Research article

The role of rosemary extract in degeneration of hippocampal neurons induced by kainic acid in the rat: A behavioral and histochemical approach

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Abstract

Systemic kainic acid administration has been used to induce experimental temporal lobe epilepsy in rats. The aim was to evaluate the neuroprotective effect of rosemary extract (40% Carnosic acid) against kainic acid-induced neurotoxicity in hippocampus and impaired learning and memory. Subjects received a single dose of kainic acid (9.5 mg/kg) intraperitoneally, were observed for two hours, and scored from 0 (for normal, no convulsion) to five (for continuous generalized limbic seizures). Rosemary extract (100 mg/kg, orally) was administered daily for 23 days, starting a week before kainic acid injection. Neuronal degeneration in hippocampus was demonstrated by using Fluoro-Jade B immunofluorescence. The number of pyramidal cells in hippocampus was evaluated by Nissl staining. Also, the Morris water maze and shuttle box were used to assess spatial memory and passive avoidance learning, respectively. Results revealed that, after treatment with rosemary extract, neuronal loss in CA1 decreased significantly in subjects in the kainic acid + rosemary extract group. Morris water navigation task results revealed that spatial memory impairment decreased in subjects in the kainic acid + rosemary extract group. Furthermore, results in shuttle box testing showed that passive avoidance learning impairment significantly improved for subjects in the kainic acid + rosemary extract group. These results suggest that rosemary extract improves spatial and working memory deficits and also, due to its antioxidant activities, neuronal degeneration induced by the toxicity of kainic acid in rat hippocampus.

Keywords

Epilepsy; kainic acid; neurotoxicity; rosemary; learning and memory; rosemary extract

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1. Introduction

Epilepsy is a neurological disorder characterized by the periodic occurrence of spontaneous seizures and learning and memory deficits [1, 2]. Seizures appear to cause neuronal death by overactivating glutamate receptors [3]. Despite the crucial role of glutamate in synaptic plasticity and cognitive functions such as learning and memory, high concentrations of this neurotransmitter and overactivation of its receptors cause neurodegeneration in the central nervous system (CNS) [4]. Kainic acid (KA), a glutamate neurotransmitter analog, is a potent neuroexcitatory amino acid. It has been widely used to induce temporal lobe epilepsy (TLE) in animal models [5, 6]. Systemic administration of KA induces repetitive seizures that eventually result in neuronal damage [7]. Neural damage in epilepsy as a result of extreme glutamatergic activity has been implicated in several human nervous system disorders, including, Alzheimer's disease [8], Huntington's disease [9], amyotrophic lateral sclerosis [10], and ischemia [11]. Several reports indicate acute, selective KA-induced neural damage to limbic structures, including CA1 and CA3 cell layers of the hippocampus [12]. Given the integral role of these regions in learning and memory, the toxic effects of KA have been used to investigate the protective role of certain drugs against epilepsy and associated cognitive disabilities [13]. According to several proposals, glutamate neurotoxicity is due to the generation of reactive oxygen species (ROS) and damage to cellular components, such as mitochondria [14]. Therefore, compounds that neutralize ROS potentially protect neurons and prevent subsequent death. *Rosmarinus officinalis*, commonly known as rosemary, is used in cosmetics and medicine, and is also a popular herb [15]. Rosemary extract (RE) has antioxidant properties [16] owing to its phenolic diterpenes, carnosic acid, and carnosol. These compounds play an important role in neutralizing harmful agents by interacting with the free radical chain reaction as hydrogen donors [17]. Some studies have also reported that RE extract has a number of pharmacological properties, and may play a hepatoprotective [18], antibacterial [19], anti-ulcerogenic [20], antidiabetic [21], antiinflammatory [22], and anti-depressant [23] role. The aim of the present study was to evaluate the protective role of RE in the context of hippocampus KA neurotoxicity.

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2. Materials and methods

2.1. Materials, animals and treatments

KA (SigmaK-0250) and Fluoro-Jade B (FJB: B-AG310) were purchased from Sigma Chemical Co. (Saint Louis, Missouri USA) and the Millipore company (Billerica, Massachusetts, USA), respectively. Hydroalcoholic RE (40% Carnosic acid; RAP20-110401) was prepared by Hunan Geneham Biomedical Technology Ltd(China).

