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# Luteolin protects the hippocampus against neuron impairments induced by kainic acid in rats

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ABSTRACT

Glutamatergic excitotoxicity is crucial in the pathogenesis of numerous brain disorders. Luteolin, a flavonoid compound, inhibits glutamate release, however, its ability to affect glutamate-induced brain injury is unknown. Therefore, this study evaluated the protective effect of luteolin against brain damage induced by kainic acid (KA), a glutamate analog. Rats were treated with luteolin (10 or 50 mg/kg, intraperitoneally) 30 min before an intraperitoneal injection of KA (15 mg/kg). Luteolin treatment reduced the KA-induced seizure score and elevations of glutamate levels in the hippocampus. A histopathological analysis showed that luteolin attenuated KA-induced neuronal death and microglial activation in the hippocampus. An immunoblotting analysis showed that luteolin restored the KA-induced reduction in Akt phosphorylation in the hippocampus. Furthermore, a Morris water maze test revealed that luteolin effectively prevented KA-induced learning and memory impairments. The results suggest that luteolin protected rat brains from KA-induced excitotoxic damage by reducing glutamate levels, mitigating inflammation, and enhancing Akt activation in the hippocampus. Therefore, luteolin may be beneficial for preventing or treating brain disorders associated with excitotoxic neuronal damage.


## 1. Introduction

Glutamate is a major excitatory neurotransmitter in the mammalian brain and acts on ionotropic (*N*-methyl-D-aspartate [NMDA], α-amino-3-hyroxy-5-methylisoxazole proprionic acid [AMPA], and kainate) and metabotropic receptors (Sattler and Tymianski, 2001; Lau and Tymianski, 2010). However, excessive stimulation of glutamate receptors induces excitotoxicity and is associated with the pathology of numerous brain disorders, such as ischemia, epilepsy, stroke, Alzheimer's disease, and Parkinson's disease (Meldrum, 2000). Kainic acid (KA), an analog of glutamate, causes neuron depolarization and excessive calcium influx, resulting in reactive oxygen species production, mitochondrial dysfunction, oxidative stress, and inflammatory responses. These events are known to cause epileptiform seizures, neurodegeneration, memory loss, and neuronal cell death (Kim et al., 2010; Golechha et al., 2011; Han et al., 2012). Because of its behavioral and pathological similarities with human brain disorders, KA has extensively been used as a tool for experimentally mimicking human brain injuries (Wang et al., 2005; Zheng et al., 2011).

Flavonoids are potential candidates for treating brain disorders (Spencer, 2012; Solanki et al., 2015). Flavonoids are plant polyphenolic compounds abundantly present in fruits and vegetables and possess a wide range of pharmacological effects, including antioxidant, free-radical scavenging, antiinflammatory, anticancer, cardioprotective, and neuroprotective effects (Middleton et al., 2000; Havsteen, 2002; Romagnolo and Selmin, 2012; Daglia et al., 2014). The flavonoid luteolin (3′,4′,5, 7-tetrahydroxyflavone) is abundant in pepper, celery, broccoli, thyme, and chamomile tea, and has been shown to enter the brain, producing numerous beneficial effects (Lopez-Lazaro, 2009; Nabavi et al., 2015). For instance, administering luteolin to rats attenuated ischemia-, oxidative stress-, and trauma-induced brain damage (Qiao et al., 2012; Nazari et al., 2013; Zhang et al., 2013; Xu et al., 2014), reduced *N*-methyl-4-phenyl-pyridinium-induced neuroinflammation (Patil et al., 2014), exerted anxiolytic- and antidepressant-like effects (Coleta et al., 2008; Ishisaka et al., 2011), and improved cerebral hypoperfusion- or streptozotocin-induced cognitive dysfunction (Xu et al., 2010; Liu et al., 2013).

Despite several studies that demonstrate the neuroprotective properties of luteolin and the role of excessive release of glutamate

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in numerous central nervous system diseases, no study has investigated the role of luteolin in attenuating glutamate-mediated excitotoxicity. In our previous study, we demonstrated that luteolin inhibits glutamate release in the cortical nerve terminals (Lin et al., 2011). Therefore, this study investigated the effect of luteolin on KA-induced seizure behavior, elevated glutamate levels, neuronal death, microglial activation, and cognitive impairment in rats. In addition, we evaluated the effect of luteolin on Akt activation, which is associate with KA-induced neuronal death (Lee et al., 2006; Bhowmik et al., 2015).

## 2. Materials and methods

### 2.1. Chemicals

Rabbit polyclonal antibodies against phospho-Akt (Ser 473) was purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-OX-42 was purchased from AbD Serotec (Oxford, UK). The avidin-biotin-peroxidase complex (ABC) kit and biotinylated anti-mouse IgG were purchased from Vector (Burlingame, CA, USA). Luteolin with purity of >98%, KA, and all other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

