บทความวิจัย (Research Article)

Neuroprotective effect of *Alpinia galanga* against neurodegeneration in the rat hippocampus induced by kainic acid

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Abstract

Injection of kainic acid (KA) can induce neurodegeneration and epilepsy in the experimental study. *Alpinia galanga (A. galanga)* exerted various biological activities including potent antioxidant and anti-inflammatory activities. However, the neuroprotective effect in KA model has not been elucidated. Thus, the authors examined the neuroprotective effect of *A. galanga* extract in the hippocampal rat's brain. Young adult male Wistar rats were randomly assigned into three groups including vehicle (sodium carboxymethyl cellulose, NaCMC) plus normal saline injection, vehicle plus KA injection and the *A. galanga* extract (200 mg/kg BW) plus KA injection. The rats were treated with either *A. galanga* extract or vehicle for two weeks before and two weeks after the injection of KA (0.8 µg) into the right hippocampus. At the end of the experiment, the rats were sacrificed and their brains were collected to determine the expression of glial fibrillary acidic protein (GFAP) and neuron density in the hippocampal CA3 subregion. KA injection into the hippocampus significantly induced neuronal loss and increased the expression of GFAP. The *A. galanga* extract treatment significantly attenuated the neurodegeneration induced by KA, as evident by the significant increase of neuron density but decrease the percent of GFAP immunoreactive expression compared with the vehicle-treated group with KA injection. The *A. galanga* extract showed the neuroprotective effect in KA-induced neurodegeneration. However, further studies are needed to explore the mechanisms of the extract to protect against neuronal loss.

Keywords: *Alpinia galanga*, glial fibrillary acidic protein, kainic acid, neurodegeneration

Introduction

According to the World Health Organization report, around 50 million people worldwide have epilepsy. Epilepsy is characterized by recurrent spontaneous seizures. They are brief episodes of involuntary movement that may involve a part of the body or the entire body. [1] Temporal lobe epilepsy (TLE) is the most common type of epilepsy in adults. A third of epileptic patients are resistant to existing antiepileptic drugs. [2] Therefore, studies are searching for new possible therapeutic herbal substances for this neurological disorder.

Animal models of TLE have been very useful in finding the basic cellular mechanisms of epileptogenesis particularly kainic acid (KA) induced neurodegeneration. [3, 4] Kainic acid is a potent agonist of kainate subtype glutamate receptors. In rodents, KA injection resulted in recurrent seizures, behavioral changes and consequent degeneration of selective populations of neurons in the brain. [5]

KA-induced oxidative stress has been a significant mechanism leading to neuronal loss. [6,7] Activation of KA receptor has been shown to elicit several cellular events, including the increase in intracellular $Ca^{2+,}$ production of reactive oxygen species (ROS), and other biochemical events leading to neuronal cell death. [8, 9] KA exerts its neural excitotoxicity by binding to kainate receptors (KAR), which have presynaptic modulatory and postsynaptic excitatory actions. [10] Many types of kainate subtype glutamate receptors are distributed in the hippocampus. The high affinity KAR receptors (KAR1 or GluK4) are highly expressed in cornu ammonis three (CA3), while KAR2 or GluK5 are highly expressed in both CA1 and CA3. This is partially why CA3 is highly susceptible to excitotoxic damage induced by KA. [11-13] The former study demonstrated dramatic neuronal loss in the CA3 in KA-induced neurodegeneration model. [5]

Astrocytes activation is another important characteristic of KA-induced neurodegeneration. [14] KA-lesion induced reactive astrocytes are often identified by increased immunoreactivity of glial fibrillary acidic protein (GFAP). It was demonstrated a significant increase of GFAP in the rat's hippocampus and the neuronal loss stained by Nissl in the CA3 subfield. [5] The consequences of activating astrocytes were the production of inflammatory mediators including interleukin (IL)-1b and tumor necrosis factor α (TNF-α) resulted in neurodegeneration. [5, 15] In recent years, studies demonstrated that the substances possess antioxidant and anti-inflammation properties can attenuate neuronal loss in a KA model. [16] For instance, it was reported that curcumin, a main active compound identified in *Curcuma longa* L., was of benefit for treating epilepsy. [17] Thus, the plants possess antioxidant and anti-inflammatory effects may also attenuate or even prevent KA-induced neuron cell death.

