Rosmarinic Acid Mitigates Apoptosis and nNOS Immunoreactivity but not MAPK and COX-2, Following Intrahippocampal Kainic Acid in the Rat

Running Title: Neuroprotective effect of Rosmarinic acid

Authors: Safoura Khamse1*, Seyed Shahabeddin Sadr1,2, Mehrdad Roghani3*, Mina Rashvand1, Maryam Mohammadian4, Narges Marefati3, Fatemeh Ebrahimi3, Elham Harati1

1. Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
2. Electrophysiology Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran.
3. Neurophysiology Research Center, Shahed University, Tehran, Iran.
4. Department of Physiology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.

* To whom correspondence should be addressed:

Dr. Mehrdad Roghani, Neurophysiology Research Center, Shahed University, Tehran, Iran. Tel: +98-21-88964792, Fax: +98-21-88966310. E-mail: mehjour@yahoo.com
Dr. Safoura Khamse, Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Tel: +98-21-66581560. E-mail: s.khamseh90@yahoo.com

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Abstract

One of the agonists for the ionotropic glutamate receptor is Kainic acid (KA). Kainic acid can induce neuronal overactivity and excitotoxicity. Rosmarinic acid (RA) is a normal polyphenolic compound with antioxidant, anti-apoptotic, anti-neurodegenerative and anti-inflammatory features. We designed this research to assess the influence of RA on apoptosis, nNOS-positive neurons number, and COX-2 and MAPK immunoreactivity, following intrahippocampal Kainic acid in the rat. Rats were randomly assigned to three groups Sham, Kainic acid (KA was inserted to the right part of the hippocamp) and Kainic acid + RA (dose of 10 mg/kg/day through a gavage needle for 1 week before injected of KA). Then, histopathological changes included apoptosis (TUNEL assay), nNOS-positive neurons number, MAPK and COX-2 immunoreactivity were evaluated in the hippocampus. In the RA pretreated group, nNOS-positive neurons and TUNEL-positive cells were significantly reduced compared to Kainic acid group (p<0.05). MAPK and COX-2 immunoreactivity showed no significant changes as compared to Kainic acid group and a significant higher reactivity for COX-2 (p<0.01) and MAPK (p<0.005) against sham. As a result, RA has neuroprotective effect against Kainic acid through reduced apoptosis and nNOS-positive neurons but not MAPK and COX-2.

**Keywords:** Kainic acid, Rosmarinic acid, nNOS-positive neurons, TUNEL-Positive cells, MAPK and COX-2 immunoreactivity
Introduction

Kainic acid (KA) is a potent analogue of glutamate leading to increased activity and toxicity of the neurons (Hsieh et al., 2011) via enforcing strong depolarizations that cause cell death. Sometimes this substance is used for modeling of the temporal lobe epilepsy (Levesque & Avoli, 2013). An unrestricted wide spectrum of neuropathological changes can be resulted from acute and sub-acute forms of activity due to KA. Therefore, its generate epileptic disorders that is connected with necrotic cell death and apoptotic (Swamy, Yusof, Sirajudeen, Mustapha, & Govindasamy, 2011). KA also enhances mitogen-activated protein kinase (MAPK) and cyclooxygenase-2 (COX-2) expression (Hsieh et al., 2011).

Labiatae family Plants such as *perilla frutescens*, *mint*, *sage*, *oregano*, *perilla*, and *sweet basil* (Scheckel, Degner, & Romagnolo, 2008) have medical uses for infection, inflammation, depression, indigestion, weakness, enhancement of memory, improvement of circulation and strengthening of fragile blood vessels in traditional medicine. These plants have several compounds with various beneficial effects. These properties are ascribed to their phenolic structure “particularly Rosmarinic acid (RA)” (Shekarchi et al., 2012). RA has several biological functions and anti pathological effect as astringent, anti-bacterial, anti-mutagen, anti-oxidant, anti-cholinesterase, anti-inflammatory, anti-tumor and hepato and cardio protective. Its anti-inflammatory activity can be seen by inhibition of lipoxygenases and cyclooxygenases (Tepe, 2008). Its anti-oxidant and anti-inflammatory property has made it well recognized as a therapeutic agent (Al-Sereiti, Abu-Amer, & Sen, 1999). Furthermore, the neuroprotective effects of RA can be associated with its power to transmogrify some intracellular cascade events participating in neuronal death (Fallarini et al., 2009). RA has shown long-standing benefits for neuronal function, probably due to its ability to overcome with inflammatory response (Luan, Kan, Xu, Lv, & Jiang, 2013) and decrease the expression of proinflammatory molecules (Gamaro et al., 2011).

According to other studies, compounds like RA could reduce DNA damage through their scavenging ability, this suggests a neuroprotective effect for this compound. Which can prevents and manages various neurological disorders, such as epilepsy (Rodrigues Coelho et al., 2015). In our prior research, we showed that RA pretreatment could decrease oxidative stress and score seizure, increases the action of protective systems and prevents the neuronal death of the hippocampus and mossy fiber sprouting (khamse et al., 2015).
The goal of this research was to assess the influence of RA on apoptosis, nNOS-positive neurons number, and COX-2 and MAPK immunoreactivity, following intrahippocampal kainic acid in the rat.