Adult male Wistar rats (n = 34) weighing 200–250 g were used (Pasteur's Institute, Tehran, Iran or animal lab of Iran University of Medical Sciences). They were housed at room temperature, 21 $\pm$ 2°C, with a 12/12 h light/dark cycle and, except during experiments, had unlimited access to food and water.

Behavioral experiments were conducted between 9.00 and 14.00. Subjects were assigned to four groups: Control, n = 10; RE, n = 10; KA, n = 7 and KA+RE, n = 7. Subjects in RE groups received daily RE (100 mg/kg) dissolved in distilled water, orally for 23 days, starting a week before KA injection. Subjects in KA groups were administered, by intraperitoneal (i.p.) injection, a single dose of KA (9.5 mg/kg) dissolved in normal saline.

2.2. Behavioral tests

After KA injection, seizure signs of subjects were evaluated and graded. Also, one week after KA administration, a passive avoidance task was performed over two days of training and testing. On day 11, the Morris water maze (MWM) test was performed for six days. All animal procedures were approved by the animal care committee of Chancellor for Research of Iran University of Medical Sciences (Tehran, Iran), according to international rules to minimize animal suffering and reduce the number of animals used.

The seizure signs of subjects were evaluated and graded according to the Racine scale for two hours after injecting KA. The following criteria were used: 0 for normal, rare wet dog shakes (WDS), no convulsion; 1 for intermediate number of WDS, rare focal convulsions affecting the head and extremities; 2 for frequent WDS, frequent focal convulsions, generalized convulsions (no rearing, no salivation); 3 for frequent WDS, focal convulsions, frequent appearance of generalized convulsions with rearing (but not falling) salivation; 4 for frequent WDS, focal convulsions, frequent generalized convulsions with falling, salivation; 5 for continuous generalized limbic seizures, death within two hours [24].

The passive avoidance task was performed using a shuttle box to study the learning and working memory status of subjects. The shuttle box consisted of two equally-sized compartments, with a guillotine (execution) door and a grid floor for the release of an electric foot shock. An electric light bulb illuminates one compartment, while the other remains dark. During the training session, each subject was placed in the lit chamber, facing away from the execution door. When the subject entered the darkened compartment, the door was quietly lowered. Then a 0.5 mA foot shock was applied for two seconds through the grid floor. During the test session, the subject was placed in the light compartment again and, thereafter, entered the dark compartment, but the foot shock was not applied. The latency to enter the dark compartment was recorded for up to 300 seconds [25].

The MWM test is comprised of a monitor, a video camera set in the ceiling, a computerized tracking system (DMS-2), and a black round metal tank (136 cm in diameter, 130 cm in height) filled with water (20 $\pm$ 1°C). Four start locations with white marks were located equidistantly around the edge of the maze, dividing it into four similar quadrants. During training and testing, a black escape or hidden platform (10 cm in diameter) was placed 2 cm below the surface of the water. Water maze tests were performed on 11 days after treatment side-effects. After a single habituation experiment (day 0, 60 s), the escape platform was placed in the middle of the southwest quadrant on five subsequent days. Acquisition test (day 1–5): Each day, subjects were situated in the pool at four different starting points. Entrance from the North, South, East, or West point was varied in a quasi-random manner. The escape latency of the subject was monitored by video and a computerized tracking system (DMS-2) for up to 60 s to measure the spatial learning score of the subject for five subsequent days. Subjects that could not find the platform within 60 s were located there within 10 s by the experimenter. The escape latency was recorded at 60 s. Data from the four trials each day were averaged for each subject. Probe Test (day 6): The hidden platform was removed on the 6th day of experimental tests. The time spent in the target quadrants (TQ) during the 60 s, which revealed the spatial memory score, was recorded.

2.3. Histology

Twenty-three days after RE administration, rats were anesthetized with ketamine (150 mg/kg) and xylazine (15 mg/kg) i.p. through the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brain tissue was removed and embedded in paraffin. The paraffin slides were mounted onto gelatin-coated slides and stained with Nissl and FJB stains.

5-µm thick, Nissl stained (0.1% Cresyl violet acetate) coronal sections of the dorsal hippocampus were studied. At least five sections for each subject, representative planes between -3.3 and -3.8 interval from Bregma, according to the Paxinos and Watson atlas (1986), were examined by scanning the entire extent of the CA1 cell layer. The mean number of intact pyramidal cells in this layer was expressed as the total count obtained from the representative sections. Counters were blind to the treatment received. All sections were visually inspected using an Olympus light microscope (magnification x400) and an OLYSIA Bio Report Soft Imaging System GmbH, Version: 3.2 (Build 670).

