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Original Research Article

Effects of Tualang Honey Pre-Treatment on Cerebellar and Striatal Neuronal Changes and Excitatory Amino Acid Transporter-2 (EAAT2) Expression Following Kainic Acid Exposure in Rats

Nurdarina A. Zulkifli[1], Sirajudeen K.N.S.[2]*, Muzaimi Mustapha[1], Anani A. Mat Zin[3], Hidani Hasim[4], Suk Peng Tang[5]*

[1]*Department of Neurosciences, School of Medical Sciences, Health Campus, Universiti Sains Malaysia*
[2]*Department of Basic Medical Sciences, Kulliyyah of Medicine, International Islamic University Malaysia*
[3]*Department of Pathology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia*
[4]*Department of Chemical Pathology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia*
[5]*Department of Pharmacology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia*

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ABSTRACT

Excitatory amino acid transporter-2 (EAAT2) is the predominant glutamate transporter that helps in maintaining low extracellular glutamate levels in the brain. Defect in EAAT2 causes impaired clearance and accumulation of this excitatory neurotransmitter, leading to excitotoxicity and neuronal cell death. The cerebellum and striatum play an important role in motor functions. This study aimed to evaluate the effects of Tualang honey (TH) on cerebellar and striatal neuronal changes as well as EAAT2 expression following kainic acid (KA) exposure in rats. Male Sprague-Dawley rats (n=48) were divided into four groups depending on the respective treatment. Each group was further divided into two subgroups based on time-point of sacrifice at 24-hour or at 5-days after KA injection. The rats were initially treated orally with distilled water, TH (1.0 g/kg) or topiramate (40 mg/kg), 12-hourly for five times. Then, the rats were injected with saline or KA (15 mg/kg) 30 minutes after the fifth oral dose. Before the rats were sacrificed, an open field test was conducted. Locomotor activity significantly increased in all KA-injected groups 5 days after KA administration. TH pre-treatment significantly reduced cerebellar neuronal death 5 days after KA injection. TH pre-treatment also showed to reduce KA-induced loss of striatal neurons 24 hours after KA injection as well as increases EAAT2 expression in 24 hours and 5 days groups. These results imply that pre-treatment with TH may mitigate the KA-induced excitotoxicity in the cerebellum and striatum partly via modulation of EAAT2 expression.

***Keywords*:** EAAT2 expression, Tualang honey, kainic acid, cerebellum, striatum

Introduction

Neurodegenerative disorders are characterized by the progressive loss of neuronal structure and function. Numerous factors can cause neuronal cell death, including death induced by stimulus or through various mechanisms that execute cell death.[1,2] For instance, glutamate is the most prevalent amino acid and a major excitatory neurotransmitter in the brain. Excessive glutamate release is known to induce excitotoxic neuronal cell death via overactivation of glutamate receptors, which causes a high influx of calcium. The disturbance of calcium homeostasis subsequently triggers a series of neurotoxic cascades that include mitochondrial dysfunction, excess formation of reactive oxygen or nitrogen species and oxidative stress that eventually result in neuronal damage. Various neurodegenerative disorders including epilepsy have all been linked to glutamate excitotoxicity.[3]

*Corresponding author. E mail: sukpeng@usm.my; knssiraj@iium.edu.my
Tel: +6097676128; +6095704490

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Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Excitatory amino acid transporters (EAATs), also known as glutamate transporters, are essential in the reuptake of glutamate from the synaptic cleft to maintain low levels of extracellular glutamate and prevent excitotoxicity. Approximately 90% of extracellular glutamate reuptake in the brain is carried out by the EAAT2 [also known as glutamate transporter-1 (GLT-1)], a major EAAT subtype which is found predominantly in astrocytes.[4] Impaired glutamate clearance and subsequent glutamate buildup in the synaptic cleft have been associated to dysfunction or reduced expression of EAAT2.[3,5] A decrease in the expression and function of EAAT2 may lead to increased glutamate excitotoxicity and has been linked to the development of seizures and epilepsy.[6,7] Studies have shown that upregulation of EAAT2 attenuates neurotoxicity and exerts protective effects against seizures.[4,8] Therefore, treatment targeting functional EAAT2 and restorations of EAAT2 expression level have gained considerable interest as a potential strategy to prevent neurodegenerative disorders such as epileptic seizures.[7,9]

