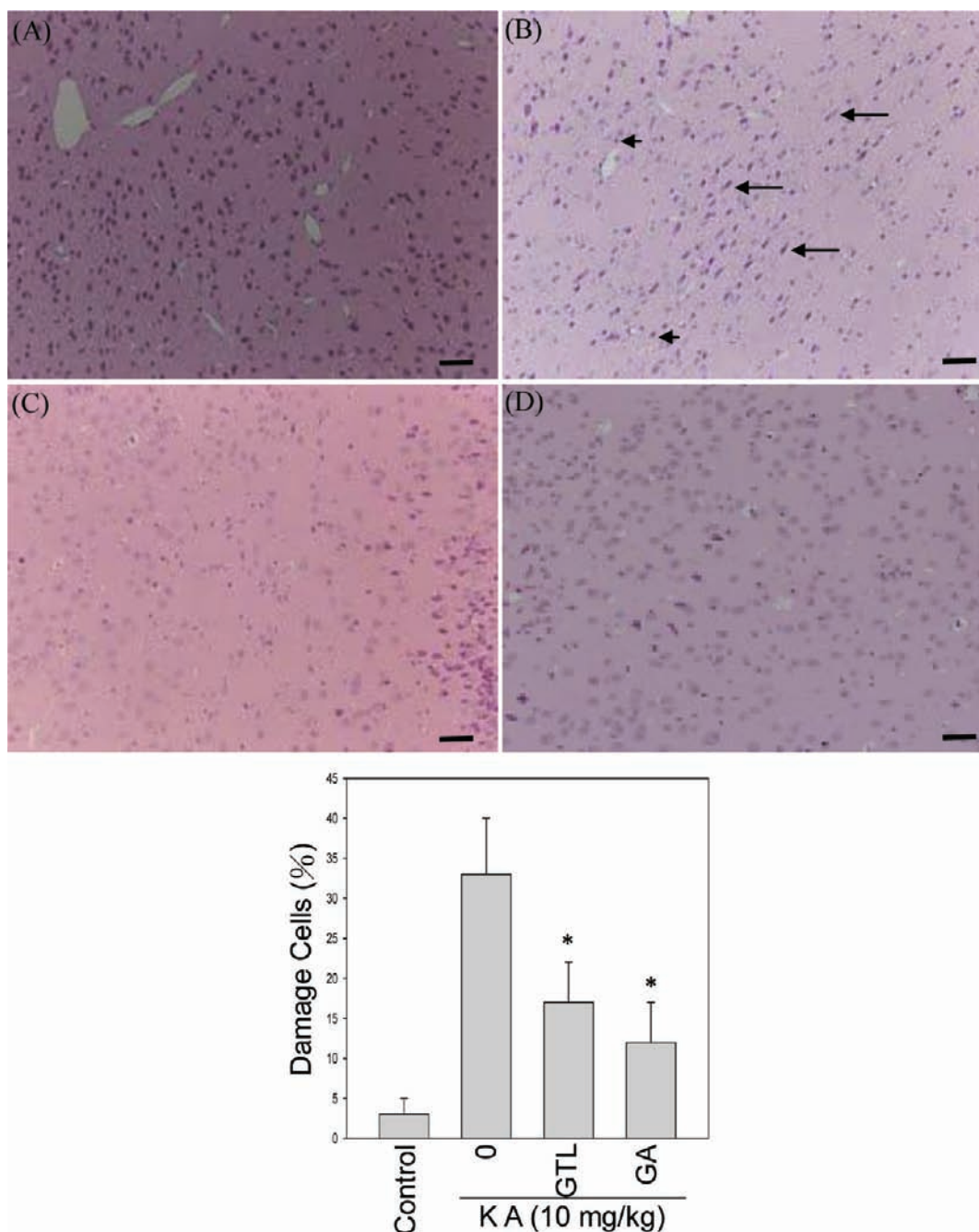
**Figure 1.** H&E staining of KA-stressed FVB mice cortex. KA (10 mg/kg) caused neuronal damage. After 5 h of KA-induced SE in FVB mice, cell shrinkage and lengthening were observed in the cortexes (B). GTL (10 mg/kg) (C) or GA (1 mg/kg) (D) significantly reduced KA-induced neuronal damage in the cortex of the FVB mice as compared to control (A). The black bar represents 50 $\mu$m, the arrow indicates a long shape neuron, and the arrowhead indicates a shrinking neuron (100×).