FJB is an anionic fluorescent with excitation peaks at 362 and 390 nm and an emission peak at 550 nm. It is an effective marker by which to detect neuronal degeneration. The staining protocol followed Schmued [26]. Briefly, brain sections were immersed in xylene and then rehydrated by alcohol solutions. Slides were then placed into a solution of 0.06% potassium permanganate for 10 min, then rinsed in distilled water. Subsequently, they were incubated for 20 min in the FJB staining solution (0.0004%). Finally, dried slides were cleared with xylene, mounted in DPX (water-free mounting medium), and examined with a x40 objective, using the FITC filter.

2.4. Statistical analysis

Results were presented as mean $\pm$ standard error of the mean (S.E.M.) and calculations were performed using the SPSS statistical software package (version 16). Statistical significance between experimental groups was analyzed using one-way analysis of variance (ANOVA). For multiple comparisons, when appropriate, a post-hoc Tukey's test was performed. The level of statistical significance was

set at $p < 0.05$. All graphs were prepared using Sigma Plot (10) software.

3. Results

3.1. Behavioral tests

Fig. 1 illustrates the effect of administering a single dose of KA with or without RE on the occurrence and intensity of typical seizures over two hours. Animals in the KA and KA+RE groups scored between 0 and 5 on a modified version of the scale devised by Racine. A one-way ANOVA [$F(3,30) = 70.80, p < 0.001$] indicated that there were significant differences among the groups of subjects that received KA (the KA and KA+RE groups) and groups of subjects that received saline or RE (the control and RE group). Administration of KA alone (9.5 mg/kg, i.p.) induced seizures in rats. Post-hoc analysis revealed that treatment with rosemary extract (100 mg/kg, orally) significantly reduced seizure signs and delayed the onset of KA-induced seizures ($p < 0.01$). Additionally, no seizures were observed in the groups that received saline (10 ml/kg) or RE alone.

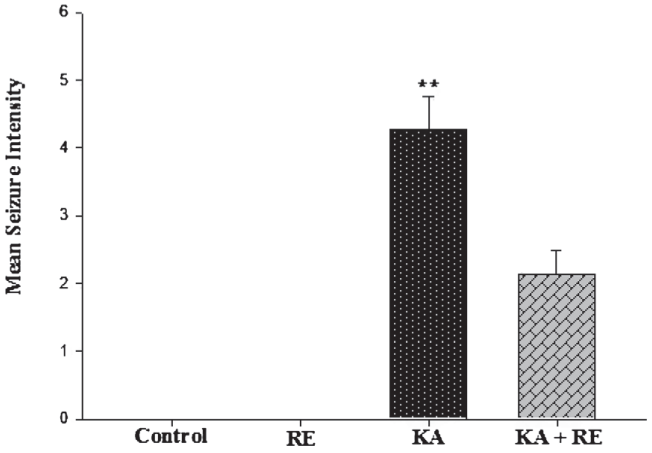

Fig. 1. Effect of KA (kainic acid) with or without RE (rosemary extract) on occurrence and intensity of seizure. Subjects in the control and RE groups received vehicle and rosemary extract, respectively. There was no sign (score 0) of the occurrence of seizure in these subjects. Other groups received KA alone (KA group) or with RE (KA+RE group) and were evaluated for signs of seizure. $^{**}p < 0.01$ compared with the KA+RE group.

As illustrated by Fig. 2, statistical analysis revealed that performance in the passive avoidance task for subjects that received KA administration differed significantly to performance in the other groups. A one way ANOVA ($F(3,27) = 14.92, p < 0.001$) revealed a significant decrease in step-through latency in the KA group compared to the control ($p < 0.001$) and RE ($p < 0.001$) groups. Diminished step through latency for subjects in the KA group indicates impaired passive avoidance memory formation. Further analysis indicated a significant increase in step-through latency in the RE+KA ($p < 0.01$) group, compared to the KA group (Fig. 2). However, RE alone did not significantly influence passive avoidance memory in rats compared to its influence in the control group.