Kainic acid (KA) is a potent agonist of ionotropic glutamate receptors, including the kainate receptor and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor.[10] Binding of KA on kainate receptors induces a series of cellular events that lead to neuroexcitation and epileptogenic effects. In animal model, KA shown to induce hyperactivity as well as impairment of spatial learning and object exploration task impairments in rats.[11–13] Administration of KA causes neurotoxic effects in different brain regions including the cerebellum and striatum.[14–16] The cerebellum and striatum, together with the cerebral cortex, form interconnected networks that play essential roles in motor functions. Any changes that occur in one of

the regions may influence the neural networks and the related node, thus affecting motor or movement performances.[17,18]
Apitherapy using various type of bee products such as honey has long been used as a complementary medicine against various diseases.[19,20] Tualang honey (TH) is a wild multifloral honey found in Malaysian rainforest and rich with bioactive compounds when compared to other types of local honey.[21] The compounds that have been identified in TH include flavonoids such as catechin, kaempferol, luteolin, naringenin, quercetin and vitexin, as well as phenolic acids (gallic, chlorogenic, coumaric, caffeic, benzoic, ferulic and salicylic acid).[21] Previous studies showed protective effects of TH against oxidative stress and inflammation in different brain regions of KA-induced epileptic seizures in rats.[22–24] However, little is known about the effects of TH on EAAT2. Therefore, this study aims to evaluate the effects of TH supplementation on KA-induced neuronal changes and EAAT2 expression in the rat cerebellum and striatum.

Materials and Methods

Animals
The method conducted was reviewed and approved by the Institutional Animal Ethic Committee, Universiti Sains Malaysia (USM) [No. of Animal Ethics Approval: USM/ IACUC/2018(111) (904)]. A total of 48 male Sprague-Dawley rats, aged two-month-old weighing between 260 and 320 g were purchased from the Animal Research and Service Centre (ARASC), Health Campus, USM, Malaysia. All the rats were housed in polypropylene cages, individually, with free access to a standard rodent diet and drinking water. The housing facility was maintained in a controlled environment with a 12-hour light/dark cycle at room temperature of 25 ± 1°C. All of the rats were acclimatized for at least one week before the experiments started.[25]

Tualang honey (TH)
AgroMas® TH from the Federal Agricultural Marketing Authority, Kedah, Malaysia was used in this study. After being filtered and dried in an oven (40°C) to reduce moisture content to 20% (w/v), the TH was gamma irradiated at a dose of 25 kGy for sterilization [SterilGamma (M) Sdn. Bhd., Selangor, Malaysia]. Then, the sterilized honey was stored at room temperature (23 ± 2°C). The same batch of honey was used throughout the study. The amount of TH was calculated based on the body weight (b.w.) of the rats and diluted in distilled water to a final volume of 0.5 mL honey solution. The honey solution was prepared freshly before use.
a
Study design
The rats were randomly separated into four groups depending on the treatment given: control, KA, TH+KA and TPM+KA (Table 1). Then, the rats were further divided into two subgroups based on the time-point of sacrifice (24 hours or 5 days after KA injection). Topiramate (TPM; T2755, Tokyo Chemical Inc., Japan), a known antiseizure medication was used as a treatment control in this study. Briefly, the rats were pre-treated orally with TH (1.0 g/kg b.w.), TPM (40 mg/kg b.w.) or distilled water (0.5 mL) five times, 12-hourly. All oral treatment was performed between 8-10 a.m. (morning session) and 8-10 p.m. (night session). The dosage of TH was chosen based on earlier research reporting TH's neuroprotective effects against KA-induced oxidative and inflammatory stress in several brain regions.[22] The rats were injected subcutaneously with KA (15 mg/kg b.w.) (K0250, Sigma-Aldrich, Missouri, USA) 30 minutes after the last (fifth) oral treatment. The control group received a saline injection. The selected KA dose was previously shown to induce significant injury in different brain regions.[22] Topiramate, a known antiseizure medication was used as a treatment control in this study.

Open-field test (OFT)
OFT was performed before sacrifice (24 hours or 5 days after KA injection) (n=6 per group, respectively) according to the previously described method to assess locomotor activity.[22] The apparatus contains a platform that is 90 cm long, 90 cm wide with 25 squares (18 cm x 18 cm each) and has a Perspex wall around it (27 cm high). The rats were originally placed in the middle of the platform, and the rat's locomotor activity was recorded for duration of five minutes using a camera installed at 1.0 m above the platform. The apparatus was cleaned thoroughly using 30% ethanol and air-dried before continuing to the next assessment. The test procedures were carried out between 8-10 a.m. in a dark, quiet animal behavioral testing room located at ARASC. The OFT was assessed by an observer who was unaware of the experimental groups. The number of lines crossed with four paws placed in each square was measured to assess the rat's locomotor activity.

Collection and preparation of brain samples
The rats were deeply anaesthetized with sodium pentobarbital (0.1 mL/ 100 g b.w.; i.p. injection) (Dolethal injection Vetoquinol, United Kingdom). Then, the rats were transcardially perfused for five to ten minutes with phosphate buffer saline (pH 7.4), and then with paraformaldehyde (PFA) solution (4% w/v in 0.1 M sodium phosphate buffer, pH 7.4).[26] The harvested brains were postfixed in a 4% (w/v) PFA solution at 4°C for three days before tissue processing. The cerebellum and striatum [antero-posterior (AP) position from bregma: -11.40 to -12.84 mm for cerebellum; -0.12 to 1.08 mm for striatum) were identified and sectioned according to the rat brain atlas.[27] The automated tissue processor was used to process the sectioned brain tissues, followed by the preparation of paraffin-embedded brain tissue blocks. Coronal slices (5 µm thick) of each brain region were prepared using a rotary microtome for subsequent histological and immunohistochemical staining procedures. All staining was performed in duplicate for each tissue sample. Prior to staining procedures, the slides were placed on a slide warmer set at 60°C for 45 minutes to increase tissue adhesion. All stained slides were viewed and examined under a light microscope (Olympus BX41, Olympus Scientific, USA) equipped with a digital camera (Olympus cellSens standard imaging software version 2.2, Olympus Scientific, USA).

