Regular Article

Echinacoside, an Active Constituent of *Cistanche Herba*, Exerts a Neuroprotective Effect in a Kainic Acid Rat Model by Inhibiting Inflammatory Processes and Activating the Akt/GSK3β Pathway

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Echinacoside is a major compound of *Cistanche Herb* and has glutamate release-inhibiting activity in the brain. Given the involvement of excitotoxicity caused by massive glutamate in the pathophysiology of epilepsy, we explored the antiepileptic effect of echinacoside on kainic acid-induced seizures in rats. The rats were intraperitoneally administrated echinacoside for 30 min prior to intraperitoneal injection with kainic acid. The results showed that kainic acid induced seizure-like behavioral patterns, increased glutamate concentrations, caused neuronal loss and microglial activation, and stimulated proinflammatory cytokine gene expression in the hippocampus. These kainic acid-induced alternations were found to be attenuated by echinacoside pretreatment. Furthermore, decreased Akt and glycogen synthase kinase 3β (GSK3β) phosphorylation as well as Bcl-2 expression in the hippocampus was reversed by the echinacoside pretreatment. These results demonstrate that echinacoside exert its antiepileptic and neuroprotective actions in a kainic acid rat model through suppressing inflammatory response and activating the Akt/GSK3β signaling. Therefore, the present study suggests that echinacoside is the potentially useful in the prevention of epilepsy.

Key words echinacoside; kainic acid; epilepsy; neuroprotective effect; hippocampus

Epilepsy is a prevalent brain disorder worldwide. It affects more than 65 million people, nearly 1% of the world's population.[1] Synthetic antiepileptic drugs are widely available in the pharmaceutical market; however, these drugs are only effective in 60–70% of patients and have adverse side effects.[2–4] Thus, there is an unmet need to search a drug with more efficacious and safer. Medicinal plants represent a potential source of such compounds.[5,6]

In traditional Chinese herbal medicine, *Cistanche Herba* has long been used for the treatment of epilepsy.[7,8] Echinacoside is a major component of *Cistanche Herba*[9] and has many pharmacological properties, such as antioxidant, anti-inflammatory, antineoplastic, hepatoprotective, and immune modulatory activity.[10,11] Moreover, it has been reported that echinacoside can entry the brain and elicit neuroprotection.[12–16] We previously demonstrated that echinacoside reduces glutamate release in rat cerebrocortical nerve terminals.[17] Because glutamate is a major excitatory neurotransmitter in the mammalian brain and plays a crucial role in the pathophysiology of epilepsy,[18,19] we suggested that echinacoside has antiepileptic activity. To elucidate this hypothesis, a kainic acid rat model was used in this study.

Kainic acid is a glutamate derivative and its single systemic injection to rodents results in seizures, neuroinflammation, and neuronal degeneration or death on selective population of neurons in the brain.[20–22] These pathological alternations are similar to human temporal lobe epilepsy.[23,24] Administering kainic acid to rodents is generally considered to induce an adequate model of epilepsy. To date, no study has investigated the antiepileptic and neuroprotective properties of echinacoside in a kainic acid-injected animal model. Therefore, our study aims to evaluate the effect and its possible mechanism of echinacoside preadministration in kainic acid-treated rats.

MATERIALS AND METHODS

Animals and Seizure Activity Male Sprague–Dawley rats (BioLASCO, Taipei, Taiwan) weighing 150–200 g were used throughout the study. The rats were randomly assigned to four groups as follows: i) Group 1: dimethylsulfoxide-treated group (control); ii) Group 2: kainic acid-treated group; iii) Group 3: echinacoside 10 mg/kg+kainic acid group; iv) Group 4: echinacoside 50 mg/kg+kainic acid group. Echinacoside (ChemFaces, Wuhan, P.R.C.) was injected (intraperitoneal administration) 30 min prior to kainic acid (15 mg/kg) intraperitoneal injection. Seizure activity was rated during a 4 h period after kainic acid injection according to the Racine's scale.[25] The dose and schedule of administration were chosen on the basis of previous studies.[22,26,27] The experiments on animals were approved by the Institutional Animal Care and Use Committee at the Fu Jen Catholic University, and carried out in accordance with the protocols issued, which followed National Institutes of Health Guide for the Care and Use of Laboratory (NAC 2011).

Glutamate Levels Determination Four hours after the kainic acid treatment, the rats were sacrificed by decapitation. The whole brain was dissected and the hippocampus of each rat was collected for analysis. The glutamate concentration of brain hippocampus tissue was determined according to the methods described by previous studies.[28,29]

Neutral Red and Fluoro-Jade B Staining Three days after the kainic acid injection, rats were anesthetized using chloral hydrate (650 mg/kg) and perfused transcardially with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS, pH 7.4). Brains were removed immediately after perfusion and stored in 4% paraformaldehyde overnight at 4°C. After post-fixation, the brains were immersed with 30% sucrose at 4°C for 24 h. The specimens were frozen rapidly and 30-μm-thick sections were cut by using a frozen microtome. Free-floating sections were stained with neutral red (Sigma-Aldrich, St. Louis, MO, U.S.A.) and Fluoro-Jade B (Millipore, CA, U.S.A.). Fluoro-Jade B-positive cells in the CA3 region of hippocampus was determined as previously described.[30,31]

Immunohistochemistry The sections were washed with 0.1 M PBS and blocked with 0.5% normal goat serum in 0.1 M PBS for 1 h at room temperature. Primary antibody (mouse monoclonal anti-OX-42 antibody, 1:1000; AbD Serotec, Oxford, U.K.) incubation was performed two overnight at 4°C. After repeated washes with PBS, the sections were incubated with biotinylated anti-mouse immunoglobulin G (IgG) (1:200) for 90 min, treated with the avidin–biotin complex (ABC) solution (1:1000) for 1 h at room temperature, and reacted with 0.025% 3,3′-diaminobenzidine and 0.0025% H_2O_2 until the desired stain intensity was achieved. Sections were mounted on slides using standard protocols. The stained slides were examined and imaged under a microscope (Olympus, Tokyo, Japan).[31] Analysis of microglial cell body and process length was carried out with ImageJ.[32]

Transmission Electron Microscopy Transmission electron microscopy was performed as previous described.[33] Briefly, the CA3 region of hippocampus was removed and fixed in a buffer containing 4% paraformaldehyde and 2.5% glutaraldehyde at 4°C for 24–36 h. After washes with PBS, CA3 regions were postfixed in 1% osmium tetroxide for 2 h, dehydrated with ethanol, and embedded in epoxy resin. Ultrathin sections (70 nm) were prepared by using an ultramicrotome (EM UC7, Leica Microsystems, Wetzlar, Germany). The sections were stained with uranyl acetate and lead citrate, and observed through transmission electron microscopy (Model JEM-1400, JEOL Ltd., Tokyo, Japan).

