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Original Article

TUALANG HONEY ATTENUATES KAINIC ACID-INDUCED OXIDATIVE STRESS IN RAT CEREBELLUM AND BRAINSTEM

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ABSTRACT

Objective: The present study examined the protective effect of tualang honey (TH) against kainic acid (KA)-induced oxidative stress in the cerebellum and brainstem of rats.

Methods: Male Sprague-Dawley rats were randomly divided into four groups: Control, KA-treated, TH+KA-treated, and topiramate (TPM, an antiepileptic agent)+KA-treated groups. Rats were pretreated orally with drinking water, TH (1.0 g/kg body weight), or TPM (40 mg/kg body weight), respectively, five times at 12 h intervals. Saline or KA (15 mg/kg body weight) were injected subcutaneously 30 min after last oral treatment. Rats were sacrificed at 2 h, 24 h, and 48 h after KA administration. Oxidative stress markers were analyzed in different brain regions (cerebellum and brainstem) 2 h, 24 h, and 48 h after KA administration.

Results: KA caused significant ($p<0.05$) elevation in the thiobarbituric acid reactive substances level, protein carbonyl contents, and nitric oxide production, impairment of glutathione system, and a significant reduction in the total antioxidant status in the rat cerebellum and brainstem at multiple time-points, as compared to control groups. Pretreatment with TH significantly ($p<0.05$) reduced the elevation in the thiobarbituric acid reactive substances level, protein carbonyl contents, and nitric oxide production and increasing a reduction in the total antioxidant status in the rat cerebellum and brainstem induced by KA at multiple time-points, as compared to KA only-treated group.

Conclusion: Taken together, this study suggests that TH has therapeutic potential in reducing oxidative stress in the cerebellum and brainstem of KA-induced rats via its antioxidant property.

Keywords: Rat Brainstem, Rat Cerebellum, Excitotoxicity, Kainic Acid, Oxidative Stress, Tualang Honey

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INTRODUCTION

Excitotoxicity is a process triggered by the excessive activation of ionotropic glutamate receptors which cause excitotoxic neuronal degeneration. Kainic acid (KA) has been extensively used to study the mechanism of excitotoxicity-induced neurodegeneration and to explore the pharmacological intervention of neurodegeneration. KA causes neuron depolarization and an increase in the intracellular calcium ions, leading to the production of free radicals [1]. Free radicals are likely to attack cellular structures and cause oxidative damage to DNA, lipid, and protein, thereby disrupt cellular functions. The increase in the production of free radical can give threat to cellular homeostasis and neuronal survival. This will give rise to oxidative stress in the brain. Cumulative evidences strongly suggest that oxidative stress may be a possible mechanism of neurodegeneration [2-6]. This could led to the subsequent brain damage, which similar to human brain disorder [7].

Excitotoxicity is a global insult to brain tissue, though different parts of the brain are affected varying degree [8]. Several studies have reported that excitotoxicity-induced by KA has caused neuro-degeneration in the hippocampus, basal ganglia, piriform cortex, thalamus, amygdala, and parietal cortex [9-11]. Up to our knowledge, there has been a little information concerning the excitotoxic effect of kainic acid on the cerebellum and brainstem. The cerebellum is a brain structure that involves the coordination of movement, cognitive functions, motor learning and maintenance of balance. Damage to the cerebellum can lead to loss coordination of motor movement, loss of ability to walk, movement tremors, and dizziness [12]. There are two types of neurons that play dominant roles in the cerebellum: Purkinje cells and granule cells. Cerebellum granule cells are widely used as a cellular model to study the mechanisms of kainate receptor-mediated neuronal cell death and are vulnerable to excitotoxins [13-15]. Purkinje cells is highly susceptible to the pathological condition that involved excitotoxicity induced by glutamate and is selectively vulnerable to ischemia [16-17]. For the brainstem, it comprises of the medulla oblongata, the pons, and the midbrain. The medulla oblongata is a control centre for cardiovascular, vasomotor, respiratory function, and reflex activities including vomiting, sneezing, and coughing. The pons is involved in the sensory roles in hearing, taste, and balance and in the regulation of deep sleep and arousal. The midbrain serves as a centre for visuals, hearing, body movement, and eye movement. Damage to brainstem causes sleep difficulties, dizziness, reduced vital capacity in the respiration, difficulty in swallowing food and water, loss of consciousness, difficulty with the organizational perception of the environment [18]. The substantia nigra, which is a basal ganglia structure located in the midbrain, plays a critical role in brain function and in the development of many neuro-degenerative diseases.