During five consecutive days of testing, escape latency scores in all groups decreased followed by time points on most days. As shown in Fig. 3, on days 1, 2, 3, 4, and 5, escape latency scores for subjects in the KA group increased compared to those in the other groups. On day 2, a one-way ANOVA ($F(3,30) = 4.55, p < 0.01$) indicated that

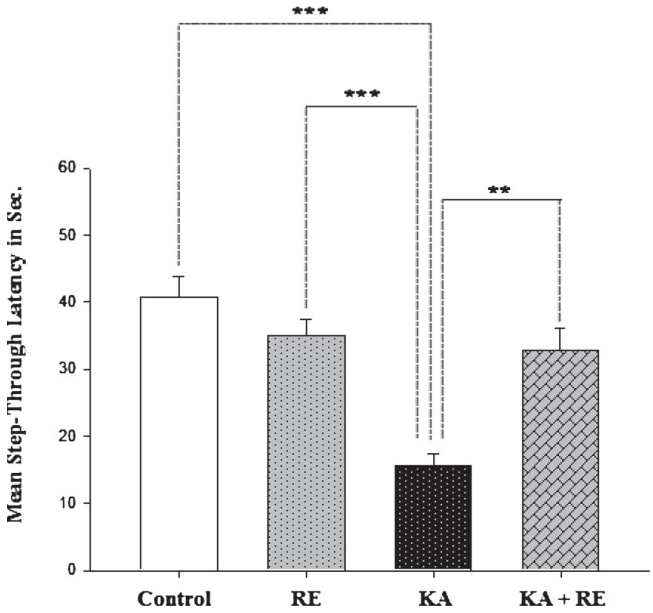

Fig. 2. Effects of systemic administration of KA alone or in combination with RE administration on memory retrieval in the passive avoidance task. On the test day, step-through latency for subjects was assessed as a measure of passive avoidance learning. $^{***}p < 0.001$ compared with the control and RE group, $^{**}p < 0.01$ compared with the KA+RE group.

increased escape latency induced by KA is statistically significant compared to that in the RE group ($p < 0.01$) and the control group ($p < 0.05$). Post hoc analysis also revealed that escape latency score decreased in KA+RE group compared to the KA group, but this difference was not significant ($p = 0.06$). On day 3 ($F(3,30) = 3.29, p < 0.05$), comparison of the groups indicated that escape latency was significantly higher in the KA group compared to the control and RE groups (both $p < 0.05$). On day 4, ($F(3,30) = 3.26, p < 0.05$), escape latency scores were significantly increased compared to the RE group ($p < 0.05$) but not to the control group. Escape latency also decreased in the KA+RE group compared to the KA group on day 4 ($p < 0.05$). No significant differences were found among the other groups at single time points during the trial. Although, these data indicate a tendency for learning in all groups, there is an increase in escape latency time in KA group subjects compared to subjects in the control and RE groups. To explore spatial memory in all groups, a Spatial Probe Test was conducted to measure time spent in the TQ ($F(3,30) = 3.72, p < 0.05$), which is shown in Fig. 4. Post-hoc analysis indicated that the subjects in the KA group spent less time in the TQ compared to subjects in the control group ($p < 0.05$). These data indicated a spatial memory disability under KA treated subjects that spent a long time in the TQ.

3.2. Histology

Results (Fig. 5), compare the mean number of intact pyramidal neurons in CA1 cell layer between groups, loss of neurons was observed in KA groups. alternatively, following RE treatment, neuronal loss significantly decreased in the KA+RE group compared to the KA group ($F(3,16) = 44.849, p < 0.001$). In conclusion, the present results suggest that RE can reduce neuronal damage in the CA1 cell layer after KA-induced seizures. As illustrated by Fig. 6 (density of CA1 neurons in groups), slices from the brain of subjects in the