Quantitative Real-Time PCR RNA extraction and real-time quantitative PCR were performed as previously described.[34,35] Total RNA was extracted from the hippocampus using *mir*Vana™ miRNA Isolation Kit (Life Technologies, Grand Island, NY, U.S.A.) following the manufacturer's protocol. Genomic DNA was removed by incubation with deoxyribonuclease (DNase) I. The cDNA was synthesized by (GoScript reverse transcription system kit; Promega, Madison, WI, U.S.A.) and used for the SYBR Green (Roche Diagnostics, Mannheim, Germany) quantitative real-time PCR amplification with specific primers using the LightCycler 480 System (Roche Diagnostics). The protocol consisted of an initial denaturation step at 95°C for 3 min, followed by 45 cycles of denaturing at 95°C for 10 s, annealing at 56°C for 30 s. The mRNA levels of each target gene were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Fold-induction was calculated using the $2^{-\Delta\Delta CT}$ method as described in a previous study.[34]

Immunoblotting Twenty micrograms of protein extracts from the hippocampi were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrophoretic transfer onto polyvinylidene difluoride (PVDF) membrane. Membrane was blocked using 5% skimmed milk for 1 h at room temperature and incubated with the appropriate antibodies in 1% skimmed milk overnight at 4°C, followed by incubation with a secondary antibody for 1 h. Primary antibodies were rabbit monoclonal antibodies directed against Akt (1:2000), $pAkt_{Ser473}$ (1:2000), glycogen synthase kinase 3β (GSK3β) (1:2000), $pGSK3\beta_{Ser9}$ (1:500), Bcl-2 (1:1000), and β-actin (1:2000) obtained from Cell Signaling Technology (MA, U.S.A.). The secondary peroxidase-conjugated goat anti-rabbit antibody (1:5000) was obtained from Santa Cruz (CA, U.S.A.). Bands were visualized with enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.) using a gel imaging analysis system. The intensity of each band was measured using the Syngene software (Synoptics, Cambridge, U.K.).

Statistical Analysis Data are presented as the mean±standard error of the mean (S.E.M.). Statistical analysis was carried out by the two-tailed Student's *t* test when comparing two groups and by one-way ANOVA with Tukey's multiple comparisons *post hoc* tests when comparing more than two groups. Analysis was completed using SPSS software (Version 17.0; SPSS Inc., Chicago, IL, U.S.A.). $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Echinacoside on Kainic Acid-Induced Seizures and Elevation of Hippocampal Glutamate Levels To evaluate the neuroprotective effect of echinacoside against kainic acid-induced excitotoxicity, rats were pretreated with echinacoside (5, 10 and 50 mg/kg, intraperitoneal administration) for 30 min before kainic acid (15 mg/kg) intraperitoneal injection. Kainic acid (15 mg/kg) intraperitoneal injection resulted in seizures with latency and score of 92.5±5.3 min and 4.9±0.1, respectively (Figs. 1A, B). A significant increase in seizure latency[$F_{(2, 43)} = 40.2$, $p < 0.01$; Fig. 1A] and a significant decrease in seizure score [$F_{(2, 40)} = 21.5$, $p < 0.001$; Fig. 1B] were observed in echinacoside (10 or 50 mg/kg)-pretreated rats. However, echinacoside at 5 mg/kg had no significant effect on seizure latency and score ($p > 0.05$; Figs. 1A, B). Given the robust attenuation of seizure activity that was seen with 10 or 50 mg/kg echinacoside, these two dose of echinacoside were used in subsequent experiments to evaluate the mechanisms that underlie the ability of echinacoside to inhibit kainic acid-induced seizures. Figure 1C shows that elevation of hippocampal glutamate levels was noted in kainic acid-treated rats at 4 h when compared with dimethylsulfoxide-treated group (control; $p < 0.001$). However, decreased hippocampal glutamate levels were obtained in the echinacoside group (10 or 50 mg/kg) [$F_{(3, 16)} = 6.1$, $p < 0.01$; Fig. 1C].

Effect of Echinacoside on Kainic Acid-Induced Neuronal Injury in the CA3 Region of Hippocampus The degree of neuronal damage was evaluated 3 d after kainic acid injection
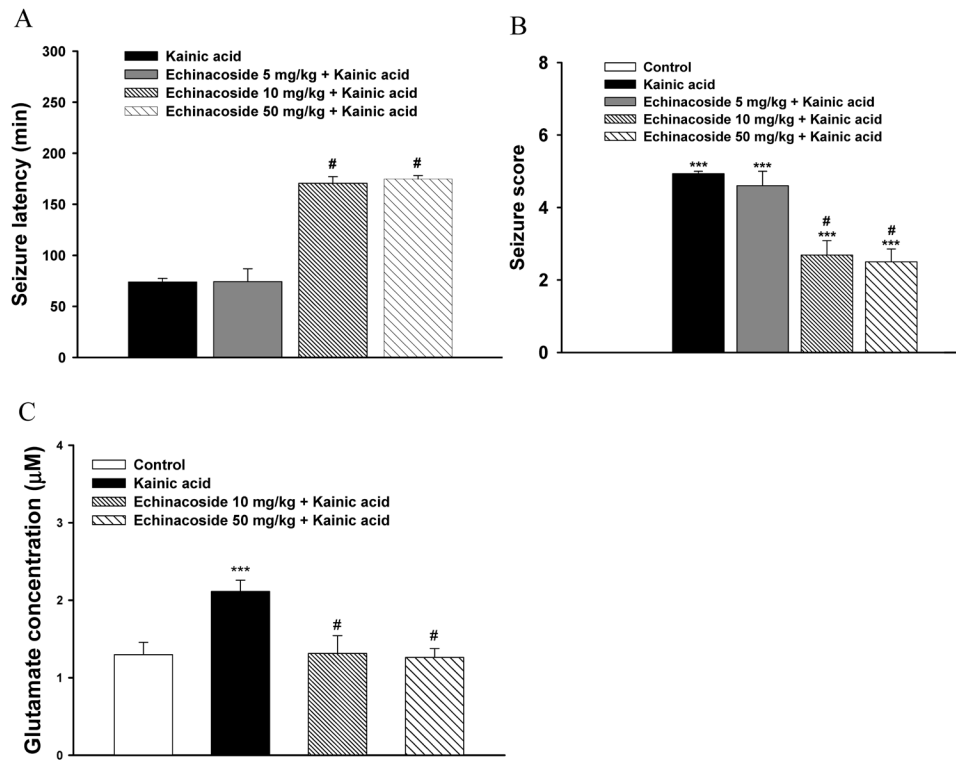
Fig. 1. Echinacoside Attenuates Kainic Acid-Induced Seizure Behavior in Rats