Since, the mechanism of KA-induced excitotoxicity involves the production of free radicals and the involvement of oxidative stress, it is plausible that treatment against excitotoxicity-induced neurodegeneration should be a substance that has strong antioxidant properties and scavenging activity. Many of natural products possess antioxidant activity that enables them to protect against oxidative stress-related diseases. Honey is known to contain substantial antioxidant compounds. In Malaysia, honey has been used as a supplementation and in the traditional medicine among the local community. Tualang honey (TH) has been reported to have the highest phenolic compound and flavonoid contents among other Malaysian honey [19-23]. From literature findings, there have been a quite numbers of studies reported on its medicinal benefit, including

antioxidant effect in diabetic rats [24], protective effect in animal model of menopause [25] and in cigarette smoke-induced in rat male reproductive system [26], and also in the management of the wound-healing [27]. In addition, the neuroprotective effect of TH has been previously reported attenuating the cognitive impairment caused by chronic cerebral hypoperfusion-induced neuro-degeneration [28] and ameliorate oxidative stress in the rat midbrain against repeated paraquat exposure [29]. TH supplementation also improved the hippocampus and medial prefrontal cortex morphological impairment in stressed ovarie-ctomized rats [30-31]. Our previous study has demonstrated that TH has attenuated oxidative stress in the cortex of KA-induced excitotoxicity rat and thereby reduced the neurodegeneration induced by KA in the rat cortex KA [32]. These findings led us to hypothesize that the protective effect of TH could be partly attributed to its antioxidant properties.

Despite numerous studies that demonstrated the antioxidant and anti-inflammatory actions of TH, its neuroprotective activity in the animal model of kainic acid-induced excitotoxicity has remained unexplored. Therefore, this study was conducted to investigate the excitotoxic effect of kainic acid on oxidative stress-related markers in the rat cerebellum and brainstem at multiple time points as well as to investigate the potential neuroprotective effect of tualang honey in this model.

MATERIALS AND METHODS

Ethics approval

The ethical approval was obtained from the Institutional Animal Ethic Committee, Universiti Sains Malaysia (USM) [Approval No.: USM/Animal Ethics Approval/2011/(68) (305)]. All procedures performed in this study were in accordance with the Institutional Guidelines for the Care and Use of Animals for Scientific Purposes. Eight-weeks-old male Sprague-Dawley rats (200-250 g) were purchased from the Animal Research and Service Center (ARASC), Health Campus USM, Kota Bharu, Kelantan, Malaysia. Animals were individually housed in a well-ventilated room maintained at 21±2 °C under a 12-h light/dark cycle. Animals had free access to drinking water and food pellets *ad libitum.* The rats were acclimatized for at least a week and they were observed closely for any abnormality before the experiments started. No abnormalities were observed.

Chemicals

KA was purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA. Topiramate (TPM) was purchased from Tokyo Chemical Industries Co., Ltd., Tokyo, Japan. Fluoro-Jade C (FJC) was purchased from Histo-Chem Inc., Jefferson, Arkansas, USA. Diazepam was purchased from Atlantic Laboratories Corp. Ltd., Bangkok, Thailand. All chemicals and reagents were of analytical grade.

Tualang honey

Tualang honey (AgroMas®) was supplied by the Federal Agricultural Marketing Authority (FAMA), Kedah, Malaysia. The honey was previously filtered, evaporated to 20 % (w/v) water content at 40 °C and then sterilized by gamma irradiation (25 kGy) by SterilGamma (M) Sdn. Bhd., Selangor, Malaysia. The same batch of TH was used throughout the study. The dosage of TH (1.0 g/body weight) was selected based on the previous studies that demonstrated the protective effect of TH in an animal model of diabetic [24] and an animal model of menopause [25]. The physicochemical characterization of TH has been studied and reported by previous studies [23, 33].

Experimental design

A total of 144 adult male Sprague-Dawley rats weighing between 260-320 g (aged 9-11 w) were selected and divided them for two experiments: experiment 1 for biochemical analysis (N = 72) and experiment 2 was for histology analysis (N = 72). For each experiment, animals were randomly divided into four groups (n = 18/group).

1. Animals in group 1 served as normal control group. Animals were treated orally with drinking water five times every 12 h.

2. Animals in group 2 served as KA only-treated group. Animals were treated orally with drinking water five times every 12 h.

3. Animals in group 3 served as a TH+KA-treated group. Animals were treated orally with TH (1.0 g/kg body weight) five times every 12 h. The dosage of TH was selected based on other studies [24-25].

4. Animals in group 4 served as a TPM+KA-treated group. Animals were treated orally with TPM (40 mg/kg body weight) five times every 12 h. The dosage of TPM was selected as described in previous studies [34-36].

All treatments were conducted between 8:00 a. m.-10:00 a. m. and between 8:00 p. m.-10:00 p. m. for each treatment day. Thirty minutes after the last oral administration, animals in all groups were received a subcutaneous injection (s. c.) of KA (15 mg/kg body weight; 10 mg/ml in saline). For the control groups, a subcutaneous injection of saline was administered instead.

Considering the dosage of KA (15 mg/kg body weight) used was associated with high mortality rate, special efforts were made to improve the survival rate of KA-induced animals [37]. Approximately 90 min after the first generalized seizure, which was categorized as stage 4 by Zhang *et al.* study [38], diazepam (10 mg/kg body weight) was injected intraperitoneally to animals of 24 h and 48 h subgroup. For the control groups, an equal amount of saline was administered.