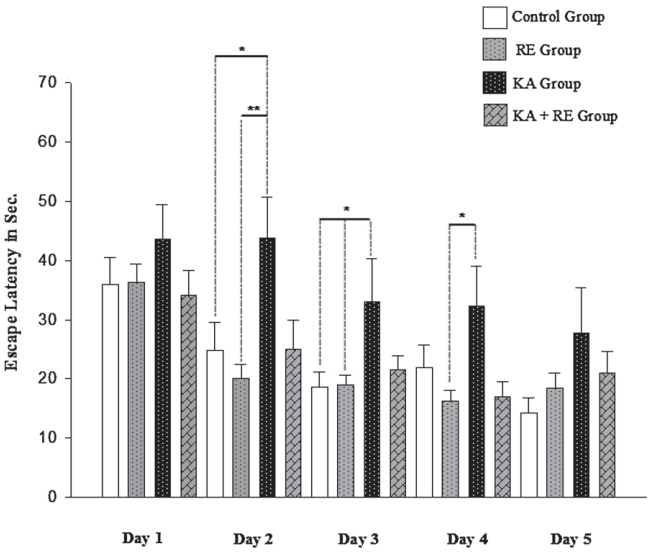

Fig. 3. Effects of systemic administration of KA alone or in combination with RE on spatial memory formation in the Morris water maze test. Animals were classified into four groups according to the administration (Control, RE, KA or KA+RE) and spatial memory formation was evaluated over 5 days. On days 1 and 5, there were no significant differences among groups in Morris water maze performance. On days 2 and 3, KA administration impaired spatial learning in the Morris water maze and caused a significant increase in escape latency when compared to both the control and RE groups. On day 2, post hoc analysis also revealed that escape latency decreased (not significantly) for the KA+RE group compared to the KA group ($p = 0.06$). On day 4, significant differences were observed between the KA group and the control group but not with the RE group. $^{**}p < 0.01$ compared to the RE group, $^{*}p < 0.05$ compared to the control and RE groups.

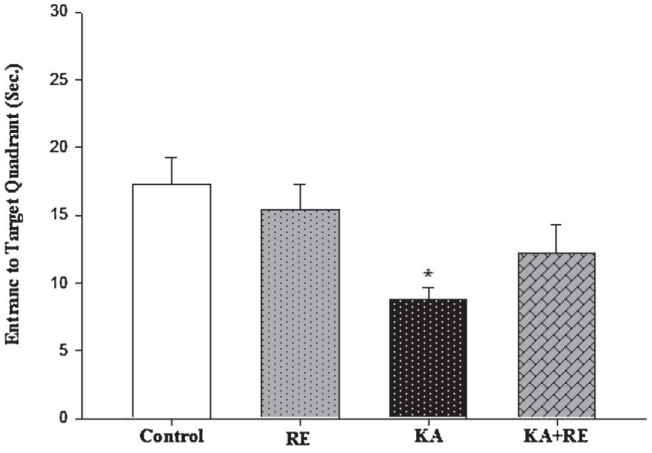

Fig. 4. The spatial probe test measured the time spent in the target quadrant (TQ). KA (kainic acid) administration caused subjects in the KA group to spend less time in the TQ compare to subjects in the control group. This performance by subjects demonstrated a spatial memory disability induced by KA. $^{*}p < 0.05$ compared to control groups.

KA group showed decreased neuronal density in hippocampal CA1 cell layer, compared to the control group. In the other hand, slices from the brain of subjects in the KA+RE group showed less change in neuronal density in this layer.

FJB staining was done to show neuronal loss in CA1 was due to degeneration caused by KA toxicity. Fluorescent micrographs

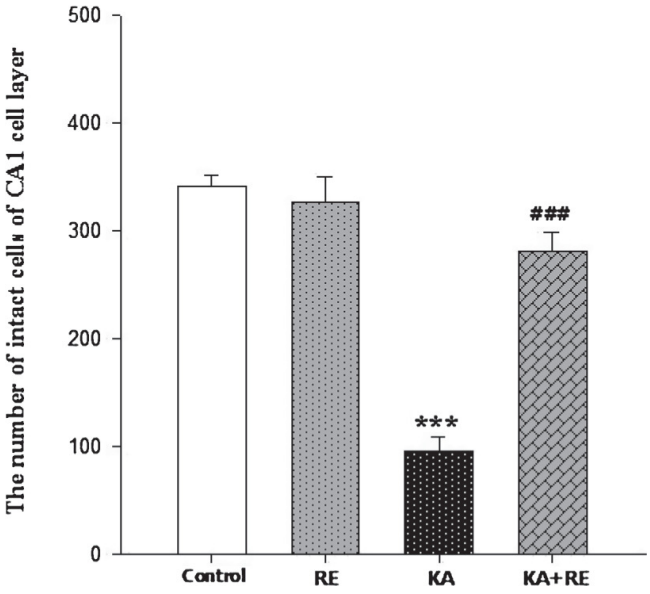

Fig. 5. Improvement effects of RE on hippocampal neurons survival influenced by KA (kainic acid). KA administration significantly decreased the number of intact neurons in CA1 cell layer. RE administration alone had no effect on neuronal survive in this layer compared to the control group, but RE reversed the KA-neurotoxicity in CA1 in the KA+RE group. $^{***}p < 0.001$ compared with the control group, $^{###}p < 0.001$ compared with the KA group.