Echinacoside (5, 10 or 50 mg/kg) was intraperitoneally administered 30 min prior to kainic acid (15 mg/kg) intraperitoneal injection. The seizure behavior tests [seizure latency (A) and seizure score (B)] were evaluated during 1–4 h after kainic acid injection. (C) Glutamate levels in the hippocampus were measured with HPLC at 4 h after kainic acid treatment. Data are expressed as mean±S.E.M. ***$p<0.001$ compared with the control group. #$p<0.05$ compared with the kainic acid-treated group.
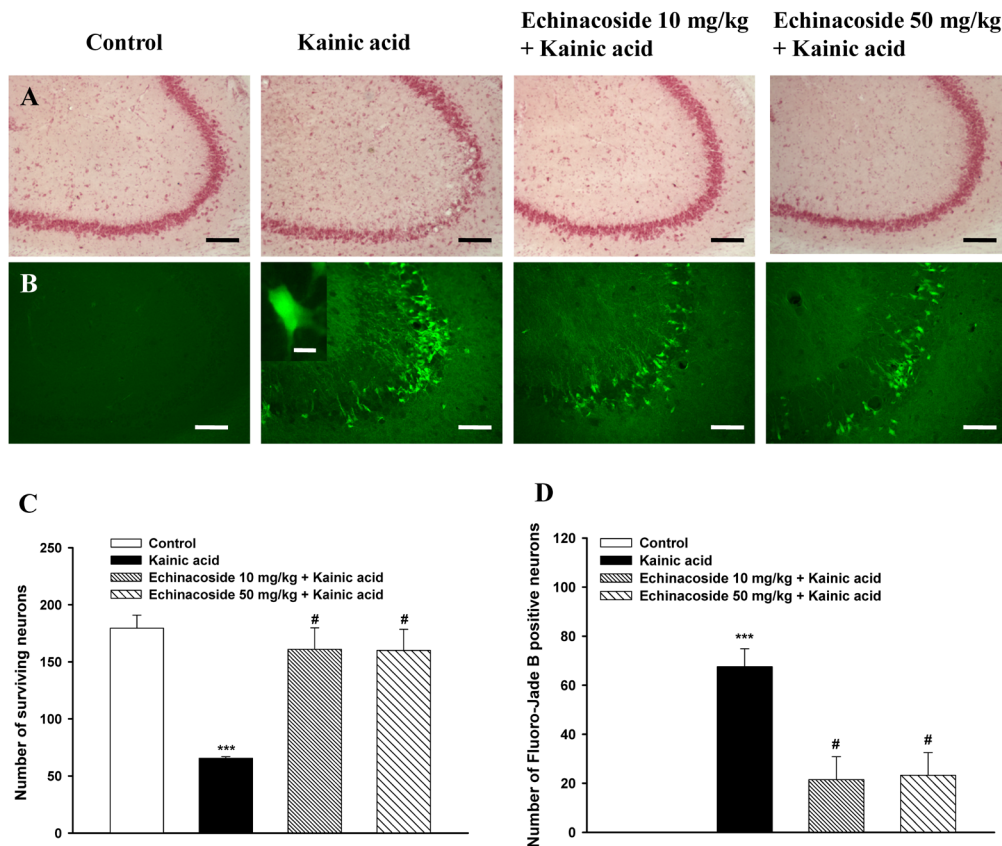

Fig. 2. Protective Action of Echinacoside against Kainic Acid-Induced Neuronal Damage in CA3 Region of Hippocampus

Determination of neuronal damage was examined by neutral red (A) and Fluoro-Jade B (B) staining. Scale bar, 100 μm. The insets are high magnification micrographs of neurons. Scale bar, 25 μm. Numbers of surviving neurons (C) and Fluoro-Jade B-positive neurons (D) were counted in the CA3 regions of hippocampus and presented as mean±S.E.M. ($n=6$–11). ***$p<0.001$ compared with the control group. #$p<0.05$ compared with the kainic acid-treated group. (Color figure can be accessed in the online version.)
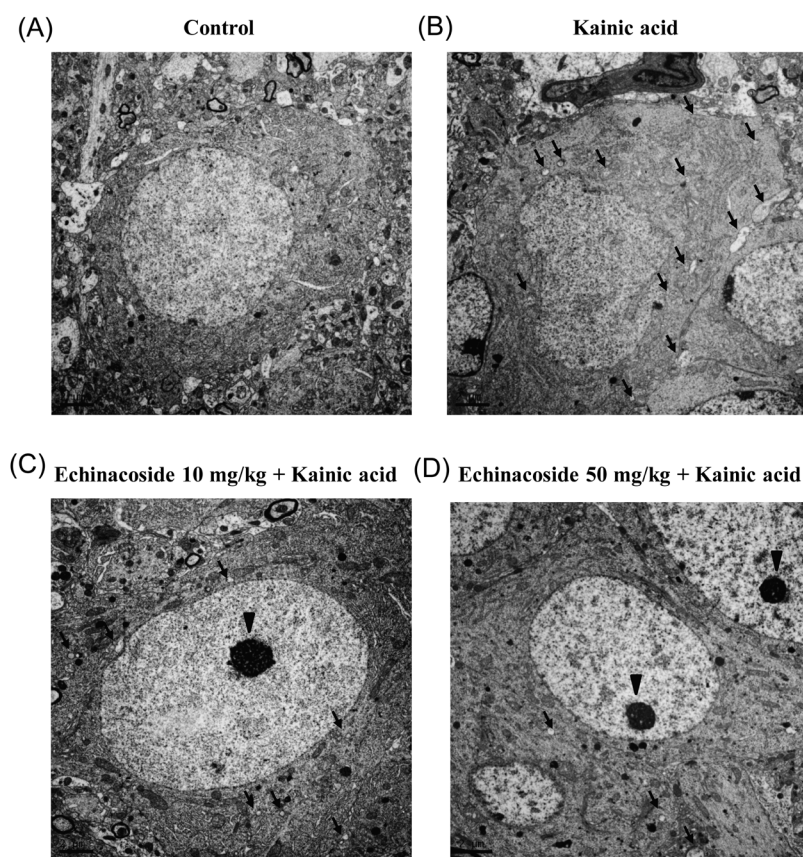
Fig. 3. The Hippocampal CA3 Nerve Cell Injury Measured by Transmission Electron Micrograph