Biochemical analysis

Sample collection and tissue homogenate preparation

The rats were decapitated using guillotine depending on the time of sacrifice; 2 h, 24 h, and 48 h after KA administration with the respective control groups (n = 6 rats per groups for each time point). The brain was quickly removed. The cerebellum and brainstem were rapidly dissected, weighed and homogenized in an ice-cold sodium phosphate buffer (0.1 M, pH 7.4) to make 7.5 % (w/v) homogenates using a motor-driven Teflon-glass homogenizer. The homogenates were then centrifuged in a refrigerated centrifuge at 1000 x g for 15 min at 4 °C. Aliquots of the resulting supernatant were used for the determination of biochemical parameters.

Determination of thiobarbituric acid reactive substances level

The lipid peroxidation was estimated colourimetrically by measuring thiobarbituric acid reactive substances (TBARS) level using the method previously described [39] with some modifications. Briefly, a 100 µl of 7.5 % (w/v) homogenates was added to the reaction mixture, containing 0.2 ml of 8.1 % (w/v) sodium dodecyl sulfate, 1.5 ml of 20 % (v/v) acetic acid (pH 3.5), 1.5 ml of 0.8 % (w/v) thiobarbituric acid and 0.7 ml of distilled water.

The sample was incubated at 95 °C for an hour. After cooling in an ice-bath for 5 min, the sample was centrifuged at 1 000 x g for 10 min. The absorbance reading of supernatant (1 ml) was measured at 532 nm. Using 1,1,3,3-tetraethoxypropane as standard, TBARS level was calculated and expressed as nanomoles of malondialdehyde equivalent per gram wet tissue.

Determination of total antioxidant status level

Total antioxidant status (TAS) of the homogenate sample was determined as previously described [40]. TAS level was calculated and expressed as nanomoles of uric acid equivalent per gram wet tissue.

Determination of protein carbonyls contents

Protein oxidation was estimated colourimetrically by measuring protein carbonyls contents using Protein Carbonyl Colorimetric assay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA) following the manufacturer's protocol. Protein carbonyls contents were expressed as nanomoles per gram wet tissue.

Determination of nitric oxide concentration

Nitric oxide (NO) concentration was estimated as a total nitrate/nitrite (NOx) using Nitrate/Nitrite Colorimetric assay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA) following

the manufacturer's protocol. The concentration of NOx was expressed as nanomoles per gram wet tissue.

Determination of reduced glutathione and oxidized glutathione level

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined using Glutathione assay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA) following the manufacturer's protocol. The concentration of GSH and GSSG were expressed as nanomoles per gram wet tissue and the ratio of GSH/GSSG was calculated.

Histology analysis

Perfusion and sample collection

The rats were sacrificed depending on the time of sacrifice; 2 h, 24 h, and 48 h after KA administration with the respective control groups. The rats (n = 6 rats per groups for each time point) were deeply anesthetized with ketamine/xylazine mixture (90 mg/kg body weight ketamine and 5 mg/kg body weight xylazine), and subsequently were perfused transcardially with ice-cold phosphate buffer saline, pH 7.4 and followed by 4 % paraformaldehyde in sodium phosphate buffer (0.1 M, pH 7.4). The cerebellum and brainstem were post-fixed in the same fixative solution for overnight at 4 °C. After post-fixation, the cerebellum and brainstem were processed, embedded in paraffin, and sectioned into serial 5-µm-thick coronal sections.

Fluoro jade C staining

To identify degenerating neurons in the cerebellum and brainstem of rats, Fluoro Jade C (FJC) staining was performed using method previously described [41]. The sections were visualized under 200x magnification using an Olympus BX41 fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan) using a filter system designed for visualizing fluorescein isothiocyanate (Excitation peak: 485 nm, Emission peak: 525 nm) equipped with a high resolution digital camera system and desktop computer preinstalled with image analysis software, analySIS FIVE.

Statistical analysis

All data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) for Windows software version 22.0 (SPSS Statistics IBM, Chicago, USA). For the normally distributed data with equal variances, the statistical significance of differences was determined by one-way analysis of variance (ANOVA) test followed by Tukey post-hoc test. Results are expressed as mean±standard deviation (SD). Meanwhile, for the non-normally distributed data, the statistical significance of differences was determined by Kruskal-Wallis test and followed by Mann-Whitney U (MW) test. Results are expressed as median (Interquartile Range; IqR). Data were considered statistically significant difference when the p-value was less than 0.05 (p<0.05).

RESULTS

KA administration induced epileptic seizures.

KA (15 mg/kg body weight; s. c.) induced epileptic seizures in all KA-treated rats and KA-treated rats that received pretreatment of TH and TPM, in contrast to the control group, which showed no seizure activity.