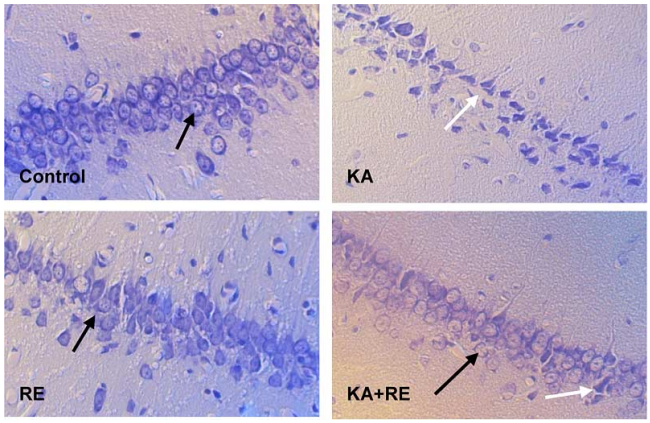

Fig. 6. Light micrographs of hippocampus with cresyl violet staining in the control, KA, RE, and KA+RE groups in CA1 cell layer of hippocampus (400 ×). Black and white arrows indicate intact and degenerating neurons, respectively.

of hippocampus in Fig. 7, showed no stained neurons in the CA1 cell layer of hippocampus in control subjects. Strong FJB staining (indicating degenerating neurons) was observed in the pyramidal cells of the CA1 layer in the KA group. In RE pretreated subjects, the effect of KA was greatly reduced.

4. Discussion

In the present study, neuroprotective effects of RE in KA-induced neurotoxicity was evaluated in a rat model of KA-induced seizure. Previous studies have shown that systemic KA injection causes severe convulsions, seizure-induced brain damage, and increased

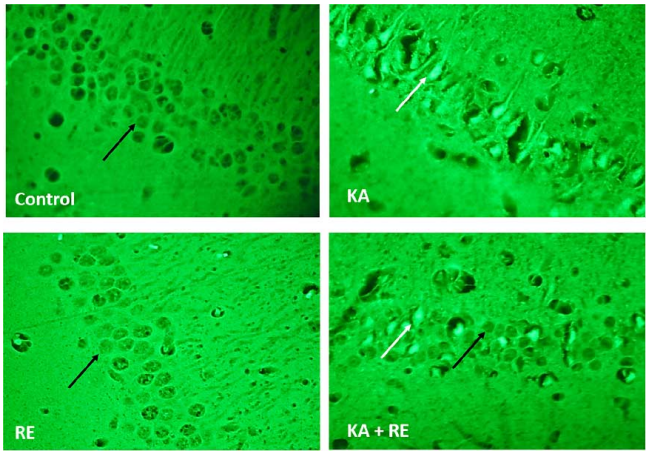

Fig. 7. Fluorescent micrographs of hippocampus with Fluoro-Jade B (FJB) from control, KA, RE, and KA+RE groups in CA1 cell layer of hippocampus (x400). Black and white arrows indicate the intact and fluorescent positive neurons, and white arrows indicate the neurons, respectively. Numerous FJB-stained positive neurons in the CA1 cell layer are seen in KA group. Rare FJB-stained fluorescent cells are detected in CA1 cell layer by pretreatment of RE.

seizure susceptibility in rats [27, 28]. This finding confirms these reports and demonstrates that KA administration (9.5 mg/kg) can induce seizures in rats. Additionally, the comparison of seizure severity in subjects in the KA group and KA+RE group indicated that RE significantly decreases both seizure onset and severity in rats. Seizure severity and signs were scored according to a modified version of a scale devised by Racine [24]. In addition to the epileptic effect of KA, seizure-induced cognitive dysfunction, particularly learning and memory deficits, have been observed following KA administration [29, 30]. Several animal studies indicate that KA administration causes significant memory impairment [31, 32]. This finding confirms these reports and indicates decreased step-through latency for subjects in the KA group compared to the control group. The current results also reveal significant differences in step-through latency for the KA group compared with both the RE and KA+RE groups. Therefore, RE could reverse KA-induced memory impairment in the passive avoidance task. The mechanism of this improving effect of RE has not been well recognized. Pearson and colleagues [33] reported that both KA and pilocarpine (Pilo) induced significant deficits in long-term recognition memory. In the present study, intraperitoneal KA administration significantly impaired cognitive performance on the MWM, as indicated by the delayed time latency for reaching the platform (escape latency) and reduced time spent in the TQ. To the author's knowledge, this is the first study to indicate that RE partially decreased escape latency in the KA+RE group compared to the KA group on days two and four of a MWM test.