(A) The control group, (B) the kainic acid group, (C, D) the echinacoside group. Arrows indicate autophagosomes and arrowheads indicate nucleolus. Scale bar, 2 μm.
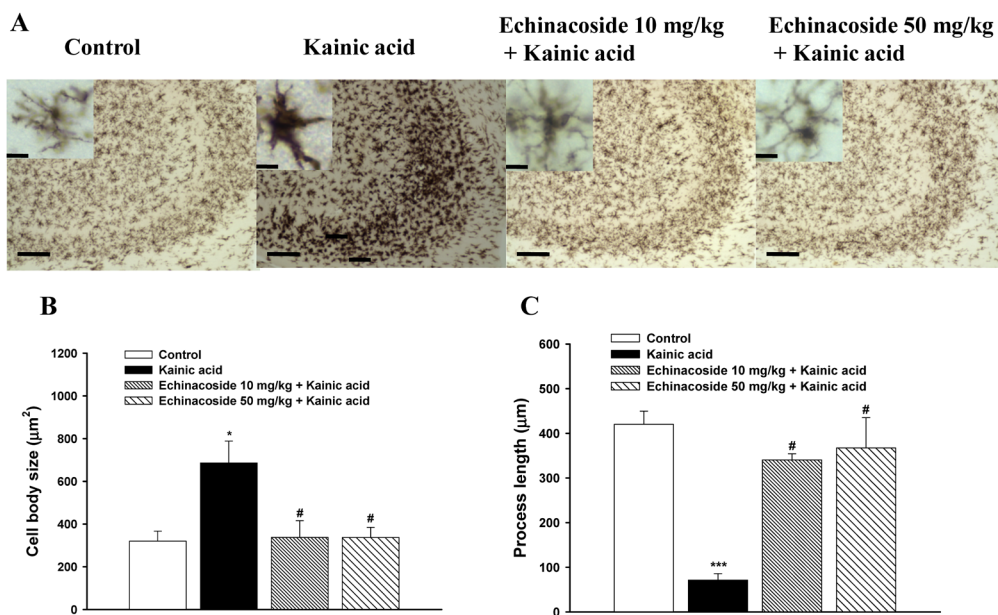

Fig. 4. Echinacoside Suppresses Kainic Acid-Induced Microglial Activation in the Hippocampal CA3 Region

(A) Representative coronal sections of the hippocampal CA3 region following OX-42 immunostaining 3 d after kainic acid treatment. Representative picture from five independent experiments were presented. Scale bar, 100 μm. The insets are high magnification micrographs of microglial cells. Scale bar, 25 μm. Measurements of cell body size (B) and longest processes (C) and presents as mean±S.E.M. (n=5–11). ***p<0.001, *p<0.01 as compared with the control group. #p<0.05 as compared with the kainic acid-treated group. (Color figure can be accessed in the online version.)

through neutral red staining or Fluoro-Jade B to detect surviving neurons and degenerating neurons, respectively. Neutral red staining revealed an apparent neuronal loss in CA3 of kainic acid-injected rats, compared with dimethylsulfoxide-treated rats (control; p<0.001; Figs. 2A, C). However, echinacoside pretreatment (10 or 50 mg/kg) effectively reduced kainic acid-induced neuronal loss in CA3 [$F_{(2, 13)}$=11.6, p<0.001; Figs. 2A, C]. Similarly, echinacoside-treated animals presented a
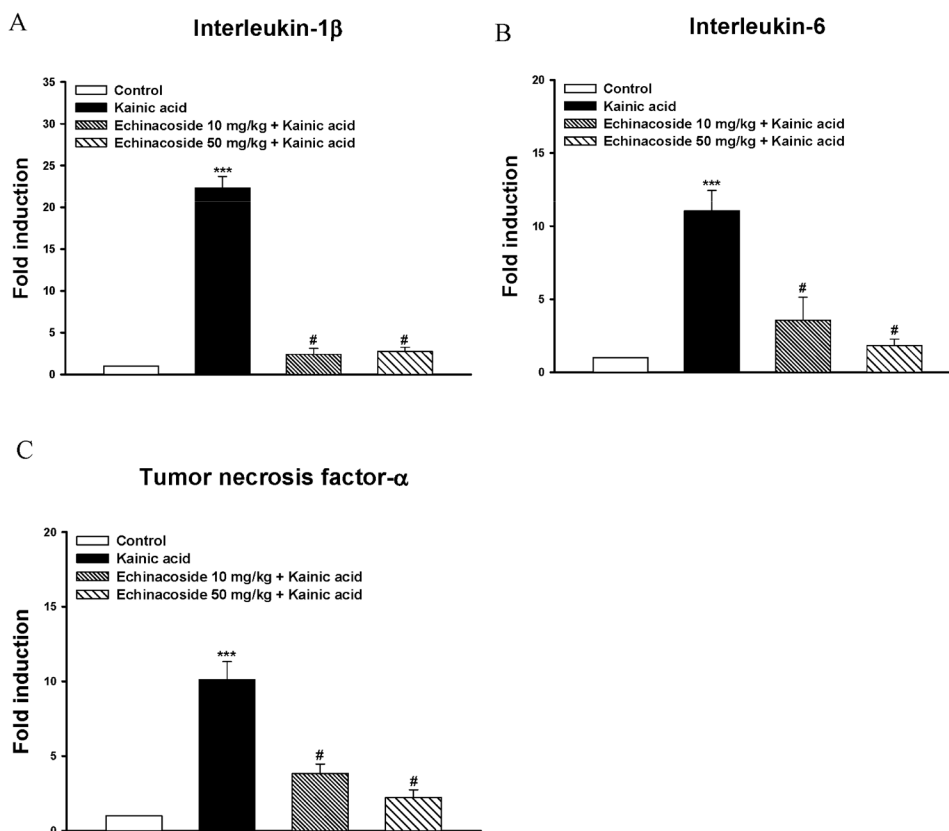
Fig. 5. Echinacoside Reduces Kainic Acid-Induced Proinflammatory Cytokine Expression in the Hippocampus

Relative quantification of interleukin-1β (A), interleukin-6 (B), and tumor necrosis factor-α (C) mRNA expression in the hippocampus were measured at 1 d after kainic acid treatment with RT-PCR. The expression levels of interleukin-1β, interleukin-6, and tumor necrosis factor-α were normalized to that of control. Data are expressed as mean±S.E.M. ***$p<0.001$ compared with the control group. #$p<0.05$ compared with the kainic acid-treated group.

diminished Fluoro-Jade B fluorescence in the CA3 regions of hippocampus after kainic acid injection ($p<0.001$), compared with the strong damage observed in kainic acid-injected rats [$F_{(2, 24)}=6.9$, $p<0.001$; Figs. 2B, D]. In addition, transmission electron microscopy shows that neuronal injury in CA3 was not obvious in the dimethylsulfoxide-treated (control) group. However, in the kainic acid-treated group, nerve cells had ultrastructural changes. Nerve cells exhibited autophagosome formations, deformed nuclei with condensed, and disruptive cell plasma membrane. In contrast, the neuronal injury in the echinacoside-pretreated group was apparently attenuated compared with the kainic acid-injected rats (Fig. 3).