**Caspase-3 Activation.** The death of nerve cells during SE is partly due to apoptosis. GTL and GA significantly reduced KA-induced apoptosis in hippocampus cells of the mice brain (Figure 2). Therefore, we further evaluated whether the apoptotic signaling pathways was involved in the apoptosis of KA-treated PC12 cells. The results showed that KA or GA affected caspase-3 activation (Figure 7). Cells were treated with KA (150 $\mu$M) alone or with GTL extract or GA in various concentrations for 24 h. Both GTL and GA decreased the caspase-3 activity significantly in KA-treated PC12 cells.

**COX-2 and MAPKs Activation.** The effect of GA or GTL extract on KA-induced activation of signaling pathways in PC12

cells was evaluated by Western blot assay. KA induced the activation of MAP kinases (JNK, ERK, and P38MAP kinase), RhoA, and COX-2 in PC12 cells at 30 min, but only the activated COX-2 and MAPKs expression, not RhoA, was suppressed by GA and GTL extract as compared to KA controls. GA suppressed ~95% COX-2 expression, whereas GA and GTL suppressed 80−90% p38 MAPK expression as compared to KA controls (Figure 8).

**Effect of GA on PGE$_2$ Production in PC12 Cells.** Since COX-2 control PGE$_2$ production, we then examined whether the KA-induced COX-2 expression would affect PGE$_2$ production. The result showed that GTL extract and GA
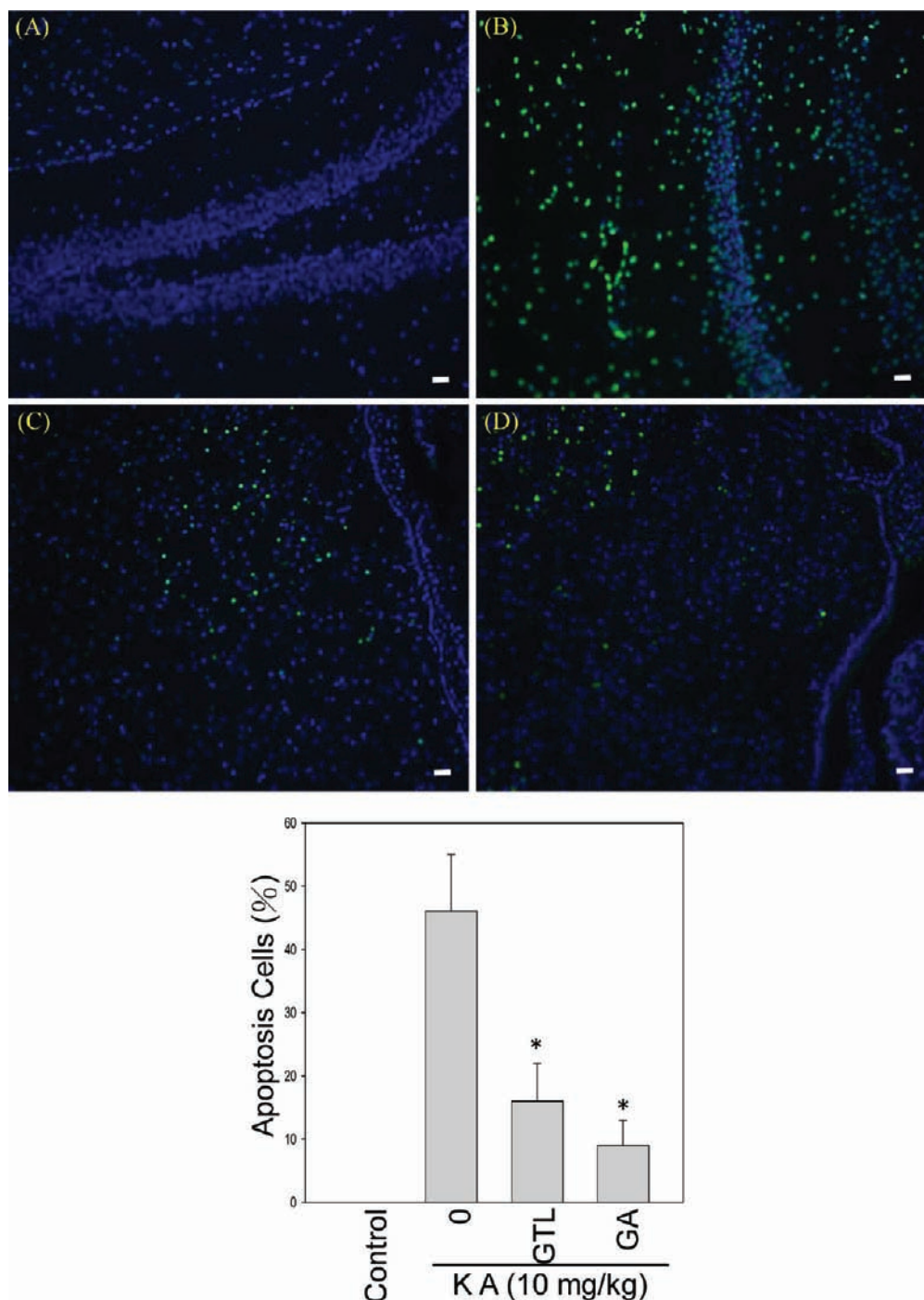
**Figure 2.** DAPI and TUNEL staining of hippocampi form KA-stressed mice. KA-induced apoptosis (green fluorescence) of hippocampus neurons on vehicle control mice (B). The TUNEL staining showed that 10 mg/kg GTL (C) and 1 mg/kg GA (D) significantly reduced KA-induced apoptosis in hippocampus of the FVB mice brain as compared to control (A). The white bar represents 50 $\mu$m (200×).