KA caused increase in TBARS levels in the cerebellum and brainstem

KA caused significant increases ($p<0.05$) in TBARS levels in the cerebellum and brainstem of KA-induced rats at all-time points, as compared to control groups (table 1) indicating the lipid peroxidation. TBARS levels in the cerebellum and brainstem were significantly reduced ($p<0.05$) in the TH+KA-treated group after 2 h, 24 h and 48 h of KA administration, as compared to KA only-treated group (table 1). This result suggested that the pretreatment with TH reduced lipid peroxidation in the cerebellum and brainstem induced by KA. The effect of TH in reducing the TBARS level was better than TPM at 48 h after KA administration in the cerebellum.

Table 1: Effect of KA on TBARS levels in the cerebellum and brainstem

Brain regions	TBARS level (nanomoles of malondialdehyde equivalent/gram wet tissue) median (IqR), (n = 6/group)					Kruskal-Wallis test (N = 24)
	Subgroups	CONTROL	KA	TH+KA	TPM+KA	p-value
Cerebellum	2 h	18.02(2.98)	26.20(2.66)[a]	22.86(3.13) [a,b]	24.12(3.28) [a,b]	0.0007
	24 h	19.26(2.68)	118.15(10.36)[a]	86.11(16.76) [a,b]	87.61(16.93) [a,b]	0.0002
	48 h	20.05(1.48)	92.24(8.82)[a]	71.78(3.93) [a,b,c]	67.16(3.50) [a,b]	0.0001
Brainstem	2 h	26.20(2.66)	36.69(1.84)[a]	33.68(2.38) [a,b]	36.69(2.14) [a]	0.0005
	24 h	27.52(4.93)	140.38(11.17)[a]	95.89(16.91) [a,b]	98.66(7.84) [a,b]	0.0002
	48 h	28.74(1.19)	110.27(14.57)[a]	86.51(11.37) [a,b]	90.25(21.50) [a,b]	0.0001

The results were expressed as the median (IqR) (n = 6 rats per group for each time point). The significant difference was determined by non-parametric test, Kruskal-Wallis test followed by Mann-Whitney U (MW) post-hoc test with $p<0.05$ indicates statistically significant difference. [a]$p<0.05$ versus control group (MW); [b]$p<0.05$ versus KA group (MW); [c]$p<0.05$ versus TPM+KA group (MW). KA, kainic acid; TH, tualang honey; TPM, topiramate; IqR, interquartile range; TBARS, thiobarbituric acid reactive substances.

KA caused decrease in TAS levels in the cerebellum and brainstem

There were significant ($p<0.05$) decreases in TAS levels in the cerebellum and brainstem of KA-induced rats at all-time points, as compared to control groups (table 2). Pretreatment with TH significantly ($p<0.05$) attenuated the decrease of TAS levels in the cerebellum and brainstem of KA-induced rats at all-time points, as compared to KA only-treated group (table 2). The effect of TH in increasing the TAS level was better than TPM at 24 h after KA administration in the cerebellum and at 24 h and 48 h after KA administration in the brainstem.

Table 2: Effect of KA on TAS levels in the cerebellum and brainstem

Brain regions	TAS level (nanomoles of uric acid equivalent/gram wet tissue) median (IqR), (n = 6/group)					Kruskal-Wallis test (N = 24)
	Subgroups	CONTROL	KA	TH+KA	TPM+KA	p value
Cerebellum	2 h	1034.40 (22.36)	726.92 (48.20) [a]	838.68 (52.53) [a,b]	811.77 (42.91) [a,b]	0.0002
	24 h	1033.40 (25.66)	528.16 (72.70) [a]	746.76 (33.28) [a,b,c]	699.82 (73.30) [a,b]	0.0001
	48 h	1045.57 (22.22)	813.12 (51.93) [a]	916.39 (49.58) [a,b]	880.18 (42.60) [a,b]	0.0002
Brainstem	2 h	1211.24 (29.36)	991.53 (30.40) [a]	1108.24 (35.46) [a,b]	1092.73 (73.88) [a,b]	0.0002
	24 h	1240.92 (41.65)	637.02 (59.22) [a]	830.76 (49.22) [a,b,c]	800.20 (20.09) [a,b]	0.0001
	48 h	1226.18 (37.73)	799.80 (63.40) [a]	1062.24 (57.52) [a,b,c]	1000.28 (35.46) [a,b]	0.0001

The results were expressed as the median (IqR) (n = 6 rats per group for each time point). The significant difference was determined by non-parametric test, Kruskal-Wallis test followed by Mann-Whitney U (MW) post-hoc test with $p<0.05$ indicates statistically significant difference. [a]$p<0.05$ versus control group (MW); [b]$p<0.05$ versus KA group (MW); [c]$p<0.05$ versus TPM+KA group (MW). KA, kainic acid; TH, tualang honey; TPM, topiramate; IqR, interquartile range; TAS, total antioxidant status.

KA caused increase in protein carbonyl content in the cerebellum and brainstem

KA caused significant increases (*p<0.05*) in the protein carbonyl contents in the cerebellum and brainstem at all-time points, as compared to control groups (table 3), indicating the protein oxidation. Pretreatment with TH significantly (*p<0.05*) attenuated the increase of protein carbonyl contents in the cerebellum and brainstem of KA-induced rats at all-time points, as compared to KA only-treated group (table 3). This result suggested that the pretreatment with TH reduced protein oxidation in the cerebellum and brainstem induced by KA.