Alpinia galanga (L.) Willd *(A. galanga)*, or Kha in Thai, is a plant in the Zingiberace family. In Thailand, it has long been used as herbal spice for cooking and in medicinal folklore. Results from a growing number of experimental studies have shown that *A. galanga* exert antimicrobial, [18] anti-cancer, [19] anti-viral, [20] anti-inflammatory, [21] and antioxidant effects. [22] The methanolic extract of *A. galanga* exerts central nervous system stimulation. [23] In addition, the extract also attenuates memory impairment and exhibited neuroprotective effects in an amnesia model induced by Aβ. [24] Since *A. galanga* exerted antioxidant, anti-inflammatory and neuroprotective effects, the administration of *A. galanga* extract might be attenuate

neurodegeneration induced by KA. However, the available data about the neuroprotective effect of *A. galanga* in an epilepsy model induced by KA has not yet been reported. Therefore, the experiment was set up to elucidate the issue.

Material and Method

Plant materials and extract preparation: Plant samples, rhizomes of *Alpinia galanga* (L.) Willd (*A. galanga*) were bought from the farmer in Ban So, Mae Narua Subdistrict, Mueang District, Phayao Province between November 2016 and March 2017. The samples were cleaned and cut to small pieces then dried with a hot air oven at 60°C for 48 h. Dried samples (5 kg of *A. galanga*) were ground and extracted with 95% v/v ethanol by maceration. A vacuum was used to remove the ethanol from the extract.

Animals: Male Wistar rats, 8 weeks old, were derived from the Nomura Siam International Company Limited, Bangkok. The weight of the animals at the beginning of the experiment was 200 to 220 grams, and at the time they have received the KA injection the weight was more than 300 grams. They were housed 3 per cage and maintained in 12:12 light: dark cycle and given access to food and water ad libitum. All experiments were performed with the approval of the Ethical Committee of the Laboratory Animal Research Center, University of Phayao (5801040011). The rats were randomly divided into three groups including Group I-vehicle (sodium carboxymethyl cellulose; NaCMC) treated with sterile normal saline solution (NSS) injection, Group II-vehicle treated with KA injection and Group III-*A. galanga* extract (200 mg/kg BW) treated with KA injection, each group consisting of five animals. The dose of *A. galanga* extract was selected based on the previous study of Hanish Singh et al. [24]

Kainic acid injection: The previous evidence reported that a dose higher than 0.8 µg induced neuronal loss in the hippocampus together behavioral seizures. [25, 26] Therefore, KA of 0.8 µg was selected to induce neurodegeneration. After two weeks of either the extract or NaCMC administration, the rats were subjected to unilateral KA injection by using the stereotaxic apparatus. The operation was performed after anesthetizing the rats by intraperitoneal injection of sodium pentobarbital (50 mg/kg BW). The target area of injection was the right hippocampus, coordinates was -5.5 (anteroposterior; AP), 4.8 (mediolateral; ML) and -5.0 (dorsoventral; DV), as per the process used by previous study. [27] The rats received either KA injection at dose of 0.8 µg with the volume 1 µl (Sigma) or sterile normal saline solution (NSS) injection for the vehicle-treated with NSS injected group.

Histology and immunohistochemistry study: At the end of the experiment, the rats were sacrificed by using transcardial perfusion with 1 M phosphate buffer saline (PBS) solution followed by 4% paraformaldehyde. Then, the brains were processed with paraffin embedding method, cut 5-µm thickness with microtome and processed further histology and immunohistochemistry studies.

1) GFAP immunohistochemistry: Paraffin sections were deparaffinized in xylene and hydrated in a series of graded alcohols and distilled water. Sections were then boiled with a 10-mM sodium citrate buffer (pH 6.0) for 20 min in a microwave (750 W) and treated with 3% hydrogen peroxide (H_2O_2) to retrieve antigens. Then, sections were incubated for 60 min in 10% normal horse serum to block nonspecific binding and followed by incubating in primary antibodies (Mouse anti-GFAP, 1:100; Millipore) at 4°C overnight. Sections were then rinsed with 0.1 M PBS for 30 min and

incubated at room temperature for 2 h with biotinylated donkey anti-mouse secondary antibody (1:500; Jackson Immunoresearch). Sections were rinsed, incubated in 0.1% extravidin peroxidase for 60 min and then rinsed again. Immunolabelling was performed using a nickel-enhanced 3, 3'-diaminobenzidine (DAB) reaction with the addition of glucose oxidase (1:1000).

2) Cresyl violet staining: Slides were treated sequentially with the following steps. First, the sections were deparaffinized with xylene and a series of descending grade of ethanol (100%, 95% and 70%) and then brought to running tap water and distilled water. The sections were stained with cresyl violet (0.2%) for 10 min and then rinsed with distilled water. Then, sections were dehydrated through ascending graded of alcohol (70, 95 and 100%) and cleared in xylene and then coverslipped with mounting media.