significantly reduced the $PGE_2$ production in KA-stressed PC12 cells as predicted (Figure 9).

### ■ DISCUSSION

The present results showed that GTL and GA protected male FVB mice from KA-induced brain injury. MDA and apoptosis were significantly reduced in the GA and GTL-treated animals as compared with the vehicle control. This could also be confirmed by the GA and GTL effect in vitro that showed a decrease in LDH release, ROS generation, lipid peroxidation, caspase-3 activation, and an increase in cell viability of KA-

stimulated PC12 cells. We found that GA was the major bioactive components from our fresh tea leaves. GA has been long advocated for protection against cancer, oxidative stress, inflammation, and diabetes, but few studies have evaluated its efficacy and possible mode of action. The present study demonstrates that GA effectively protected PC12 cells from KA-induced injury in a dose-dependent manner. The GA and GTL extract decreased the KA-induced $Ca^{2+}$ and ROS releases and lipid peroxidation from PC12 cells and brain neuron tissue of FVB mice. Western blot analysis revealed that MAPKs, RhoA, and COX-2 expression were increased in PC12 cells
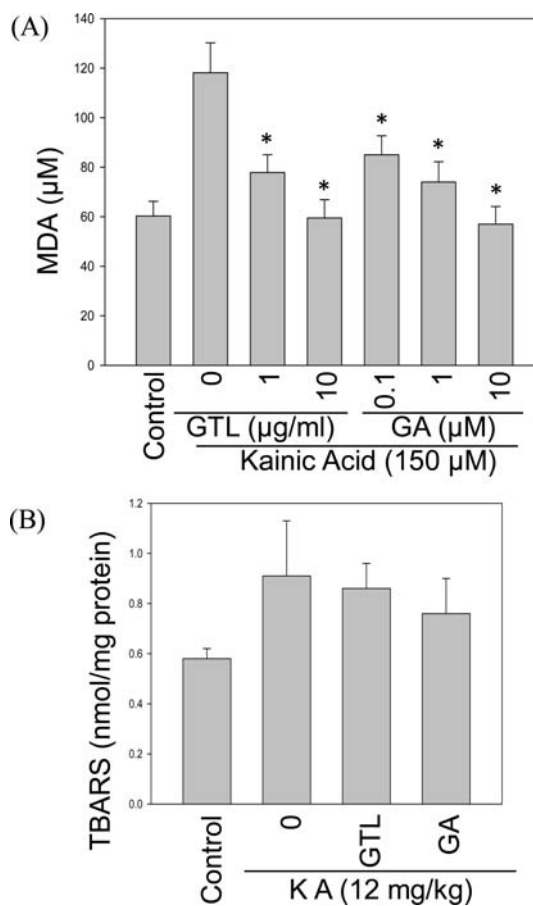
(A)