**Materials and Procedures**

The study, thirty adult male rats weighing between 200-250 grams were used. They kept three per cage in a temperature of 22 ± 2 °C under a 12-hour light-dark cycle, with water and food accessible ad libitum. In this study, all attempt were made to diminish number of animals and their pain. Ethical protocols used in this study were approved by the Ethics Committee of the Tehran University of Medical Sciences.

**Experimental technique**

Animals were randomly assigned to three groups Sham, Kainic acid and Kainic acid + RA. For intrahippocampal injections, they were anesthetized with ketamine 60 mg/kg and xylazine 5 mg/kg, i.p, put into the stereotaxic apparatus (Stoelting Co., USA) by the incisor bar fixed at 3.3 mm under the interaural line. The dorsal level of the cranium was visible and a burr cavity was penetrated by the subsequent matching according to the stereotaxic atlas of Paxinos and Watson (Paxinos G, 1986) with the bregma spot as the guideline: anteroposterior; 4.2, mm lateral; 4.2 mm and ventral to the dura; 4.1 mm. Kainic acid (Sigma-Aldrich, USA) solution (4 μg of KA in 5 μL of normal saline) was prepared and inserted to the right part of the hippocamp at a rate of 1 μL/min expending a microsyringe of Hamilton. The syringe was gently out off and the rat head skin was sewed. The sham group was given the same normal saline volume. For one week before surgical procedure Rosmarinic acid (Sigma, USA) gavaged at a dosage of 10 mg/kg/day was solved in propylene glycol (Karthikkumar, Sivagami, Vinothkumar, Rajkumar, & Nalini, 2012), and the last treatment was 1h pre- surgical procedure.

**Histochemical evaluation**

The animals were anesthetized with ketamine, perfused with heparinized normal saline for 4–7 min and then with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. The animal's brain was then removed and stored during the night in a fixed container of 4% as postfixative, then deep in 30% sucrose phosphate buffer at 4°C.

**MAPK, COX-2 and nNOS immunohistochemistry**

The hippocampal blocks were prepared, segments were incision at a width of 20 μm on a freezing microtome (Leica, Germany). Segments were washed with phosphate buffer saline (PBS). Then performed a H2O2 treatment for 10 min, subsequently permeabilization with 0.4 % Triton X-100/PBS for 15 min, nonparticular coloring was obstructed.
via incubation for 1 h with 10 % normal goat serum in PBS at room temperature. After that, sections exposure to the primary polyclonal antibody against MAPK or COX-2 or nNOS (Abcam, USA) in a dilution of 1/500 in a humid atmosphere during the night at room temperature. Slides then were rinsed in PBS and incubated for 2 h with goat anti-rabbit antibody conjugated with HRP (Abcam, USA) at a dilution of 1/500 in PBS. After some wash in PBS, slides were incubated with 3, 3'-diaminobenzidine (Sigma-Aldrich, Germany) and PBS for 5–10 min in the dark. Afterward slides were rinsed, counterstained with 0.1 % Cresyl violet, the samples dehydrated with entered alcohol 70, 90, 100 and xylol 1 and 2, respectively. After the above steps, the lamellas were carefully and without bubbles, using antelan adhesive. Then the high-magnification photographic samples were taken.

**Test of apoptosis**

To determine fragmentation of DNA and apoptotic cell death, a transferase dUTP nick-end labeling (TUNEL) test was done by the in situ cell death detection kit (Roche, Germany). In this examine, the sections were incubated with proteinase K, washed, incubated in 3% H2O2, permeabilized with 0.5% Triton X-100, washed and incubated in the TUNEL reaction mixture. Sections were washed and visualized using a converter-POD and next incubation with DAB (3, 3'-diaminobenzidine tetrachloride) and H2O2, counterstained with hematoxylin, dehydrated and coverslipped and assessed. A dark brown color demonstrating DNA breaks expanded. TUNEL reactivity density was determined in at least 10 islets for each animal and its mean was obtained as the ultimate value.

**An analysis of Statistical**

whole amounts were given as means ± SEM. For analysis of morphometric and densitometric, at least 5 sections for each histochemical reaction were used and UTHSCSA image tool software (version 3, 2002) was used. Regarding immunoreactivity intensity, the following scoring was used: no reactivity 0, very mild reactivity 1, mild reactivity 2, moderate reactivity 3, strong reactivity 4, and very strong reactivity 5. Whole counting and calculations were accomplished blind to the therapy received. Statistical study of data was approved by repeated measure and one-way ANOVA with Tukey post-hoc test. A statistical p value fewer than 0.05 considered meaningful.