Image acquisition, thresholding and cell count analysis: The images of hippocampal images were captured at 10x using a bright-field microscope connected to a computer running Olympus digital camera. The GFAP immunoreactive signal was determined using Image J software. The images were cropped into the CA3 area. These cropped regions were thresholded and the data were presented as the percentage of thresholded area (% thresholded area). The numbers of neuronal cells in the CA3 (40x) were thorough manual counted by using SXView program and data were expressed as the number of neurons (cells/200 μm^2).

Statistical Analysis: All data are presented as mean ± standard deviation (SD). Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by a post-hoc Bonferroni test. The significant difference was set at p-value <0.05.

Results

GFAP immunoreactive expression: Kainic acid injection into the right hippocampus caused an increase in GFAP expression as demonstrated in **Figure 1**. The GFAP expression in the hippocampal CA3 area of the vehicle-treated and *A. galanga*-treated groups with KA injection were higher than the vehicle-treated group with NSS injection (p-value <0.001). However, in the group treated with *A. galanga* extract showed the lower GFAP expression than the vehicle-treated group (p-value <0.01).

Nissl stained neuron density: Loss of the hippocampal neurons was observed in the CA3 of the hippocampus in the rats injected with KA. The significantly lower density of neurons in the vehicle-treated group with KA injection and the *A. galanga*-treated group with KA injection as compared to the vehicle-treated group with NSS injection (p-value <0.001) was observed as **Figure 2**. The treatment with *A. galanga* extract significantly attenuated the neuronal loss induced by KA as observed the higher density of neuron in the CA3 in the *A. galanga*-treated group as compared to the vehicle-treated group with KA injection (p-value <0.01).

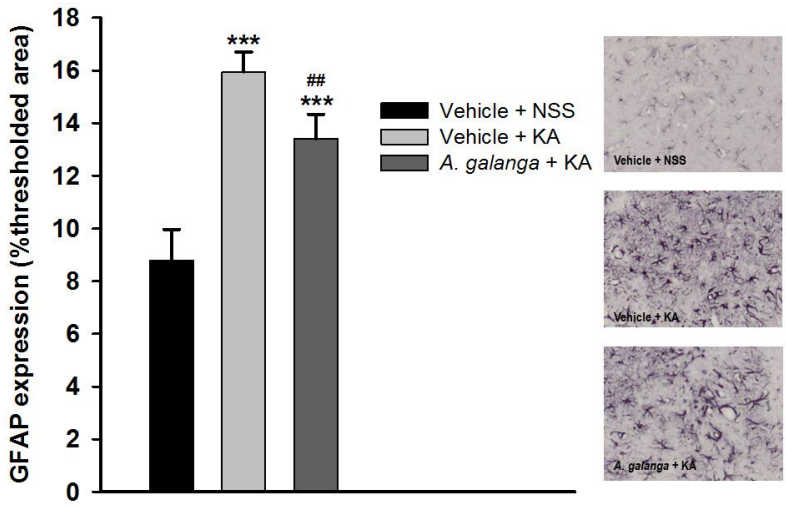

Figure 1 The effect of KA injection on the expression of GFAP in the CA3 area of the hippocampus. Data were presented as mean ± SD.

*** *p*-value <0.001 compared to the vehicle treated group with NSS injection;

p-value <0.01 compared to the vehicle treated group with KA injection. KA injection significantly increased GFAP expression in the KA injected groups but *A. galanga* treatment attenuated the increased expression of GFAP compared to the vehicle treated group.