Hematoxylin and eosin (H&E) staining for morphological assessment
The H&E staining was adapted based on the method by Feldman and Wolfe.[28] Briefly, the tissue slides were deparaffinized in two changes of xylene (Merck, Germany), followed by removal of xylene and tissue rehydration with a series of descending alcohol (HmbG Chemicals, Germany) concentrations (100%, 95% and 80% ethanol). The rehydrated tissues were stained using hematoxylin (7231, Richard-Allan Scientific, USA) for 15 minutes, rinsed under running tap water for 5 minutes, followed by staining with eosin (Tissue-Tek, 8660, Sakura Finetek Japan) for 2 minutes. The stained tissues were dehydrated using a series of ascending alcohol concentrations (80%, 95% and 100% ethanol), cleared with xylene and coverslipped with DPX mountant (VWR, Pennsylvania, USA). Microscopic examinations were performed to assess any histological features of neuronal damage.[29]

Table 1: Experimental groups (n=12 each group)

Group	Treatment[#]
Control	Distilled water + saline solution
KA	Distilled water + kainic acid solution (15 mg/kg b.w.)
TH+KA	Tualang honey (1.0 g/kg b.w.) + kainic acid solution (15 mg/kg b.w.)
TPM+KA	Topiramate (40 mg/kg b.w.) + kainic acid solution (15 mg/kg b.w.)

[#]Distilled water, Tualang honey and topiramate were given as oral treatment; saline and kainic acid were given through subcutaneous injections

Cresyl violet staining for quantification of viable neurons

The preparation of cresyl violet staining solution and staining procedure were performed manually based on the method described by Mohd Sairazi *et al*.[22] as a measurement for neuronal loss. Briefly, the tissue slides were deparaffinized using xylene, rehydrated through a sequence of descending alcohol concentrations (100%, 95% and 80% ethanol) and immersed in cresyl violet working solution. Then, the slides were rinsed with distilled water, dehydrated through a series of ascending alcohol concentrations, cleared with xylene, and mounted under a coverslip. Viable neuronal cells exhibiting normal morphology with round cresyl violet-stained nuclei were counted in six non-overlapping regions using Fiji image analysis software (version 1.51). Identification of Purkinje cells of the cerebellum and medium- to large-sized neurons in the striatum were performed according to the method described by Garman[29] and confirmed with a certified pathologist before quantification.

EAAT2 immunohistochemistry

The tissue slides were deparaffinized using xylene, rehydrated with a series of descending alcohol concentrations (100%, 95%, 80%, 70% and 50% ethanol), and then washed with 1X Tris-buffered saline (TBS, pH 7.6) (S300685, Dako, Agilent) and distilled water. The rehydrated samples were immersed in Tris/EDTA buffer (pH 9.0) (S236784, Dako, Agilent) and heated for 15 minutes in a microwave oven (NN-SM332M, Panasonic) at medium high setting for antigen retrieval, followed by blocking in peroxidase-blocking solution (S202386, Dako, Agilent) and bovine serum albumin (5% w/v, prepared in 1X TBS). The brain sections were incubated with anti-EAAT2 antibody (AB205248, Abcam; 1: 10000 dilutions) for overnight (16-18 hours) at 4°C. After overnight incubation, the brain sections were rinsed with 1X TBS buffer, followed by incubation with a horseradish peroxidase (HRP) enzyme-conjugated secondary antibody for 20 minutes, exposure to diaminobenzidine (DAB) chromogen for 5 minutes (Rabbit specific HRP/DAB Detection IHC Detection Kit, AB236469, Abcam) and counterstained with hematoxylin. The enzyme HRP reacts with the chromogenic substrate (DAB) to produce brown-colored products. Subsequently, the stained tissue sections were dehydrated through a series of ascending alcohol concentrations and cleared with xylene before coverslipped. A tissue sample from the cerebral cortex and the kidney were used as the positive and negative controls, respectively, for each round of staining. In addition, equal samples from each experimental group were used during each batch of staining to minimize batch variation. The expression level of EAAT2 was measured based on the staining intensity of DAB in six randomly selected non-overlapping fields (50 µm x 50 µm) using Fiji image analysis software (version 1.51).[30,31]

The DAB intensity was quantified using Fiji image analysis software (version 1.51) based on method described by Mahale *et al.*[30] & Mustafa *et al*.[31]. Briefly, colour deconvolution was performed to separate the brown DAB image from the hematoxylin counterstain. The optical density value was obtained using the following equation: OD=log (maximum intensity/mean intensity), in which the maximum intensity is 255 for an 8-bit image.