Although systemic KA administration can affect different regions of the brain, the hippocampus appears more sensitive to its neurotoxic effects [34, 35]. One cause of such high sensitivity is the high concentration of KA receptors on the membranes of these neurons [36]. It is known that pyramidal cells of the dorsal hippocampus, CA1, and CA3 sub-regions play a critical role in spatial memory formation [37]. It seems that enhanced NMDA-mediated excitatory and loss of GABA-mediated postsynaptic inhibition are the main reason for such an excitotoxic effect of KA [38]. Previous studies have shown that increased levels of brain-derived neurotrophic factor mRNA and protein in hippocampal neurons cause both hyperexcitability and the reduction of inhibitory synaptic transmission [39, 40]. Taken together, this shows that cognitive impairment, particularly deficiency in learning and memory, may be due to selective excitotoxic effects of KA in the hippocampus. Therefore, in patients with TLE, seizure, and subsequent hyperexcitation of neurons in the hippocampus, as a major component located in the medial temporal lobe, causes damage to these neurons and memory impairment is a common comorbidity in these patients [41, 42]. In recent years, significant findings have been obtained with respect to excitatory signaling pathways in neurons and implicate excitatory amino acids and their receptors in neurodegeneration. However, the precise mechanisms by which this occurs are still not completely clear. An increasing body of experimental evidence shows a pivotal role for mechanisms of oxidative stress and the involvement of free radical in this neuronal loss [43]. In agreement with previous findings [44, 45], here, it is confirmed that intraperitoneal administration of KA decreases neurons in the CA1 region of hippocampus. Liang and colleagues [44] reported intense consistent behavioral seizures and oxidative DNA damage in CA3 and CA1 neurons, but not dentate gyrus in aging rats following a low dose of KA administration when compared to age-matched controls. Previous studies show that oxidative stress causes increased Ca^{2+} influx from the extracellular environment into neurons [46, 47]. This rising Ca^{2+} concentration, which is also driven by endoplasmic reticulum, causes, increased Ca^{2+} concentration in mitochondria and nuclei and ultimately leads to neurodegeneration following the disruption of normal metabolism. KA, through activation of glutamate receptors, has similar effects on Ca^{2+} distribution. Indeed, it seems that KA increases the baseline adverse effects of oxidative stress and this additive impact triggers neuronal death in CA. Therefore, as mentioned, KA-induced neurodegeneration provides the possibility of assessments for a variety of interventions that reverse KA effects in the nervous system. A growing body of research suggests that most herbs and spices possess the chemical constituents that reduce oxidative stress vulnerability and the resulting neuronal loss [48, 49] as well as learning and memory impairments [50] in rat models. RE demonstrated high antioxidant activity [16] through its reversal of the activities of antioxidant enzymes [51]. Data demonstrated here that RE significantly decreased the seizure signs as well as the delayed onset of KA-induced seizure. As mentioned above, RE also improved the performance of subjects on the passive avoidance task and the MWM test. The present study showed that KA-induced memory impairments were ameliorated by the RE administered to subjects and by performing the MWM task and passive avoidance task experiences. Consistent with these findings, a previous study [52] in an experimental model of Alzheimer's disease in rats indicated that pretreatment with CA as an effective component of RE, can reduce neuronal death in hippocampus CA1. We also found that CA might ameliorate memory deficit beta-amyloid-induced toxicity in the rat hippocampus [53]. Considering that oxidative stress is one of the pathological mechanisms responsible for the beta-amyloid cascade associated with Alzheimer's disease, it appears that CA (as well as RE) plays multiple roles in the free radical chain reaction as an antioxidant.

In summary, the present study illustrated that oral administration of RE inhibits cell death in CA1 region of rat hippocampus induced

by KA administration. Although the precise mechanism underlying the neuroprotective effect of RE remains unclear, these results suggest that RE plays an important role as a natural product with neuroprotective properties, due to its antioxidant activities.

Acknowledgments

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Conflict of Interest

All authors declare no conflicts of interest.

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