Table 3: Effect of KA on protein carbonyl content in the cerebellum and brainstem

Brain regions	Protein carbonyl contents (nanomoles/gram wet tissue) mean±SD, (*n* = 6/group)					ANOVA test (*N = 24*)
	Subgroups	Control	KA	TH+KA	TPM+KA	*p* value
Cerebellum	2 h	60.61±23.47	208.33±26.57[a]	166.67±23.47[a,b]	174.24±23.47[a]	0.0000
	24 h	64.39±17.11	321.97±33.45[a]	265.15±18.56[a,b]	261.36±37.34[a,b]	0.0000
	48 h	60.61±18.56	276.52±33.45[a]	219.70±18.56[a,b]	223.48±44.11[a,b]	0.0000
Brainstem	2 h	68.18±14.37	181.82±28.75[a]	143.94±23.47[a,b]	166.67±23.47[a]	0.0000
	24 h	64.39±17.11	284.09±23.84[a]	238.64±23.84[a,b]	238.64±23.84[a,b]	0.0000
	48 h	64.39±17.11	238.64±23.84[a]	200.76±17.11[a,b]	215.91±27.84[a]	0.0000

The results were expressed as the mean±SD (n = 6 rats per group for each time point). The significant difference was determined by parametric test, ANOVA test followed by Tukey post-hoc test with *p<0.05* indicates statistically significant difference. [a]*p<0.05* versus control group; [b]*p<0.05* versus KA group. KA, kainic acid; TH, tualang honey; TPM, topiramate; SD, standard deviation; ANOVA, one-way analysis of variance.

KA caused increase in NOx levels in the cerebellum and brainstem

The NOx levels in the cerebellum and brainstem were significantly increased (*p<0.05*) in the KA only-treated group at all-time points, as compared to control groups (table 4). Pretreatment with TH and TPM significantly reduced (*p<0.05*) the increase in NOx level in the cerebellum and brainstem after 2 h, 24 h and 48 h of KA administration, as compared to KA only-treated group (table 4).

Table 4: Effect of KA on the NOx levels in the cerebellum and brainstem

Brain regions	NOx level (nanomoles/gram wet tissue) median (IqR), (*n* = 6/group)					Kruskal-Wallis test (*N = 24*)
	Subgroups	CONTROL	KA	TH+KA	TPM+KA	*p* value
Cerebellum	2 h	511.96 (26.12)	715.23 (33.56) [a]	421.77 (12.48) [a,b,c]	442.65 (26.58)[a,b]	0.0001
	24 h	513.54 (23.89)	1011.02 (29.47) [a]	626.85 (37.67) [a,b,c]	655.49 (30.17) [a,b]	0.0001
	48 h	522.56 (30.53)	813.83 (31.69) [a]	516.47 (16.23) [b,c]	549.67 (38.00) [b]	0.0008
Brainstem	2 h	409.75 (26.73)	613.50 (36.69) [a]	325.20 (28.25) [a,b,c]	357.39 (41.62) [a,b]	0.0001
	24 h	412.78 (12.50)	931.48 (65.69) [a]	533.96 (21.32) [a,b,c]	554.15 (13.59) [a,b]	0.0001
	48 h	414.74 (10.90)	712.65 (23.30) [a]	414.74 (20.75) [b,c]	445.96 (45.82) [a,b]	0.0008

The results were expressed as the median (IqR**)** (n = 6 rats per group for each time point). The significant difference was determined by non-parametric test, Kruskal-Wallis test followed by Mann-Whitney U (MW) post-hoc test with *p<0.05* indicates statistically significant difference. [a]*p<0.05* versus control group (MW); [b]*p<0.05* versus KA group (MW); [c]*p<0.05* versus TPM+KA group (MW). KA, kainic acid; TH, tualang honey; TPM, topiramate; IqR, interquartile range; NOx, total nitrate/nitrite.

Table 5: Effect of KA on GSH levels in the cerebellum and brainstem

Brain regions	GSH level (nanomoles/gram wet tissue) (*n* = 6/group)					ANOVA test/Kruskal-Wallis test (*N = 24*)
	Subgroups	Control	KA	TH+KA	TPM+KA	*p* value
Cerebellum Median (IqR)	2 h	1306.77 (239.62)	970.25 (30.41)[a]	1204.87 (288.06)[b]	1141.74 (214.03)[a,b]	0.0045
	24 h	1309.52 (138.18)	688.20 (151.39)[a]	942.97 (132.22)[a,b]	951.33 (60.25)[a,b]	0.0003
	48 h	1270.08 (203.53)	776.84 (229.71)[a]	1121.78 (115.86)[a,b]	1108.11 (54.87)[a,b]	0.0003
Brainstem mean±SD	2 h	579.60±65.89	392.14±106.09[a]	491.70±85.36	509.66±73.09	0.0089
	24 h	555.27±78.77	328.54±35.59[a]	419.61±53.40[a]	429.83±52.82[a,b]	0.0000
	48 h	619.02±79.63	326.11±135.95[a]	555.24±73.11[b]	487.67±160.90	0.0024