Statistical analysis

Data analyses were performed using GraphPad Prism 9.0 (San Diego, California). The Shapiro-Wilk test was performed to determine the normality and the homogeneity of variances of the data. Data that had a normal distribution and equal variances was presented as the mean (standard deviation). A one-way analysis of variance (ANOVA) test was used to compare the mean differences between groups, and if significant, Tukey's test was performed for multiple pairwise comparisons. For non-normally distributed data with unequal variance, the result was presented as the median (interquartile range). The median differences were tested using the Kruskal-Wallis test. If significant, Dunn's test was performed for multiple pairwise comparisons. Data is considered significant when the p-value is lower than 0.05.

Results and Discussion

Abnormal extracellular glutamate concentration levels will lead to overactivation of postsynaptic glutamate receptors, thus causing a condition known as glutamate excitotoxicity. This pathological condition can be associated with high glutamate release by presynaptic neurons or abnormalities in glial cells glutamate uptake activity, which will result in glutamate building up in the synaptic cleft.[32,33] The occurrence of neuronal cell damage and loss may cause undesirable complications in the regulation of normal brain activity. The KA-induced neurotoxicity experimental model has been widely used to comprehend the pathogenesis of excitotoxicity in various neurodegenerative disorders.[12] This study reported on the effects of TH pre-treatment on the cerebellar and striatal EAAT2 expression in rats following KA exposure.

In this study, the total number of lines crossed in the OFT was used to assess locomotor activity of rats.[22,34] The total number of line crossings in OFT for each experimental group is presented in Figure 1. KA injection increases locomotor activity but the increment was insignificant for 24-hour subgroups. A significant increase in locomotor activity was observed 5 days following KA administration, suggesting some impairments occurred in brain regions that control motor function. In comparison to group KA, pre-treatment with TH or TPM lowers the number of lines crossed. However, the effect is statistically insignificant. The subcortical cerebellum and striatum, together with the cerebral cortex, formed a complex neural network to control movement, cognition and affective functions.[17] Direct cerebellar or striatal KA administration was shown to induce a wide range of behavioral alterations including epileptic seizures and dystonia, as well as impaired spatial learning and motor performance.[35–37]

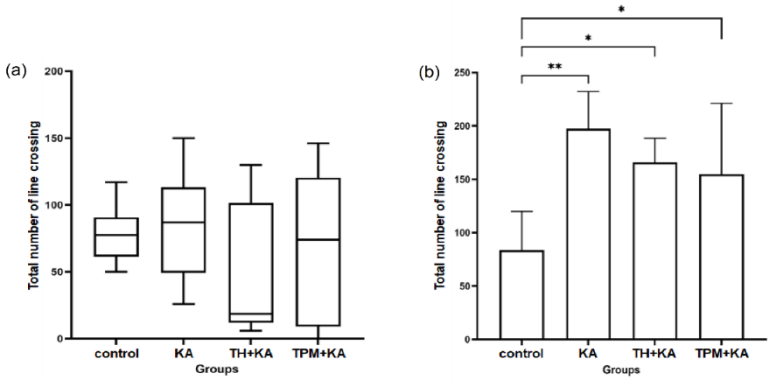

Figure 1: Total number of line crossings in the open field test for (a) 24 hours and (b) 5 days subgroups. In the 5 days subgroups, there was a statistically significant increase in the total number of line crossings compared to the control group (*p < 0.05, **p < 0.001 vs control). [KA, kainic acid; TH, Tualang honey; TPM, topiramate].

The cerebellum, one of the major structures of the hindbrain, plays a crucial part in the coordination of voluntary movements, maintaining balance, cognitive functions and motor learning activities.[38,39] KA-induced significant neuronal loss was observed in group KA for both 24 hours and 5 days subgroups in this study (Figure 2). Meanwhile, significantly reduced cerebellar EAAT2 expression in group KA was also observed in 24 hours subgroups, but the reduction was relatively insignificant 5 days later (Figure 3). TH pre-treatment significantly prevents KA-induced neuronal loss in the cerebellum for 5 days subgroups, and the results are similar to that of the antiseizure drug topiramate. However, the increases in EAAT2 expression in groups receiving TH or TPM were relatively insignificant. The results suggest that in addition to EAAT2, there might be involvement of other protective mechanisms that attenuate the neurotoxic effect of KA in the cerebellum.