Effect of Echinacoside on Kainic Acid-Induced Microglia Activation and Gene Expression of Proinflammatory Cytokines in the Hippocampus In the model for kainic acid-induced seizures and hippocampal neuronal death, microglia activation and proinflammatory cytokine production are increased in the hippocampus.[36,37] In order to investigate whether echinacoside affected inflammatory response in the hippocampus of kainic acid-injected rats, microglia activation was analyzed using the anti-OX42 antibody. In dimethylsulfoxide-treated rats (control group), the microglial cell bodies in the CA3 regions were small and their processes were long and thin (Fig. 4A). However, the number of activated microglial cells in the CA3 area of KA injected rats increased significantly, the microglial cell bodies were enlarged ($p<0.01$; Figs. 4A, B), and their cellular processes became shorter and thicker ($p<0.001$; Figs. 4A, C). In the animals pretreated with echinacoside (10 or 50 mg/kg)-pretreated rats, the number of

activated microglial cells was significantly decreased, and the microglial cell bodies were thin [$F_{(2, 13)}=6.2$, $p<0.01$; Fig. 4B] and their cytoplasmic processes were ramified [$F_{(2, 28)}=47.5$, $p<0.001$; Fig. 4C]. In addition, the expression levels of interleukin-1β, interleukin-6, and tumor necrosis factor-α mRNA in the hippocampus were apparently elevated at 1 d after kainic acid injection, compared to dimethylsulfoxide-injected animals (control) ($p<0.001$). However, decreased mRNA levels for these proinflammatory cytokines were obtained in the echinacoside group [interleukin-1β, $F_{(3, 18)}=154.7$, $p<0.001$; interleukin-6, $F_{(3, 17)}=17.9$, $p<0.001$; tumor necrosis factor-α, $F_{(3, 16)}=31.1$, $p<0.001$; Figs. 5A–C].

Effect of Echinacoside on Kainic Acid-Induced Decrease in the Levels of Hippocampal Phospho-Akt, Phospho-Glycogen Synthase Kinase 3β, and Bcl-2 The levels of phosphorylated Akt (pAkt) (Ser473), phosphorylated GSK3β (pGSK3β) (Ser9), and Bcl-2, was assayed in the hippocampus (Fig. 6). A substantial lowering in pAkt, pGSK-3β, and Bcl-2 expression in the hippocampus were observed in kainic acid-treated rats at 4 h when compared with dimethylsulfoxide-treated rats (control) ($p<0.001$). However, pretreatment with echinacoside (10 or 50 mg/kg) significantly increased the levels of pAkt, pGSK3β, and Bcl-2 compared with kainic acid treatment alone [pAkt, $F_{(3, 19)}=69.5$, $p<0.001$; pGSK3β, $F_{(3, 17)}=60.3$, $p<0.001$; Bcl-2, $F_{(3, 18)}=26.1$, $p<0.001$; Figs. 6A–C].
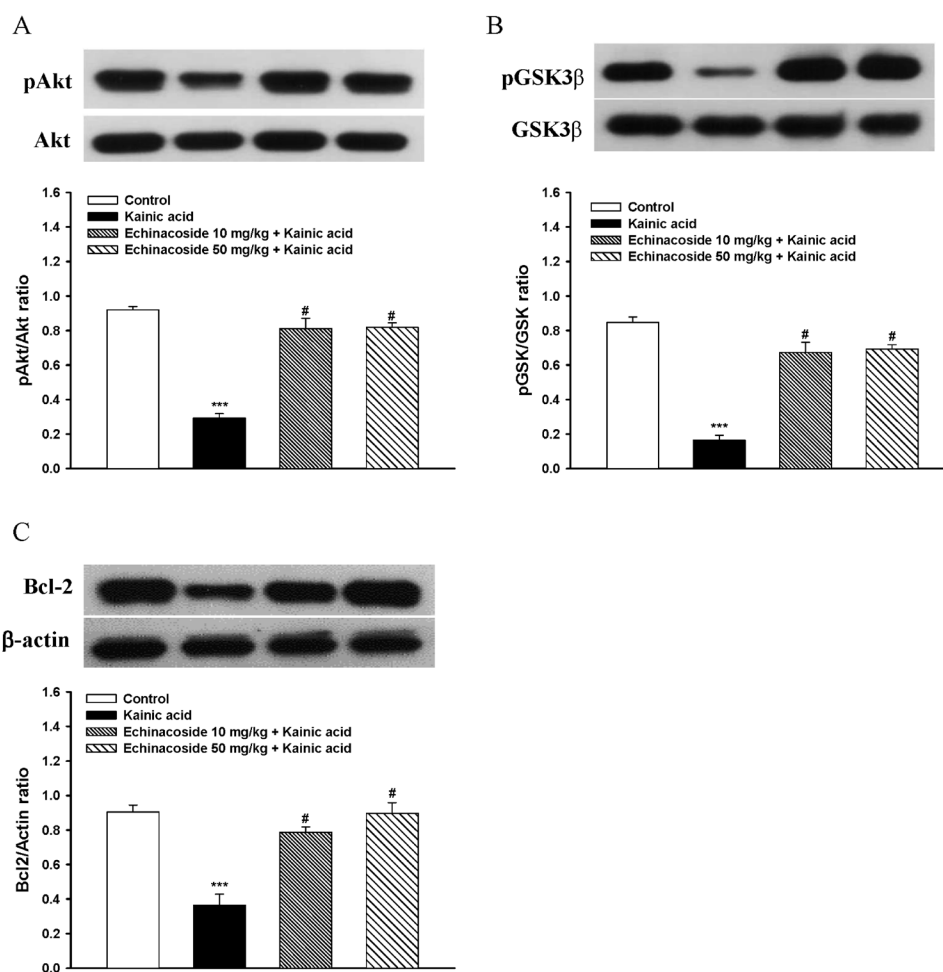
Fig. 6. Echinacoside Rescues Kainic Acid-Induced Inhibition of pAkt, pGSK3β, and Bcl-2 Expression in the Hippocampus

The expression levels of pAkt (A), pGSK3β (B), and Bcl-2 (C) in the whole hippocampus were determined with immunoblotting at 4h after kainic acid treatment. Data are expressed as mean±S.E.M. ***$p<0.001$ compared with the control group. #$p<0.05$ compared with the kainic acid-treated group.