### 2.2. Animals

Male Sprague–Dawley rats weighing 150–200 g were purchased from BioLASCO (Taiwan Co., Ltd., Taipei, Taiwan). The rats were housed in plastic cages at 22 ± 2 °C and 50% relative humidity with an alternating 12 h light-dark cycle; animal chow and water were provided ad libitum. All animal treatments adhered strictly to the institutional and international ethical guidelines for the care and use of laboratory animals. The experimental protocol was approved by the Fu Jen Institutional Animal Care and Utilization Committee. The rats were randomly divided into 4 groups: dimethylsulfoxide (DMSO)-treated group (control), KA-treated group, luteolin 10 mg/kg + KA group, and luteolin 50 mg/kg + KA group. Luteolin (10 and 50 mg/kg) was dissolved in a saline solution containing 1% DMSO and was administered (i.p.) 30 min before KA (15 mg/kg in 0.9% NaCl, pH 7.0, i.p.) injection. The rats were continuously monitored for seizure behavior for 4 h after KA injection and scored according to the scale devised by Racine (1972), as follows: stage 1 (facial clonus), stage 2 (nodding), stage 3 (forelimb clonus), stage 4 (forelimb clonus with rearing), and stage 5 (rearing, jumping, and falling). Seventy-five percent of the KA-injected rats survived, of which eighty percent developed epileptic seizures and were used in the subsequent experiments. The dose and schedule of administration were chosen based on previous experiments of our group and others (Friedman et al., 1994; Spigolon et al., 2010; Lin et al., 2013; Chang et al., 2015).

### 2.3. Glutamate levels

4 or 72 h after drug administration, the rats (n = 40) were sacrificed through decapitation and the brains were removed immediately. The hippocampi were dissected and homogenized in 300 μl of 200 mM ice-cold perchloric acid. After centrifugation (4000 rpm for 10 min at 4 °C), the supernatant (20 μl) was filtered and injected directly into a high-performance liquid chromatography (HPLC) system with an electrochemical detection (HTEC-500, Eicom, Kyoto, Japan). An Eicompak GU-GEL column, a glutamate oxidase-immobilized column (EENZYMPAK), and a platinum electrode set at 450 mV against an Ag/AgCl reference electrode were used. The mobile phase comprised 50 mM $NH_4Cl$ and 250 mg/l hexadecyltrimethylammonium bromide with pH set at 7.4. The column temperature was 35 °C, and the flow rate was 1.2 ml/min (Morishima et al., 2005; Chang et al., 2015).

### 2.4. Histological analysis of neuronal death

Three days after KA injection, the rats (n = 32) were anesthetized with chloral hydrate (650 mg/kg, ip) and perfused with an ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde infusion via the left ventricle of the heart. The brains were removed and postfixed with 4% paraformaldehyde overnight at 4 °C. After dehydrating in 30% sucrose, the brains were sectioned coronally at a thickness of 30 μm by using a frozen microtome. Three sections selected from a one-in-six series were collected from each rat and were stained separately with neutral red and Fluoro-Jade B. For neutral red staining, the sections were mounted on gelatin-coated slides, stained with 1% neutral red, dehydrated by passing through graded alcohols solutions (70, 80, 90, and 100%), placed in xylene, and covered with coverslipped. Staining for Fluoro-Jade B (Chemicon, Millipore Ltd., Billerica, MA, USA) was performed as described previously (Schmued and Hopkins, 2000). In brief, the sections were mounted on gelatin-coated slides and dried at room temperature. After rehydration in 100% ethanol (5 min), 70% ethanol (2 min), and distilled water (2 min), the sections were oxidized in 0.06% potassium permanganate for 15 min, washed with water and immersed in 0.001% Fluoro-Jade B solution for 30 min in the dark. The slides were washed with distilled water, air dried, and coverslips were placed. The hippocampal CA3 region was visualized under 100× magnification through upright fluorescence microscopy (Zeiss Axioskop 40, Goettingen, Germany); the digitized photomicrographs used for analysis were captured using a digital camera (Nikon D80, Tokyo, Japan). The number of Fluoro-Jade B-positive cells was counted in a 255 × 255 μm area of the hippocampal CA3 in 3 randomly selected sections in each rat, and the count was averaged for each rat by using a computer-assisted image analysis system (Image J; NIH Image, National Institutes of Health, Bethesda, MD, USA) by an examiner blinded to the experimental conditions. The results of labeled cells per 0.1 $mm^2$ are expressed as mean ± SEM.

### 2.5. Transmission electron microscopy

The rats (n = 12) were anesthetized with chloral hydrate (650 mg/kg, ip) and perfused with 4% paraformaldehyde 3 days after KA injection. The brains were removed and sectioned at a thickness of 200 μm by using a vibratome (VT1000S, Leica, Germany). The hippocampus was dissected into small pieces, and the pieces were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for 1 day. The specimens were then washed in PBS, postfixed in 1% osmium tetraoxide for 2 h, dehydrated, and embedded in epoxy resin. Semithin sections (70 nm thick) were prepared using an ultramicrotome (EM UC7, Leica Microsystems, Wetzlar, Germany) and examined through transmission electron microscopy (Model JEM-1400, JEOL Ltd., Tokyo, Japan) at 80 KV.

### 2.6. Immunohistochemistry for activated microglia

The free floating sections were incubated at 4 °C for 24 h in PBS containing mouse monoclonal anti-OX-42 antibody (1:1000), 0.3% Triton X-100, and 2% normal goat serum. The sections were then incubated for 90 min with biotinylated anti-mouse IgG (1:200) and treated with the ABC solution (1:1000) for 1 h at room temperature, followed by 6 min treatment with 0.025% of 3, 3′-diaminobenzidine in PBS containing 0.0025% hydrogen peroxide. After each incubation step, the sections were washed 3 times with PBS. Finally, the sections were mounted on gelatin-coated slides, dehydrated, cleared with xylene, and covered with coverslips by