Previous study by Lee *et al*.[40] suggests that a significant reduction in astrocytic EAAT2 expression may cause the Purkinje cells, the only output neurons of the cerebellum, to be more susceptible to excitotoxicity. Besides EAAT2, decreased levels of other subtypes of glutamate transporters, such as EAAT1 and EAAT4, may also contribute to the loss of Purkinje cells.[41] For example, studies demonstrated that low expression or activity of EAAT4 causes increased glutamate concentration and Purkinje cell death, which leads to impaired cerebellar function.[42,43] On the other hand, high expression of EAAT4 prevents abnormal neuron firing by restricting activation of perisynaptic and extrasynaptic metabotropic glutamate receptors.[43,44]

The striatum is the primary input nucleus to the basal ganglia, a group of subcortical nuclei that is necessary for control of voluntary movement and other cognitive and behavioral responses.[45] Recent findings showed activation of different neuronal circuits in the basal ganglia, including activation of striatal neurons, occurs during frontal lobe seizures.[46] In this study, administration of KA induced significant striatal neuronal loss for 24 hours subgroups. Pre-treatment with TH significantly attenuates KA-induced striatal neuronal loss. For 5 days subgroups, similar trends were observed but the difference between groups was insignificant (Figure 4). However, a significant decrease in the expression of striatal EAAT2 was observed in group KA at both time points. The EAAT2 expression in the striatum increased significantly in the TH pre-treated group at both time points (Figure 5).

As mentioned before, EAAT2 is predominantly expressed in astrocytes, the main glial cells in the central nervous system. Previous study showed a significant increase in the astrocyte protein marker glial fibrillary acidic protein (GFAP) in various brain regions at 2, 24 and 48 hours after KA administration.[24] Reactive astrocytes, characterized by increased GFAP, potentially induce upregulation of metabotropic glutamate receptors (mGluRs) such as mGluR5 as an acute response to injury and enhanced glutamate clearance through astrocytic glutamate uptake.[47,48] These findings may probably explain the results in this study, where significant neuronal loss in the striatum was observed initially at 24 hours but became insignificant after 5 days despite significantly reduced EAAT2 expression. Following KA exposure, activation of astrocytes may occur, followed by mGluR5 upregulation and enhanced glutamate uptake during the acute phase, thereby compensating for the neuronal loss caused by reduced EAAT2 expression.