(B)
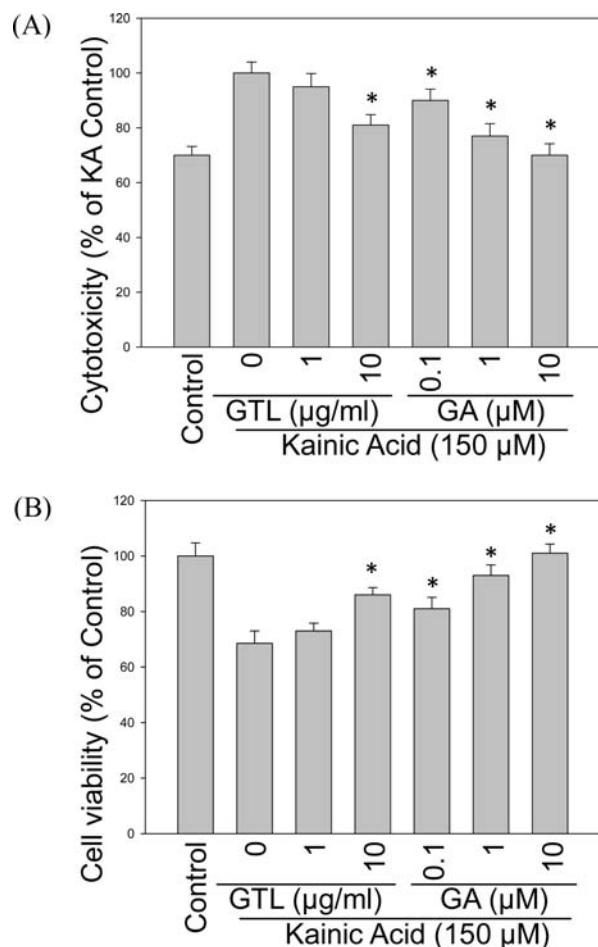
**Figure 3.** Effect of GTL extract and GA on the viability and cytotoxicity of KA-stressed PC12 cells. Cells were treated with KA (150 μM) alone or with various concentrations of GTL extract (1 and 10 μg/mL) or GA (0.1, 1, and 10 μM) for 24 h. LDH (A) release was decreased, and cell viability (B) was increased by GTL extract (only 10 μg/mL) and GA (0.1, 1, and 10 μM). *, $p < 0.01$ as compared to KA control.
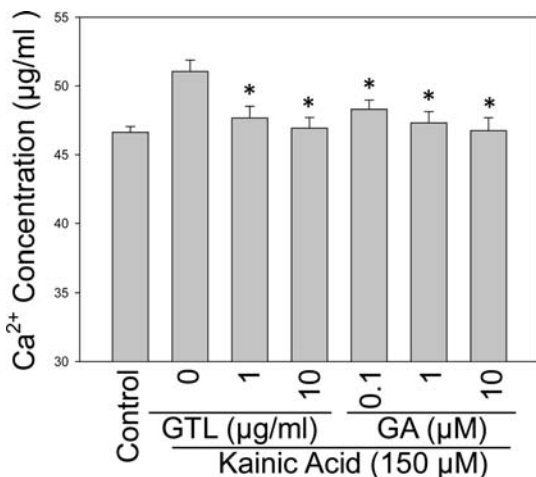


**Figure 4.** Effect of GTL extract and GA on $Ca^{2+}$ generation from KA-treated PC12 cells. Cells were treated with KA (150 μM) alone or with various concentrations of GTL extract or GA for 24 h. GTL and GA effectively reduce the release of $Ca^{2+}$ from PC12 cells under KA stress. *, $p < 0.01$ as compared to the KA control.
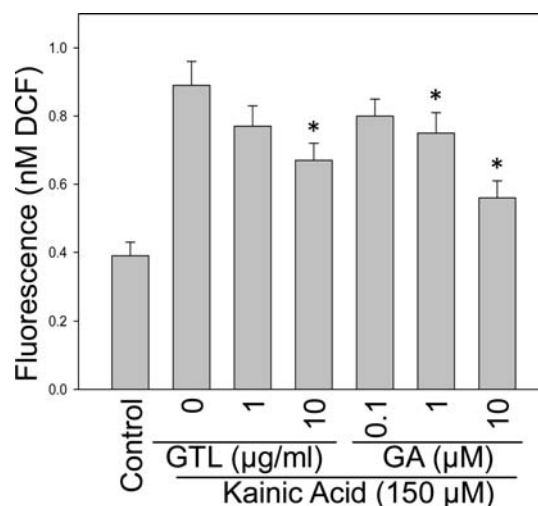