For the cerebellum, the results were expressed as the median (IqR**)** (n = 6 rats per group for each time point) and the significant difference was determined by non-parametric test, Kruskal-Wallis test followed by Mann-Whitney U (MW) post-hoc test with *p<0.05* indicates statistically significant difference. [a]*p<0.05* versus control group (MW); [b]*p<0.05* versus KA group (MW). For the brainstem, the results were expressed as the mean±SD (n = 6 rats per group for each time point) and the significant difference was determined by parametric test, ANOVA test followed by Tukey post-hoc test with *p<0.05* indicates statistically significant difference. [a]*p<0.05* versus control group; [b]*p<0.05* versus KA group. KA, kainic acid; TH, tualang honey; TPM, topiramate; IqR, interquartile range; SD, standard deviation; ANOVA, one-way analysis of variance; GSH, reduced glutathione.

KA caused decrease in GSH levels in the cerebellum and brainstem

KA caused a significant decrease (*p<0.05*) in GSH level in the cerebellum and brainstem at all-time points, as compared to control groups (table 5). Pretreatment with TH significantly (*p<0.05*) attenuated the decrease of GSH levels in the cerebellum of KA-induced rats all time-points, as compared to KA only-treated group (table 5). However, in the brainstem, TH significantly (*p<0.05*) attenuated the decrease of GSH levels only after 48 h of KA administration, as compared to KA only-treated group (table 5).

KA caused increase in GSSG levels in the cerebellum and brainstem

In the cerebellum, KA caused a significant increase (*p<0.05*) in GSSG level at 24 h after KA administration, as compared to the control group (table 6). On the other hand, KA caused significant increases (*p<0.05*) in GSSG levels in the brainstem at all-time points, as compared to the control group (table 6). Pretreatment with TH only attenuated the increase of GSSG level after 48 h of KA administration in the brainstem, as compared to KA only-treated group (table 6).

Table 6: Effect of KA on GSSG levels in the cerebellum and brainstem

Brain regions	GSSG level (nanomoles/gram wet tissue) mean±SD, (*n* = 6/group)					ANOVA test (*N = 24*)
	Subgroups	Control	KA	TH+KA	TPM+KA	*p* value
Cerebellum	2 h	35.73±14.55	57.77±13.38	46.43±16.94	50.46±15.51	0.1185
	24 h	31.64±10.42	72.61±23.39[a]	50.90±16.76	56.47±23.42	0.0130
	48 h	38.07±17.99	73.38± 28.19	49.70±25.44	60.12±23.82	0.1056
Brainstem	2 h	27.19±11.15	67.50±28.05[a]	44.97±21.52	55.89±24.97	0.0336
	24 h	26.26±7.77	72.37±9.41[a]	53.86±14.25[a]	57.05±15.77[a]	0.0000
	48 h	23.46±6.30	44.03±7.98[a]	27.34±5.55[b]	27.74±5.34[b]	0.0001

The results were expressed as the mean±SD (n = 6 rats per group for each time point). The significant difference was determined by parametric test, ANOVA test followed by Tukey post-hoc test with *p<0.05* indicates statistically significant difference. [a]*p<0.05* versus control group; [b]p<*0.05* versus KA group. KA, kainic acid; TH, tualang honey; TPM, topiramate; SD, standard deviation; ANOVA, one-way analysis of variance; GSSG, oxidized glutathione.

KA caused decrease in the ratio of GSH/GSSG in the cerebellum and brainstem

For the ratio of GSH/GSSG, administration of KA caused the ratio of GSH/GSSG to decrease (*p<0.05)* all time points in the both regions, as compared to control groups (table 7). Pretreatment with TH significantly (*p<0.05*) attenuated the decrease of GSH/GSSG ratio only in the brainstem of KA-induced rats after 24 h and 48 h of KA administration, as compared to KA only-treated group (table 7).

Table 7: Effect of KA on the ratio of GSH/GSSG in the cerebellum and brainstem

Brain regions	Ratio of GSH/GSSG median (IqR), (*n* = 6/group)					Kruskal-Wallis test (*N = 24*)
	Subgroups	Control	KA	TH+KA	TPM+KA	*p* value
Cerebellum	2 h	39.01 (36.63)	15.60 (5.40)[a]	23.32 (25.13)	23.37 (14.30)[a]	0.0088
	24 h	45.75 (25.84)	9.45 (8.52)[a]	18.62 (10.16)[a]	17.40 (14.64)[a]	0.0023
	48 h	40.43 (34.78)	12.19 (9.30)[a]	31.03 (23.10)	18.29 (19.00)	0.0161
Brainstem	2 h	23.21 (8.33)	5.57 (10.15)[a]	11.67 (14.91)	8.52 (12.99)	0.0363
	24 h	22.37 (12.71)	4.49 (1.81)[a]	7.44 (5.91)[a,b]	7.29 (6.27)[a,b]	0.0007
	48 h	29.96 (14.14)	8.14 (6.83)[a]	20.03 (6.19)[b]	17.71 (13.58)[b]	0.0029

The results were expressed as the median (IqR) (n = 6 rats per group for each time point). The significant difference was determined by non-parametric test, Kruskal-Wallis test followed by Mann-Whitney U (MW) post-hoc test with *p<0.05* indicates statistically significant difference. [a]p<0.05 versus control group (MW); [b]p<0.05 versus KA group (MW). KA, kainic acid; TH, tualang honey; TPM, topiramate; SD, standard deviation; ANOVA, one-way analysis of variance; GSH, reduced glutathione; GSSG, oxidized glutathione.