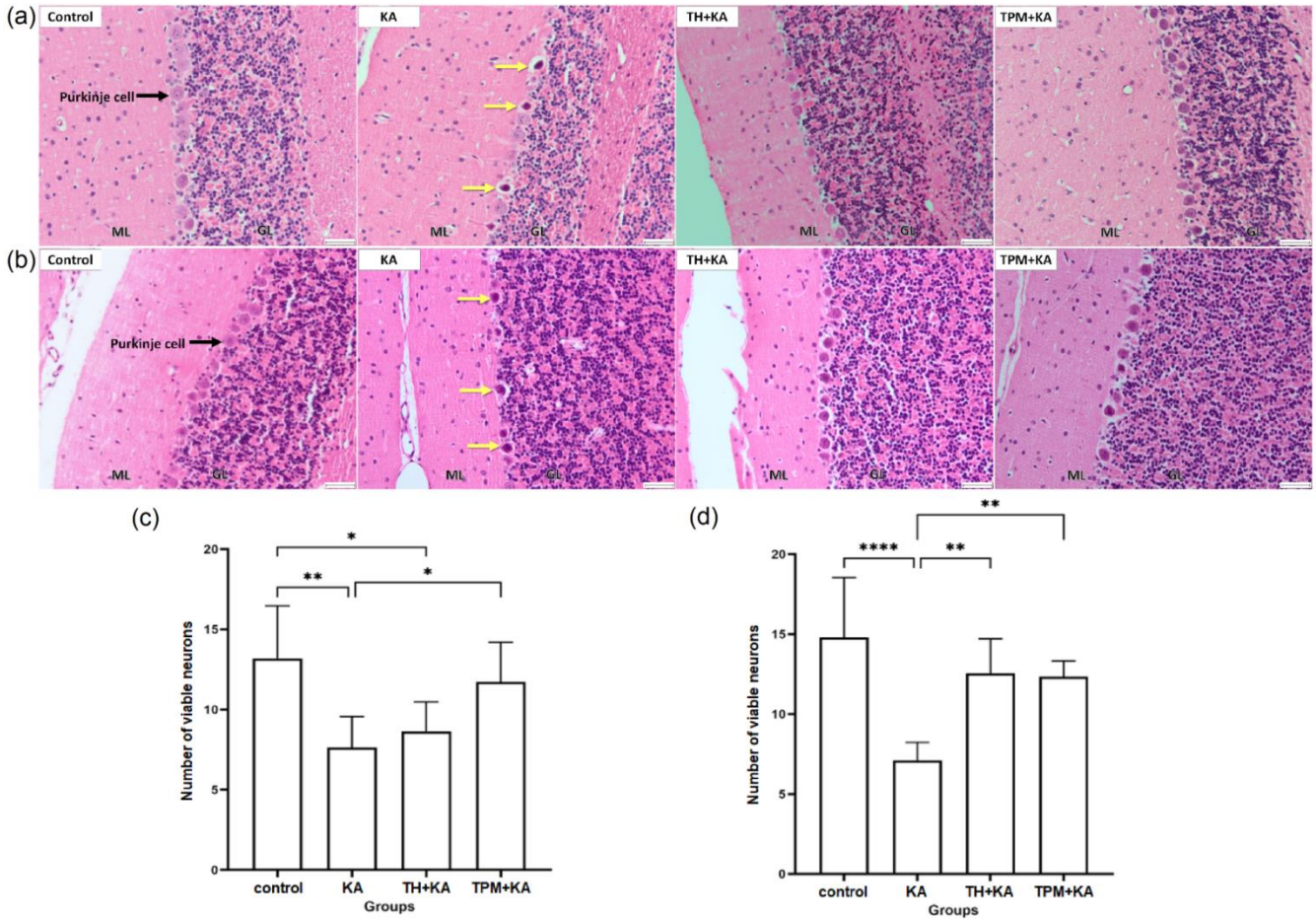

Figure 2: Representative photomicrographs of H&E-stained cerebellum for (a) 24 hours and (b) 5 days subgroups. The Purkinje cells were arranged orderly between the molecular layer (ML) and granular layer (GL) of cerebellum in control groups. Meanwhile, degenerating neurons (yellow arrow) with dense cytoplasmic staining surrounded by vacuolated neuropil and patchy loss of Purkinje cells were observed following KA injection. Histological improvements were observed in groups receiving treatment with TH or TPM (scale bar: 50 µm; magnification: ×400). The number of viable Purkinje cells for (c) 24 hours subgroups (*p<0.05, **p<0.005) and (d) 5 days subgroups (**p<0.005, ****p<0.0001) [KA, kainic acid; TH, Tualang honey; TPM, topiramate].

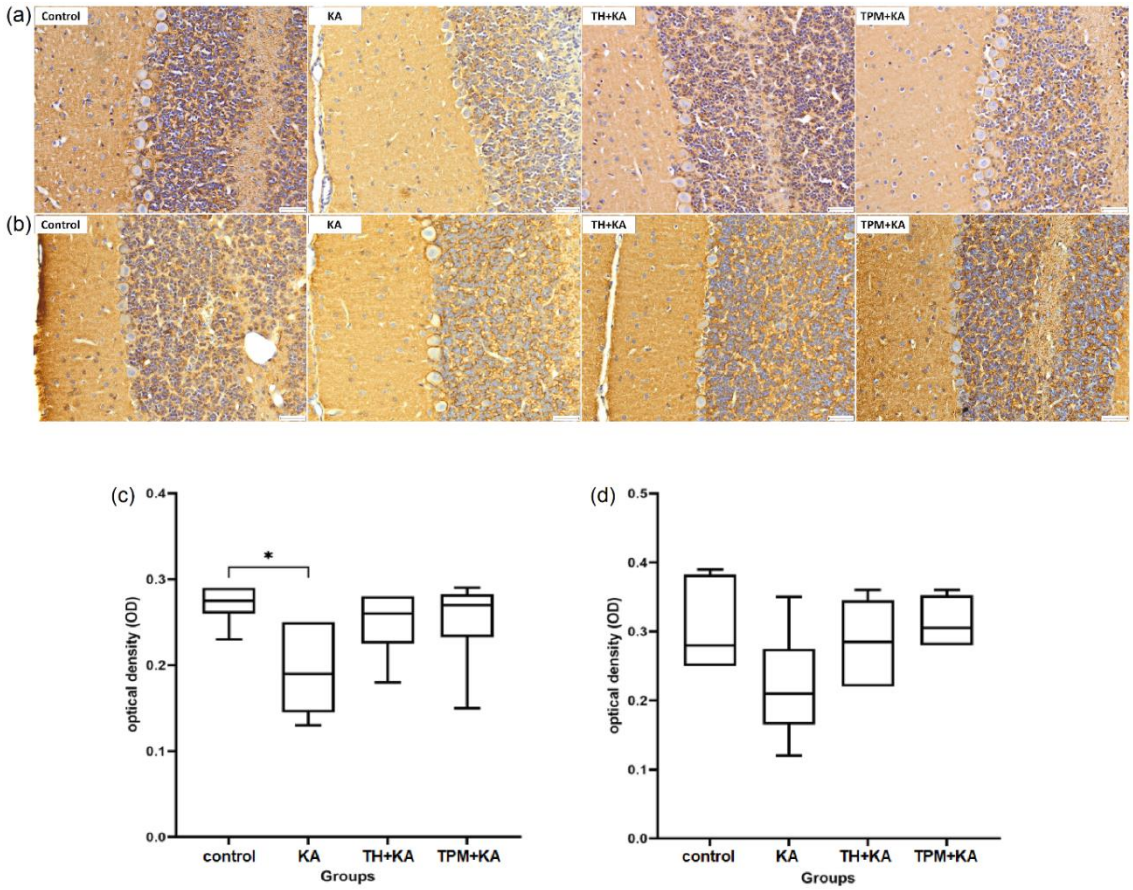

Figure 3: EAAT2 immunohistochemistry and densitometric analysis in the cerebellum (n=6 per group). (a) and (b) showed the representative photomicrographs of EAAT2 immunostained cerebellum for 24 hours and 5 days subgroups, respectively (scale bar: 50 µm). (c) and (d) showed the DAB staining intensity for EAAT2 for 24 hours and 5 days subgroups, respectively. The expression of EAAT2 in the cerebellum was significantly reduced at 24 hours after KA injection (*$p<0.05$). [DAB, 3,3′-diaminobenzidine; EAAT2, excitatory amino acid transporter-2; KA, kainic acid; TH, Tualang honey; TPM, topiramate].