DISCUSSION

Evidences indicated that glutamate plays a crucial role in the pathophysiology of epilepsy.[17,18] The glutamatergic hypothesis of epilepsy suggests that epileptogenic process is related to an increased glutamate concentration in the brain and a reduction in the concentration of this neurotransmitter is able to produce antiepileptic action.[38–40] Study from our laboratory demonstrated that echinacoside can reduce glutamate release from nerve terminals and suggested that echinacoside is likely to have an antiepileptic effect.[17] This suggestion was confirmed in the current study using a kainic acid-injected rat model. Kainic acid, a glutamate derivative, is widely used for inducing epilepsy in animal studies, because its neuropathological and biochemical characteristics are similar to those in humans.[23,24]

Intraperitoneal injection of kainic acid into rats causes seizures and neuronal damage, particularly in the CA3 regions of the hippocampus.[41–43] Furthermore, these pathological alternations produced by kainic acid are related to the massive release of glutamate.[41,44,45] In agreement with these findings, we observed that the intraperitoneal administration of kainic acid (15mg/kg) induced seizure behavior, increased glutamate levels in the hippocampus, and caused pyramidal cell loss in the hippocampal CA3 region. These kainic acid-induced

pathological alternations were attenuated by intraperitoneal pretreatment with echinacoside (10 or 50mg/kg). Furthermore, the hippocampal CA3 nerve cell injury evaluated by ultrastructural alternation with transmission electron microscopy was alleviated by echinacoside in the kainic acid rats. In addition, an increased number of autophagosomes was observed in CA3 cells after kainic acid administration. Pretreatment with echinacoside suppressed the increased formation of autophagosomes. Previous studies have suggested that autophagy activation is involved in kainic acid-induced neuronal death and inhibition of autophagy process seems to be neuroprotective.[46,47] Although the mechanism underlying the antiepileptic effect of echinacoside requires further exploration, the neuroprotective effect of echinacoside in the kainic acid animal model may be resulted from antiepileptic activity through inhibition of glutamate excitotoxicity and autophagy activation.

Neuroinflammation contributes considerably to delayed brain damage after acute injury and exerts a detrimental effect on a neurological outcome.[27] Inflammatory responses, such as microglia activation and inflammatory cytokine production, has been described in human epilepsy and in experimental models of epilepsy.[36,48] Moreover, the release of these proinflammatory cytokines from the activated microglial cells promotes kainic acid-induced neuronal damage in the hippocampus.[36,37] We showed that, while kainic acid increased the

activation of microglia and gene expression of proinflammatory cytokines (interleukin-1β, interleukin-6, and tumor necrosis factor-α) in the hippocampus, these effects were suppressed by echinacoside pretreatment. Therefore, we suggest that echinacoside through suppressing inflammatory processes, may contribute to its antiepileptic and neuroprotective effects in the kainic acid animal model. As already observed by our previous studies, several drugs and natural products also exert antiepileptic effects and neuroprotection against kainic acid-induced seizures, by means of their anti-inflammatory effect.[31,33,35]

Numerous protein kinase signaling cascades are known to be activated by kainic acid that play important roles in neuropotection. Among those that appear to have a particularly significant participation is Akt.[49,50] Akt mediates its neuroprotective effects by activating various downstream substrates, including GSK-3β and Bcl-2. Akt phosphorylates GSK-3β at Ser9, leading to its inactivation and the reinforcement of neuronal cell survival[51] because the active form of GSK-3β activates the mitochondrial death pathway.[52,53] Bcl-2 is a key member of the anti-apoptotic Bcl-2 family and plays a key role in suppressing mitochondrial-mediated apoptotic cell death. For example, Bcl-2 can protect the integrity of mitochondrial membrane and block cytochrome C release from mitochondria, leading to cell survival and the inhibition of apoptosis.[54] It has also reported that the overexpression of Bcl-2 protects neurons against excitotoxic insults *in vitro* and *in vivo*.[54,55] The present study observed a decreased Akt (Ser473) and GSK3β (Ser9) phosphorylation as well as Bcl-2 expression in the hippocampus of rats following kainic acid exposure, which is in accordance with previous studies.[56,57] Furthermore, echinacoside pretreatment rescued kainic acid-induced decrease in pAkt and pGSK3β as well as Bcl-2 expression. Thus, the neuroprotective effects of echinacoside in the kainic acid animal model might be partly mediated by preventing the downregulation of the Akt/GSK-3β/Bcl-2 pathway, thus increasing neuron survival.

Echinacoside is a potential new therapeutic candidate. This is because echinacoside can entry the brain and exhibits numerous benefits.[58] Numerous studies have demonstrated that echinacoside attenuates brain damage and alleviates motor or memory impairment in several experimental models *in vivo*.[12,14,15,59] Although the mechanisms that underlie the neuroprotective effects of echinacoside in the brain require elucidation, inhibited neuroinflammation, antioxidation, oxygen-free radical scavenging, and increased neurogenesis have been implicated.[14–16] In addition to these possible mechanisms, our previous[17] and present results suggest that the inhibition of glutamate-mediated overexcitation may partly contribute to the neuroprotective and antiepileptic effects of echinacoside in the brain. This is supported by the fact that kainic acid-induced seizure activity, neuroinflammation and brain damage are associated with the excessive release of glutamate and activation of glutamate receptors.[44,45] Furthermore, several clinically used antiepileptic drugs have been shown to attenuate the seizure activity induced by kainic acid[60,61] and decrease the glutamate release in human and rat brain tissues.[62,63] These findings pointed out that reduced glutamate levels is important for pharmacotherapeutic effects of antiepileptic drugs.

In conclusion, our results demonstrate that echinacoside, through suppressing inflammatory processes, decreasing glutamate-mediated overexcitation, as well as increasing Akt/GSK-3β activation and Bcl-2 expression, have a significant antiepileptic and neuroprotective effect in kainic acid-injected rats. Although the role of echinacoside on patients with epilepsy requires further evaluation, our findings suggest that it could be a valuable approach in epilepsy therapy.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Hesdorffer DC, Beck V, Begley CE, Bishop ML, Cushner-Weinstein S, Holmes GL, Shafer PO, Sirven JI, Austin JK. Research implications of the Institute of Medicine Report, epilepsy across the spectrum: promoting health and understanding. *Epilepsia*, **54**, 207–216 (2013).

2) Patsalos PN. Some new developments in therapeutic drug monitoring of antiepileptic drugs. *Ther. Drug Monit.*, **21**, 139 (1999).

3) Löscher W. Current status and future directions in the pharmacotherapy of epilepsy. *Trends Pharmacol. Sci.*, **23**, 113–118 (2002).

4) Rogawski MA, Löscher W. The neurobiology of antiepileptic drugs. *Nat. Rev. Neurosci.*, **5**, 553–564 (2004).

5) Grunze H, Langosch J, von Loewenich C, Walden J. Modulation of neural cell membrane conductance by the herbal anxiolytic and antiepileptic drug aswal. *Neuropsychobiology*, **42** (Suppl. 1), 28–32 (2000).