Assessment of neurodegeneration by fluoro jade C in the cerebellum and brainstem

Histological observation of the FJC staining revealed that there was no FJC-positive neuron in the cerebellum and brainstem for all groups at any time-point, including the control groups (data not provided).

This result indicated that there was no degenerated neuron in the cerebellum and brainstem 2 h, 24 h, and 48 h post-KA injection.

DISCUSSION

Considering that brain has a high amount of polyunsaturated fatty acids, a high oxygen requirement and a low capacity of antioxidant, these conditions make brain is more susceptible to oxidative damage. Oxidative stress has been suggested to be a major player in the mechanism of excitotoxicity in different brain regions in KA-induced rats [42-43]. In the present study, oxidative stress-related markers were studied in rat cerebellum and brainstem. KA caused lipid peroxidation, protein oxidation, impairment of glutathione status, an increase of nitric oxide production and a decrease in the total antioxidant status in the rat cerebellum and brainstem 2 h, 24 h, and 48 h post-KA administration. Systemic injection of KA on rats also induced epileptic seizures in rats. All previous studies using the same dosage of KA also reported that all KA-treated animals have epileptic seizures [44-47]. Administration of KA has also known to induce a sequence of well-characterized seizures syndrome [7, 38]. On top of that, this study is the first to report the protective effect of TH on the cerebellum and brainstem in KA-induced excitotoxicity model. Pretreatment with TH attenuated oxidative stress induced by KA in the cerebellum and brainstem after 2 h, 24 h and 48 h of KA administration by attenuating those KA-induced alternations.

Excitotoxicity causes the production of free radicals, leading to increases in lipid peroxidation and protein oxidation level. The overproduction of free radicals disturbed the antioxidant defence system which leads to the oxidative stress. In this study, the elevation of protein carbonyl contents and TBARS level and the reduction of TAS level in the cerebellum and brainstem indicated an increase of lipid peroxidation and protein oxidation and the

presence of low antioxidant capacity. This might be due to the formation of free radical and free radical chain reaction following KA-induced excitotoxicity. Moreover, increases of lipid peroxidation and protein oxidation as early as 2 h, suggesting both of lipid and protein are exposed to the damaging effect of oxidative stress and the involvement of free radicals during an early time-point of the KA-induced excitotoxic damage. The sustained elevation of lipid peroxidation and protein oxidation and the decrease of antioxidants status observed after 24 h post-KA administration suggested the increase of free radical production and greater oxidative damage in the cerebellum and brainstem induced by KA. These results indicate the imbalance between free radical production and brain antioxidant defence system in KA-induced rats. Previous studies have reported that KA caused lipid peroxidation and protein oxidation in the different brain regions [42, 48]. Therefore, based on this study and previous findings, it could be indicated that the production of free radical and the involvement of oxidative stress in the cerebellum and brainstem in the animal model of KA. In this study, pretreatment with TH reduced lipid peroxidation and protein oxidation level and elevated the total antioxidant status in the cerebellum and brainstem induced by KA.

Glutathione is the major low molecular weight thiol in the mammalian system and most important antioxidants in the brain. It can react directly with free radicals in non-enzymatic reaction and thus serves as an important intracellular free radical scavenger that protects cells from oxidative damage. Besides, glutathione is also co-substrate for many important enzymes and plays a role in the hydrogen peroxide degradation [49]. A severe oxidative stress can alter endogenous antioxidant function in the brain. The cellular redox status is often assessed by the ratio of GSH to GSSG and it is a reliable measurement of the cellular oxidative stress and cellular damage [50]. The present study showed that administration of KA elicited the impairment of the glutathione system, which caused the decreases of GSH level and GSH/GSSG ratio and the increase of GSSG level in the cerebellum and brainstem, suggesting the involvement of oxidative stress. Decreased GSH level may be due to the increase of free radical production, which utilized a large amount of GSH. The reduction of GSH indicated lower antioxidant capacity, which consistent with TAS level. The reduction of GSH might be resulted from the role of GSH as intracellular free radical scavenger and co-substrate for glutathione peroxidase and glutathione-S-transferase [49]. The result from this study supported earlier findings of the reduction of GSH level and the elevation of GSSG level in the cerebellum and brainstem after KA administration [42, 48]. Pretreatment with TH showed the protective effect on the impairment of the glutathione system by attenuating the decrease of GSH levels in the cerebellum of KA-induced rats at all time points. Pretreatment with TH did also show the protective effect on the impairment of the glutathione system caused by KA administration by attenuating the decreases of GSH level and GSH/GSSG ration and the increase of GSSG levels in the brainstem only after 48 h of KA administration.