Despite general protective effects against KA-induced neuronal loss and reduced EAAT2 expression, the locomotor activity remained significantly high after 5 days of KA administration in groups pre-treated with TH. Another study also showed similar findings, whereby KA-induced recurrent seizures and impaired behavioral activities were not prevented although the neuroprotective effects were observed from histological assessment.[49] Moreover, the onset of recurrent spontaneous seizures appears heterogeneous, ranging from 3 days to 6 weeks following KA exposure.[49,50] In contrast, latent or seizure-free period without behavioral seizures can be approximately 5 to 30 days and even take up to five months, but electrophysiological recordings may show some epileptic activity.[10] Therefore, a prolonged study period is recommended to confirm the protective role of TH at a later stage of disease.

Diets rich with polyphenols were linked to improved brain health and functions although their underlying cellular and molecular mechanisms are still relatively not well understood.[51–53] Honey is generally regarded as a good source of antioxidants and the combined actions of a wide range of bioactive ingredients are believed to contribute to its beneficial effects in various disease models. The beneficial effects of TH supplementation have also been reported in various animal and human studies.[21,54] Some identified compounds in TH including luteolin and gallic acid were previously reported to show neuroprotective effects against toxin-induced excitotoxicity. For instance, luteolin protects against KA-induced excitotoxicity in rat's hippocampus by reducing glutamate levels, microglial activation and increasing Akt activation.[55] Gallic acid treatment also showed to protect the cortex neurons from glutamate-induced excitotoxicity by improving the antioxidant status and inhibiting production of proinflammatory cytokines.[56] However, the composition of honey varies depending on many factors including geographical or botanical origins, thereby creating potential reproducibility challenge by other researchers.

Conclusion

In conclusion, pre-treatment with TH prevents KA-induced neuronal loss in the cerebellum and striatum of rats, partly via modulation of EAAT2 expression, and the effects are comparable to TPM, a known kainate receptor antagonist. This study only focused on the effects of TH on neuronal changes and expression of EAAT2 in the cerebellum and striatum of rats following KA administration. Further investigations, such as effects on other subtypes of glutamate transporters as well as the role of astrocyte activation and mGluRs, are recommended to better understand the protective mechanisms of TH in the brain.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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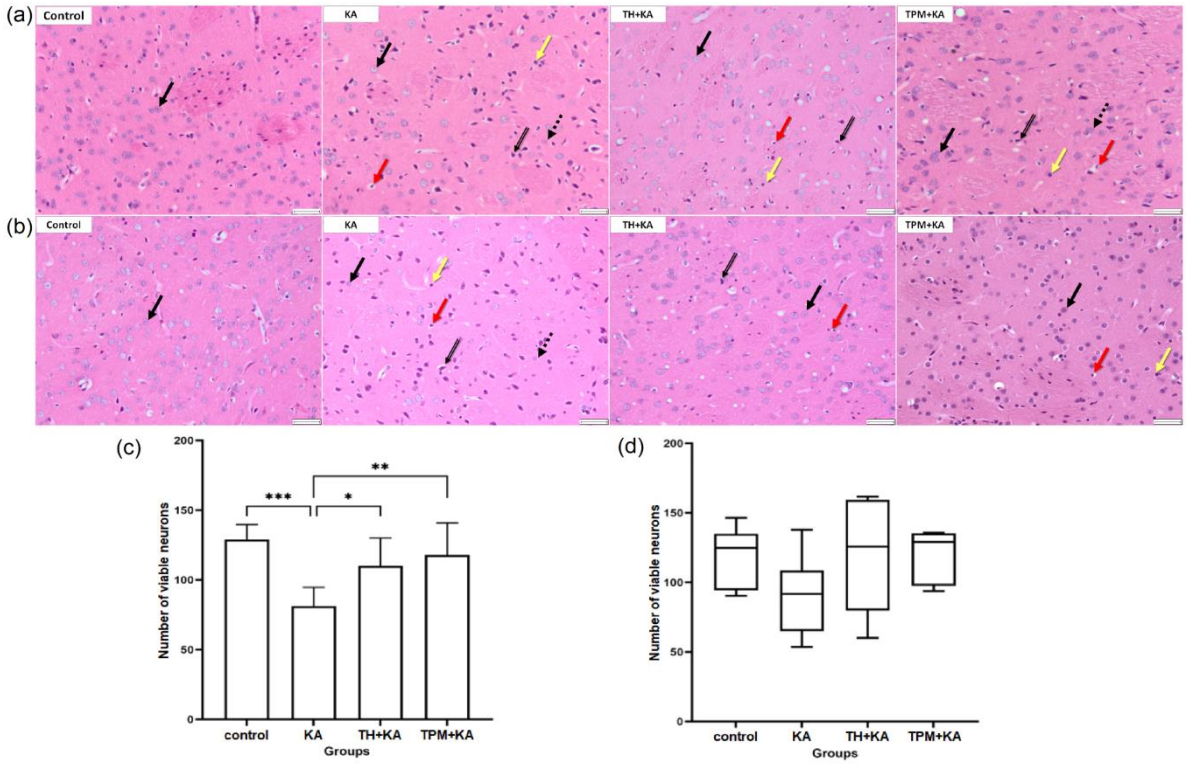