**Figure 5.** Effect of GTL extract and GA on ROS generation in PC12 cells under KA stress. The PC12 cells were treated with KA stress (150 μM) alone and GTL extract (1 and 10 μg/mL) or GA (0.1, 1, and 10 μM) for 120 min. GTL extract (10 μg/mL) and GA (1 and 10 μg/mL) effectively reduced the ROS production of KA-stressed PC12 cells at 120 min. *, $p < 0.01$ as compared to the KA control.



(A)

(B)

**Figure 6.** In vitro and in vivo effect of GTL extract and GA on the KA-induced oxidative stress. KA-induced lipid peroxidation of PC12 cells and brain neuron tissue of FVB mice were determined by ELISA and spectrophotometry, respectively. GTL or GA effectively reduced lipid peroxidation of PC12 cells by under 24 h KA stress (A) and brain neuron tissue of mice with 2 h of KA-induced SE (B). *, $p < 0.01$ as compared to the KA control.
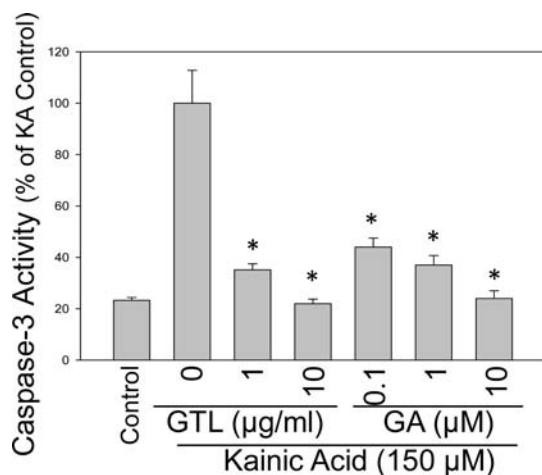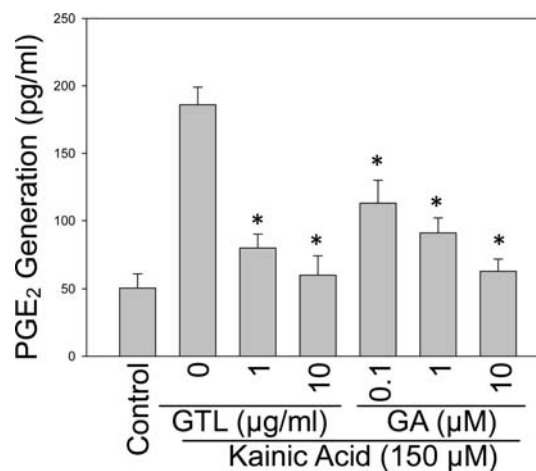
**Figure 7.** KA-induced caspase-3 activation. Cells were treated with KA (150 $\mu M$) alone or with GTL extract and GA in various concentrations for 24 h. Both GTL and GA decreased the caspase-3 activity significantly; *, $p < 0.01$ as compared to the KA control.



**Figure 8.** Effect of GTL extract and GA on KA-activated signaling pathway. COX-2, JNK, ERK, p38 MAP kinases, and RhoA expression in PC12 cell under KA stress for 30 min was determined by Western blot assay. Values represent the mean from three independent experiments. #, $p < 0.05$ as compared to the KA control.



**Figure 9.** Effect of GTL extract and GA on PGE$_2$ production. GTL extract and GA significantly reduced the PGE$_2$ production of KA-induced PC12 cells. *, $p < 0.01$ as compared to the KA control.