6) Sucher NJ, Carles MC. A pharmacological basis of herbal medicines for epilepsy. *Epilepsy Behav.*, **52** (Pt B), 308–318 (2015).

7) Tu PF, Wang B, Deyama T, Zhang ZG, Lou ZC. Analysis of phenylethanoid glycosides of Herba cistanchis by RP-HPLC. *Yao Xue Xue Bao*, **32**, 294–300 (1997).

8) Wang F, Wang WK. Chinese medicinal composition for treating epilepsy and preparation method thereof. Chinese Patent CN 101797352 B, 2012.

9) Zhao LX, Zhang CL, Zhao JX. Experience of WANG Guo-san in treating epilepsy. *China J. Trad. Chin. Med. Pharm.*, **6**, 25 (2011).

10) Karalliedde LD, Kappagoda CT. The challenge of tranditional Chinese medicines for allopathic practitioners. *Am. J. Physiol. Heart Circ. Physiol.*, **297**, H1967–H1969 (2009).

11) Lei L, Song ZH, Tu PF, Li YZ, Wu LJ, Chen FK. Metabolic regulation of phenylethanoid glycosides from Herba cistanches in dogs' gastrointestine. *Yao Xue Xue Bao*, **36**, 432–435 (2001).

12) Shiao YJ, Su MH, Lin HC, Wu CR. Echinacoside ameliorates the memory impairment and cholinergic deficit induced by amyloid beta peptides *via* the inhibition of amyloid deposition and toxicology. *Food Funct.*, **8**, 2283–2294 (2017).

13) Wu CR, Lin HC, Su MH. Reversal by aqueous extracts of *Cistanche tubulosa* from behavioral deficits in Alzheimer's disease-like rat model: relevance for amyloid deposition and central neurotransmitter function. *BMC Complement. Altern. Med.*, **14**, 202 (2014).

14) Zhang J, Zhang Z, Xiang J, Cai M, Yu Z, Li X, Wu T, Cai D. Neuroprotective effects of echinacoside on regulating the stress-active p38MAPK and NF-κB p52 signals in the mice model of Parkinson's disease. *Neurochem. Res.*, **42**, 975–985 (2017).

15) Zhao Q, Gao J, Li W, Cai D. Neurotrophic and neurorescue effects of Echinacoside in the subacute MPTP mouse model of Parkinson's disease. *Brain Res.*, **1346**, 224–236 (2010).

16) Zhu M, Lu C, Li W. Transient exposure to echinacoside is sufficient

to activate Trk signaling and protect neuronal cells from rotenone. *J. Neurochem.*, **124**, 571–580 (2013).

17) Lu CW, Lin TY, Huang SK, Wang SJ. Echinacoside inhibits glutamate release by suppressing voltage-dependent Ca^{2+} entry and protein kinase C in rat cerebrocortical nerve terminals. *Int. J. Mol. Sci.*, **17**, 1006 (2016).

18) Cavus I, Pan JW, Hetherington HP, Abi-Saab W, Zaveri HP, Vives KP, Krystal JH, Spencer SS, Spencer DD. Decreased hippocampal volume on MRI is associated with increased extracellular glutamate in epilepsy patients. *Epilepsia*, **49**, 1358–1366 (2008).

19) Lasoń W, Chlebicka M, Rejdak K. Research advances in basic mechanisms of seizures and antiepileptic drug action. *Pharmacol. Rep.*, **65**, 787–801 (2013).

20) Ben-Ari Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience*, **14**, 375–403 (1985).

21) Ben-Ari Y, Cossart R. Kainate, a double agent that generates seizures: two decades of progress. *Trends Neurosci.*, **23**, 580–587 (2000).

22) Friedman LK, Pellegrini-Giampietro DE, Sperber EF, Bennett MV, Moshé SL, Zukin RS. Kainate-induced status epilepticus alters glutamate and GABA$_A$ receptor gene expression in adult rat hippocampus: an *in situ* hybridization study. *J. Neurosci.*, **14**, 2697–2707 (1994).

23) Van Nieuwenhuyse B, Raedt R, Sprengers M, Dauwe I, Gadeyne S, Carrette E, Delbeke J, Wadman WJ, Boon P, Vonck K. The systemic kainic acid rat model of temporal lobe epilepsy: long-term EEG monitoring. *Brain Res.*, **1627**, 1–11 (2015).

24) Lévesque M, Avoli M. The kainic acid model of temporal lobe epilepsy. *Neurosci. Biobehav. Rev.*, **37**, 2887–2899 (2013).

25) Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr. Clin. Neurophysiol.*, **32**, 281–294 (1972).

26) Chang Y, Lu CW, Lin TY, Huang SK, Wang SJ. Baicalein, a constituent of *Scutellaria baicalensis*, reduces glutamate release and protects neuronal cell against kainic acid-induced excitotoxicity in rats. *Am. J. Chin. Med.*, **44**, 943–962 (2016).

27) Spigolon G, Veronesi C, Bonny C, Vercelli A. c-Jun N-terminal kinase signaling pathway in excitotoxic cell death following kainic acid-induced status epilepticus. *Eur. J. Neurosci.*, **31**, 1261–1272 (2010).

28) Chang CY, Lin TY, Lu CW, Huang SK, Wang YC, Chou SS, Wang SJ. Hesperidin inhibits glutamate release and exerts neuroprotection against excitotoxicity induced by kainic acid in the hippocampus of rats. *Neurotoxicology*, **50**, 157–169 (2015).

29) Morishima Y, Miyakawa T, Furuyashiki T, Tanaka Y, Mizuma H, Nakanishi S. Enhanced cocaine responsiveness and impaired motor coordination in metabotropic glutamate receptor subtype 2 knockout mice. *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 4170–4175 (2005).

30) Schmued LC, Hopkins KJ. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res.*, **874**, 123–130 (2000).

31) Lin TY, Huang WJ, Wu CC, Lu CW, Wang SJ. Acacetin inhibits glutamate release and prevents kainic acid-induced neurotoxicity in rats. *PLOS ONE*, **9**, e88644 (2014).

32) Young K, Morrison H. Quantifying microglia morphology from photomicrographs of immunohistochemistry prepared tissue using ImageJ. *J. Vis. Exp.*, **136**, e57648 (2018).

33) Lin TY, Lu CW, Wang SJ. Luteolin protects the hippocampus against neuron impairments induced by kainic acid in rats. *Neurotoxicology*, **55**, 48–57 (2016).

34) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2$^{-\Delta\Delta C_T}$ method. *Methods*, **25**, 402–408 (2001).

35) Chiu KM, Wu CC, Wang MJ, Lee MY, Wang SJ. Protective effects of bupivacaine against kainic acid-induced seizure and neuronal cell death in the rat hippocampus. *Biol. Pharm. Bull.*, **38**, 522–530 (2015).