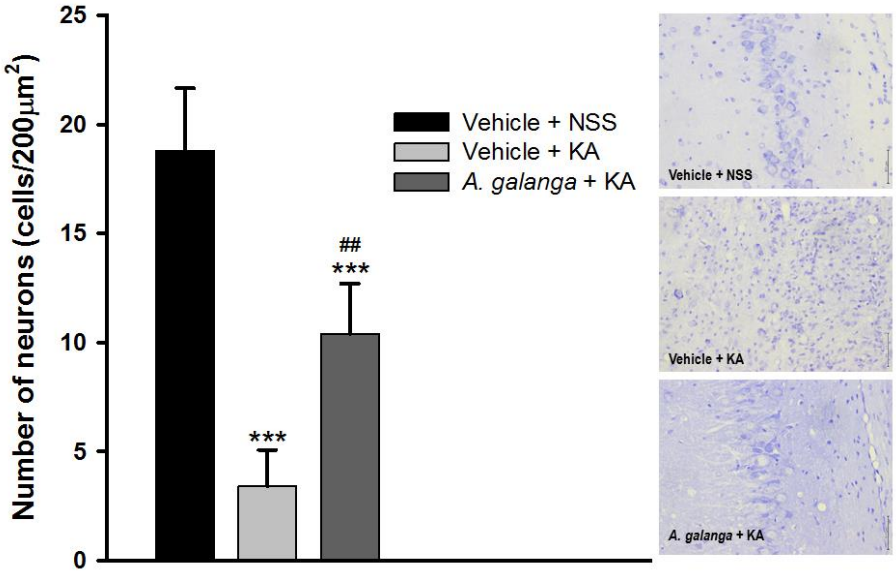

Figure 2 The effect of KA injection on the number of neurons (neuron density) in the CA3 area of the hippocampus. Data were presented as mean ± SD.

*** p-value <0.001 compared to the vehicle treated group with NSS injection;

p-value <0.01 compared to the vehicle treated group with KA injection. KA injection significantly induced neuronal loss in the groups injected with KA injection but *A. galanga* treatment attenuated the neuronal cell death compared to the vehicle treated group.

Discussion

In the study, the neuroprotective effect of ethanolic extract of *A. galanga* in KA-induced neuronal cell death. *A. galanga* extract was administered to the rats for two weeks before and two weeks after the KA injection. Kainic acid has been widely used as a model to study the mechanisms of neurodegenerative pathways induced by excitatory neurotransmitter. [28] KA injection in the hippocampus could represent a model of temporal lope epilepsy. [29, 30] The neurotoxicity is induced by the binding between KA and kainate receptors (KAR) and causes a cellular cascade and eventually leads to neuronal cell death. [31] According to the distribution of KAR is highly distributed in the CA3 area of the hippocampus. Therefore, we focused on the evaluation of neuronal survival and GFAP expression in this area.

The authors observed that the rats showed seizure syndrome following KA administration. The symptoms including immobility, facial clonus, masticatory, head nodding, wet-dog shakes, rolling toward one side and rotating were observed during 30 min to one hour after KA administration. These symptoms demonstrated the status epilepticus which corresponds with previous studies. [32-34] This indicated that the KA injection successfully developed neurodegeneration.

The results of the present study showed that KA injection significantly increased GFAP expression and neuronal loss of hippocampal pyramidal neurons in the CA3. The authors propose that these occurred due to the high distribution of KAR in the CA3. The group of rats that received the *A. galanga* extract showed lower GFAP expression but higher survival neuron density compared to the vehicle-treated group with KA injection. The suspected possible mechanisms of *A. galanga* extract to protect against neuronal death induced by KA were decreasing oxidative stress through its antioxidant effect and attenuating the inflammation process through its anti-inflammatory effect. However, these two mechanisms were not investigated in this study but required for further evaluation.

It is well known that the hippocampus plays an important role in cognitive function. [35] Previous studies demonstrated cognitive impairment in KA-treated animals. The mice were treated with a single unilateral injection of KA into the dorsal hippocampus exhibit a decrease in depression-like behavior in the forced swimming test and retarded acquisition as well as impaired retention of visual-spatial information in the Morris water maze test. [36] KA-treated Wistar rats are impaired in the water maze and object exploration tasks and hyperactive in the open field test, which can be improved by the selective cyclooxygenase (COX)-2 inhibitor administration. [37] In addition, rats that received an intraperitoneal injection of KA exhibited impaired performance on the spatial water maze and passive avoidance tasks. [38] Taken together with another study demonstrated that *A. galanga* fractions exert a neuroprotective effect in Aβ induced amnesia in mice results in improved cognitive function. [24] Therefore, future studies may also determine cognitive behavioral test along with the evaluation of neuron survival and the expression of GFAP.

Beneficial chemical compounds were identified in *A. galanga.* For instance, phenylpropanoids isolated from the rhizomes of *A. galanga* inhibited allergic cytokine IL-4 production, [39] while acetoxychavicol acetate is known to have anti-inflammation [39, 40] and p-Coumaryl alcohol-γ-O-methyl ether exerts antioxidant and anti-inflammatory activities. [41] However, we did not

isolate the lead component in the *A. galanga* extract that exerted the influence on the measured parameters in the current study. Thus, further investigations are still required.

In conclusion, *A. galanga* extract exerts a neuroprotective effect against neurotoxicity induced by KA. Nevertheless, further studies are still required to clarify the mechanisms of the extract to protect the neurons.

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