treated PC12 cells. KA-induced ROS accumulation was significantly reduced by GTL extract or GA (Figure 5). This finding is consistent with those of earlier reports that kainate induces lipid peroxidation in rat neurons.[34,35] Lipid peroxidation is essential for assessing the role of oxidative injury in pathophysiological disorders.[36,37] Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of saturated or unsaturated lipids. We found that KA induced the activation of MAP kinases (JNK, ERK, and p38), RhoA, and COX-2 in PC12 cells. However, it is surprising that the expression of KA-activated COX-2 and MAPKs was reduced by GA and GTL extract. In particular, GA significantly suppressed KA-activated COX-2 and MAPK p38 expression. This finding is supported by the results of a study, indicating that administration of tea extract (TF3) to rats with cerebral ischemia-reperfusion reduced mRNA and protein expression of COX-2, iNOS, and NF-κB activation in treated animals.[38] An in vitro study showed that antioxidants suppressed nitric oxide and PGE$_2$ production and COX-2 activity in lipopolysaccharide (LPS)-activated macrophages, RAW 264.7, and microglial cells.[39,40] Similarly, Icariin attenuated lipopolysaccharide-induced microglial activation and resultant death of neurons by inhibiting TAK1/IKK/NF-κB and JNK/p38 MAPK pathways.[40] The present results are consistent with those of previous reports that show KA-induced neuronal death is prevented either by inhibition of the enzyme xanthine oxidase, a cellular source of superoxide anions, or by the addition of free radical scavengers to the culture medium.[41] ROS generation has been shown to be correlated with KA-induced excitotoxicity in several studies.[18,20,41,42] The ability of kainate to induce lipid peroxidation is also related to the exposure of the brain to excitotoxins.[42] It is generally accepted that neuronal degeneration after KA administration is associated with a depletion of ATP levels and accumulation of $[Ca^{2+}]i$. The increase in $[Ca^{2+}]i$ may trigger $Ca^{2+}$-activated free radical formation.[41] Thus, our data showing the suppression of ROS and $Ca^{2+}$ release were consistent with the advocated protective role of GA and GTL extract.

The role of cerebral COX-2 mRNA and protein in KA toxicity has also been reported.[43−45] The KA-induced COX-2 expression parallels the appearance of neuronal apoptotic features.[43] The KA-inducted COX-2 is also involved in free radicals formation.[46] Several protease families are implicated in apoptosis, the most prominent being caspases.[47] However, we did find that KA affect the caspase-3 activation in PC12 cells. Because COX-2 may be involved in pathways leading to

under KA stress. However, COX-2 and MAPK p38, but not RhoA, expression were significantly reduced by GA (1.0 $\mu M$). Furthermore, GA and GTL were able to reduce PGE$_2$ production by PC12 cells under KA stress.

PC12 cells derived from rat pheochromacytoma have been widely used for neurological studies.[32,33] Significant increases in ROS accumulation and lipid peroxidation were observed in KA-

neuronal death, the additional mechanisms of GA could account for its neuroprotective effects, such as inhibition of KA-induced inflammatory mediators.[46] Because gallate suppresses PGE$_2$ production and COX-2 activity in LPS-activated macrophages,[39] GA and GTL extract, as predicted, reduced the PGE$_2$ production dose dependently and COX-2 activation in KA-induced PC12 cells. Taken together, these results indicate that antioxidant and anti-inflammatory effects might account for the protective mechanisms of GA on KA-induced PC12 cell injury.

Present data also showed that GA or GTL could decrease the severity of seizure behavior. However, further studies are needed to confirm whether GA has direct effects on seizure behavior and the related molecular mechanisms. The present results are consistent with those of previous reports that antioxidants such as resveratrol[13] and vitamin E[48] also provide protection against SE in various animal models in terms of the oxidative stress or convulsions. Resveratrol protects against KA-induced neuronal damage and subsequent epilepsy.[49] Stopping the seizure activity early is the best way to prevent SE-induced free radicals formation and neuronal damage. However, clinical experience shows that SE can be refractory to the commonly used medications. Therefore, intervention by antioxidants can be a potential beneficial approach in the treatment of SE.

We found that fresh summer green tea leaves (*C. sinensis*) had a higher GA content than commercial green teas. GA is also abundant in red wine, fruit, and legumes, and metabolites of GA are potent antioxidants and anti-inflammatory agents.[2−5] This suggests that natural antioxidants play an important role in neuroprotection against excitotoxins, and GA in the fresh green tea leaves was responsible for this protection. Green tea leaf extract ameliorates oxidative stress in KA-induced SE. The molecular mechanisms of GTL extract and GA on SE-induced excitotoxicity warrant further study for their therapeutic potential.

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### Notes
The authors declare no competing financial interest.

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