36) Avignone E, Ulmann L, Levavasseur F, Rassendren F, Audinat E. Status epilepticus induces a particular microglial activation state characterized by enhanced purinergic signaling. *J. Neurosci.*, **28**, 9133–9144 (2008).

37) Shin EJ, Koh YH, Kim AY, Nah SY, Jeong JH, Chae JS, Kim SC, Yen TP, Yoon HJ, Kim WK, Ko KH, Kim HC. Ginsenosides attenuate kainic acid-induced synaptosomal oxidative stress *via* stimulation of adenosine A(2A) receptors in rat hippocampus. *Behav. Brain Res.*, **197**, 239–245 (2009).

38) Chapman AG, Elwes RD, Millan MH, Polkey CE, Meldrum BS. Role of glutamate and aspartate in epileptogenesis; contribution of microdialysis studies in animal and man. *Epilepsy Res. Suppl.*, **12**, 239–246 (1966).

39) Meldrum BS. The role of glutamate in epilepsy and other CNS disorders. *Neurology*, **44** (Suppl. 8), S14–S23 (1994).

40) Carlson H, Ronne-Engström E, Ungerstedt U, Hillered L. Seizure related elevations of extracellular amino acids in human focal epilepsy. *Neurosci. Lett.*, **140**, 30–32 (1992).

41) Sperk G. Kainic acid seizures in the rat. *Prog. Neurobiol.*, **42**, 1–32 (1994).

42) Coyle JT. Neurotoxic action of kainic acid. *J. Neurochem.*, **41**, 1–11 (1983).

43) Bahn S, Volk B, Wisden W. Kainate receptor gene expression in the developing rat brain. *J. Neurosci.*, **14**, 5525–5547 (1994).

44) Ferkany JW, Coyle JT. Kainic acid selectively stimulates the release of endogenous excitatory acidic amino acids. *J. Pharmacol. Exp. Ther.*, **225**, 399–406 (1983).

45) Chittajallu R, Vignes M, Dev KK, Barnes JM, Collingridge GL, Henley JM. Regulation of glutamate release by presynaptic kainate receptors in the hippocampus. *Nature*, **379**, 78–81 (1996).

46) Wang Y, Han R, Liang ZQ, Wu JC, Zhang XD, Gu ZL, Qin ZH. An autophagic mechanism is involved in apoptotic death of rat striatal neurons induced by the non-*N*-methyl-D-aspartate receptor agonist kainic acid. *Autophagy*, **4**, 214–226 (2008).

47) Dong XX, Wang YR, Qin S, Liang ZQ, Liu BH, Qin ZH, Wang Y. p53 mediates autophagy activation and mitochondria dysfunction in kainic acid-induced excitotoxicity in primary striatal neurons. *Neuroscience*, **207**, 52–64 (2012).

48) Kim DH, Yoon BH, Jung WY, Kim JM, Park SJ, Park DH, Huh Y, Park C, Cheong JH, Lee KT, Shin CY, Ryu JH. Sinapic acid attenuates kainic acid-induced hippocampal neuronal damage in mice. *Neuropharmacology*, **59**, 20–30 (2010).

49) Piermartiri TC, Vandresen-Filho S, de Araújo Herculano B, Martins WC, Dal'agnolo D, Stroeh E, Carqueja CL, Boeck CR, Tasca CI. Atorvastatin prevents hippocampal cell death due to quinolinic acid-induced seizures in mice by increasing Akt phosphorylation and glutamate uptake. *Neurotox. Res.*, **16**, 106–115 (2009).

50) Lee SH, Chun W, Kong PJ, Han JA, Cho BP, Kwon OY, Lee HJ, Kim SS. Sustained activation of Akt by melatonin contributes to the protection against kainic acid-induced neuronal death in hippocampus. *J. Pineal Res.*, **40**, 79–85 (2006).

51) Jope RS, Johnson GV. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem. Sci.*, **29**, 95–102 (2004).

52) Frame S, Cohen P, Biondi RM. A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. *Mol. Cell*, **7**, 1321–1327 (2001).

53) Crespo-Biel N, Canudas AM, Camins A, Pallàs M. Kainate induces AKT, ERK and cdk5/GSK3beta pathway deregulation, phosphorylates tau protein in mouse hippocampus. *Neurochem. Int.*, **50**, 435–442 (2007).

54) Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene*, **22**, 8590–8607 (2003).

55) Howard S, Bottino C, Brooke S, Cheng E, Giffard RG, Sapolsky R. Neuroprotective effects of bcl-2 overexpression in hippocampal

cultures: interactions with pathways of oxidative damage. *J. Neurochem.*, **83**, 914–923 (2002).

56) Wang ZH, Mong MC, Yang YC, Yin MC. Astatic acid and maslinic acid attenuated kainic acid-induced seizure through decreasing hippocampal inflammatory and oxidative stress. *Epilepsy Res.*, **139**, 28–34 (2018).

57) Kim YS, Choi MY, Lee DH, Jeon BT, Roh GS, Kim HJ, Kang SS, Cho GJ, Choi WS. Decreased interaction between FoxO3a and Akt correlates with seizure-induced neuronal death. *Epilepsy Res.*, **108**, 367–378 (2014).

58) Wei L, Chen H, Jiang Y, Tu P, Zhong M, Liu F, Liu C. Determination of ECH on cerebral ischemia injury rat plasma and brain tissue by HPLC method. *Chin. Pharm. Bull.*, **27**, 737–738 (2011).

59) Geng X, Tian X, Tu P, Pu X. Neuroprotective effects of echinacoside in the mouse MPTP model of Parkinson's disease. *Eur. J. Pharmacol.*, **564**, 66–74 (2007).

60) Halonen T, Miettinen R, Toppinen A, Tuunanen J, Kotti T, Riekkinen PJ Sr. Vigabatrin protects against kainic acid-induced neuronal damage in the rat hippocampus. *Neurosci. Lett.*, **195**, 13–16 (1995).

61) 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*, **49**, 163–167 (2008).

62) Kammerer M, Brawek B, Freiman TM, Jackisch R, Feuerstein TJ. Effects of antiepileptic drugs on glutamate release from rat and human neocortical synaptosomes. *Naunyn Schmiedebergs Arch. Pharmacol.*, **383**, 531–542 (2011).

63) Sitges M, Guarneros A, Nekrassov V. Effects of carbamazepine, phenytoin, valproic acid, oxcarbazepine, lamotrigine, topiramate and vinpocetine on the presynaptic Ca^{2+} channel-mediated release of [3H]glutamate: comparison with the Na^+ channel-mediated release. *Neuropharmacology*, **53**, 854–862 (2007).