In addition, KA has resulted in the increase of NO production. NO has been regarded as a messenger molecule in the central nervous system [51] and has implicated in the mechanism of excitotoxicity [52]. NO is unstable and is easily to get converted to other more stable forms: nitrites and nitrates. KA-induced seizure produced a marked increase in the free radical NO because of oxidative stress and leading to depletion of high energy phosphates [46]. The present study demonstrated the increased of NOx level in the cerebellum and brainstem after 2 h, 24 h, and 48 h of KA administration. This study supported the earlier findings of NO involvement in the pathogenesis of KA-induced excitotoxicity in the brain regions [5, 46, 48] and also in the mechanism of neurodegeneration [53].

Despite there were biochemical changes associated with oxidative stress induced by KA in the cerebellum and brainstem, no degenerated neuron was detected in the cerebellum and brainstem. Several studies have reported that a marked number of degenerated neurons were detected following systemic injection of KA in the hippocampus, basal ganglia, piriform cortex, thalamus, amygdala, and parietal cortex [9-11]. From these findings, there was selective neuronal susceptibility to the neurodegeneration and differential of the sensitivity of neuronal populations to the excitatory action of KA in the brain. This might be due to the variation in the distribution of kainic acid binding sites in brain regions [54]. The cerebellum was reported to have a moderate density of KA binding sites and contains predominantly with low-affinity KA binding sites [54-55]. Meanwhile, brainstem has a lower density of KA binding sites than that in other brain regions. Most of the KA binding sites in the brainstem are low-affinity KA binding sites [55]. These might explain less vulnerable to the neurodegeneration, but not to oxidative stress in the cerebellum and brainstem of KA-induced rats in this study.

TH has been reported to contain many bioactive compounds, which include gallic acid, syringic acid, coumaric acid, cinnamic acid, caffeic acid, catechin, quercetin, pinobanksin-3-O-propionate, pinobanksin-3-O-butyratengenin and naringenin [23, 33]. Several studies have investigated the protective effects of compounds identified in TH against KA model of excitotoxicity. Bioactive compounds found in TH, like gallic acid [56] and caffeic acid [57], have been reported to reduce oxidative stress in KA-induced excitotoxicity model. Another beehive product, propolis, has been reported to attenuate KA-induced oxidative stress in the brain which is believed due to its antioxidant property [50, 58]. Thus, the protective effect of TH against KA-induced oxidative stress may be due to its antioxidant property which is attributed to the presence of flavonoids and phenolic acids.

This study also determined the significance of the modulatory effect on kainite receptors, by comparing pretreatment between TH and TPM to extend our understanding of the pharmacological mechanism of TH. Pretreatment with TH helped to reduce the oxidative stress and the protective effect of TH was better than TPM. Previous studies have reported that topiramate reduced lipid peroxidation in piriform cortex of KA-induced rat [45] and attenuated KA-induced hippocampal neurodegeneration in mice [59]. This could be due to the inhibition of the GLuR5 kainate receptors by topiramate through a postsynaptic mechanism [60-61]. Therefore, it can be suggested that the inhibition of the GLuR5 kainate receptors is essential to protect the brain against KA-induced excitotoxicity. Experimental evidence also indicated that TPM acts through multiple mechanisms of action, including modulation of voltage-dependent sodium channels and voltage-dependent calcium channels [62-63], inhibition of excitatory glutamate pathway [64] and enhancement of gamma-aminobutyric acid (GABA) activity [65]. Taken together, the protection against excitotoxicity is not based on a specific mechanism or pathway but also on several mechanisms or pathways. Further investigations are needed to elucidate the exact mechanism of action of TH on oxidative pathways in the brain. Besides, the anti-inflammatory effect of TH may be related in controlling the production of free radicals released from the inflamed tissues. This could be the cause and/or result of oxidative stress.

CONCLUSION

The findings in this study demonstrate oxidative stress as a possible mechanism of excitotoxicity. These brain regions (cerebellum and brainstem) were susceptible to the oxidative stress but less susceptible to the neurodegeneration. Pretreatment with TH attenuates oxidative stress in the cerebellum and brainstem of KA-induced rats at multiple time points. As a conclusion, TH has neuroprotective potential and it can be used as a potential therapeutic measure against oxidative stress via its antioxidant property.

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AUTHORS CONTRIBUTION

NSMS, KNSS, MM, MS, MAA, and SAM involved in the experimental design. NSMS carried out the experiments, collected the data and analyzed the data under the supervision of KNSS, MM, MS, MAA and SAM. NSMS drafted the manuscript under the supervision of KNSS,

MM, MS, MAA, and SAM. All authors have read and approved the final manuscript.

CONFLICTS OF INTERESTS

The authors declared that there are no conflicts of interest

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