Figure 4: Representative photomicrographs of H&E-stained striatum for (a) 24 hours and (b) 5 days subgroups. The control group showed normal striatum histology, in which round to oval-shaped cells with prominent nucleolus were seen (black arow). Features of neuronal damage, including irregular shape (double-lined arrow), cell shrinkage (red arrow), pyknotic nuclei (yellow arrow) or absence of nucleolus (dashed arrow), were seen in all groups receiving kainic acid (KA). Treatment with TH or TPM showed improved morphological features (scale bar: 50 µm; magnification: ×400). The number of viable neurons for (c) 24 hours subgroups (*p<0.05, **p<0.01, ***p<0.001) and (d) 5 days subgroups [KA, kainic acid; TH, Tualang honey; TPM, topiramate].

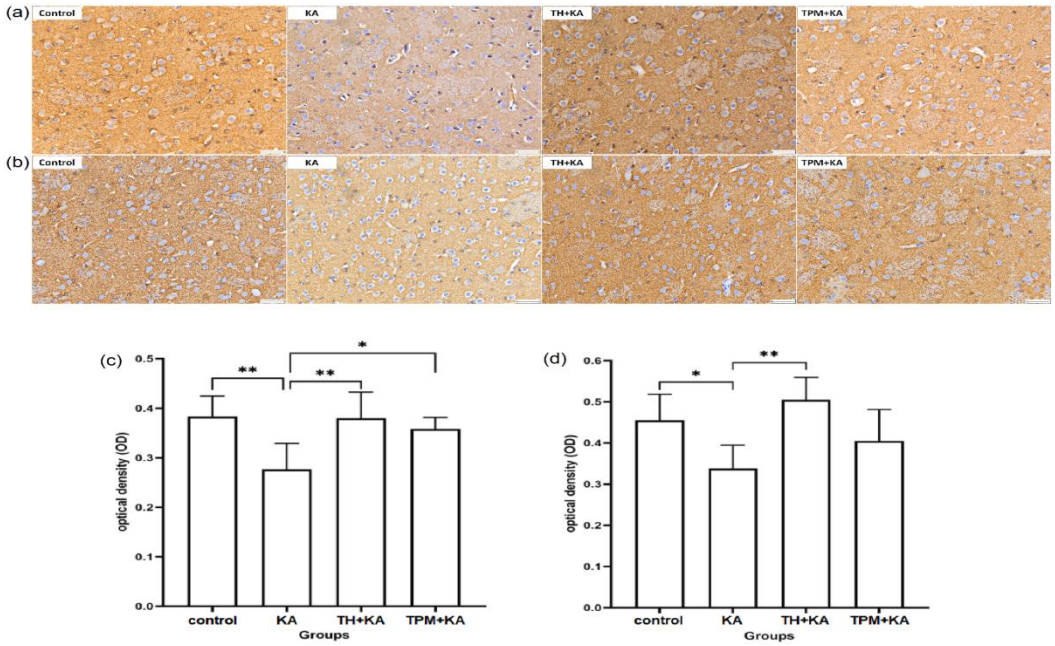

Figure 5: EAAT2 immunohistochemistry and densitometric analysis in the striatum (n=6 per group). (a) and (b) showed the representative photomicrographs of EAAT2 immunostained for 24 hours and 5 days subgroups, respectively (scale bar: 50 µm). (c) and (d) showed the DAB staining intensity for EAAT2 for 24 hours and 5 days subgroups, respectively. The expression of EAAT2 in the striatum was significantly reduced at 24 hours after KA injection (**p<0.005 vs control) and increased significantly in groups pre-treated with TH or TPM (*p<0.05, **p<0.005 vs KA). Similarly, significantly lower EAAT2 expression was observed for the 5 days subgroups (*p<0.005 vs control) and increased in group TH+KA (**p=0.001 vs KA). [DAB, 3,3′-diaminobenzidine; EAAT2, excitatory amino acid transporter-2; KA, kainic acid; TH, Tualang honey; TPM, topiramate]

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