**Outcomes**

Immunohistochemical results showed that Kainic acid group had a significantly higher number of nNOS-positive neurons (p<0.01) (Fig. 1) and a significant higher reactivity for COX-2 (p<0.01) (Fig. 2) and MAPK (p<0.005) (Fig. 3) versus sham group. In addition, regarding nNOS, Kainic acid + RA groups, only 58.2 of rats showed a significant
elevation of number of nNOS-positive neurons (p<0.05) against sham group and 51.2% of rats significantly lower number of nNOS-positive neurons relative to Kainic acid group (p<0.05) (Fig. 1).

Furthermore, that Kainic acid + RA group COX-2 immunoreactivity intensity was significant changes as compared to sham group (p<0.01). In this regaed, using RA as a pretreated could reduced about 24.7% COX-2 immunoreactivity intensity compared with Kainic acid group. Also between Kainic acid and Kainic acid + RA group did not seen any significant change (Fig. 2).

Meanwhile, MAPK immunoreactivity was significantly higher in all of the Kainic acid groups (p<0.005) (Fig. 3) versus sham group with no beneficial effect of RA.

Designation of apoptosis with TUNEL method showed that Kainic acid group has a high number of TUNEL-positive neurons apoptotic index relative to sham and RA pretreated meaningfully reduced this against Kainic acid (p<0.05). Thus, Kainic acid + RA group, the death rate of neurons decreased 42.4%. These finding indicate that RA has neuroprotective effects against KA (Fig. 4).

**Argument**

Kainic acid induced animal model of seizure is usually used as a examination model of human temporal lobe epilepsy (Kiasalari, Roghani, Khalili, Rahmati, & Baluchnejadmojarad, 2013; Tchekalarova et al., 2013). As a potent analogue of glutamate, its triggered stimulatory amino acid receptors and activates membrane depolarization of neuronal and augmented calcium inflow via voltage-dependent calcium channel unscrew through depolarization of membrane subsequent stimulation of Kainic acid receptors by following induction of the creation of reactive oxygen species (ROS), cause greater oxidative stress (Kanada et al., 2005). Therefore, the augmented production of ROS leads to malfunction of mitochondrial respiratory chain and impairment to the cell constructions, consequently resulting in neuronal injury (Shih, Chein, Wang, & Fu, 2004). The brain has an arrangement of antioxidant protective systems for example superoxide dismutase, reduced glutathione(GSH) and catalase which inhibit over-oxidative harm (Ciftci, Oztanir, & Cetin, 2014). In addition, toxicity of oxidative glutamate is begun via great concentrations of extracellular glutamate that avoid cysteine uptake toward cells, then intracellular cysteine is lowered and GSH is lost. With a reducing supply of GSH, there is an cumulation of extreme amounts of ROS and finally cell death. There is several proof that Kainic acid induced neuronal injury was the due to free radicals (Han et al., 2012). In addition, Kainic acid enhances MAPK and COX-2 expression (Hsieh et al., 2011). COX-2 is the primary isoform of cyclooxygenase in the brain and it leads to enhanced oxidative stress along with the production
of prostaglandins which may have many injurious effects. COX-2 could also contribute to certain inflammatory response, neuronal hyperexcitability and death (H. J. Zhang, Sun, Lei, Yang, & Liu, 2008). Such alterations also occurred in our study. Furthermore, nNOS upregulation is responsible for neuronal apoptosis and damage that maybe responsible for enhanced apoptosis in our study.

In this study, Portion of useful effect of RA could be associate with its neuroprotective features (Fallarini et al., 2009). In this respect, RA has been revealed that is able to safeguard N2A cells versus oxidative impairment (Ghaffari et al., 2014) and it is also a potent defensive factor versus 6-hydroxydopamine induced deterioration of the nigrostriatal dopaminergic system through adjust the ratio of Bcl-2/Bax gene expression which is involved in the pathway of apoptosis (Wang et al., 2012). Thus, RA can decrease the harmful function of neurotoxins and/or excitotoxic factors alike Kainic acid on neurons, therefore restrict cumulation of extracellular glutamate and inhibiting apoptotic neuronal death. The anti-apoptotic properties of RA may also be participated in its helpful properties. Prior studies have revealed that in the model of epileptic seizure induced by Kainic acid, the protective protein Bcl-2 is down-regulated and thus apoptosis happens (J. Zhang, Yan, Wu, Li, & Zhang, 2011) and RA trapy is capable to prevent the apoptotic cascade by enhance Bcl-2 expression (Wang et al., 2012). In this manner, RA could inhibit the apoptotic cell death induced by Kainic acid. Furthermore, the protective effect of RA on astrocytes have been proven by decrease apoptosis and increased their survival rate induced by H2O2 via augment mitochondrial membrane potential and prohibition of caspase-3 activity and decrease of cellular oxidative stress (Gao, Wei, Zhao, Xiao, & Zheng, 2005). Kainic acid induced epileptic seizure also along inflammation by augmented some prostaglandin production for instance prostaglandin E2 subsequent an enhancement levels of mRNA cyclooxygenase and prostaglandin E2 synthase in the brain tissue and anti-inflammatory factors could decrease the intensity of this situation (Ciceri et al., 2002). Also, it has been shown that Kainic acid reasons excitotoxicity via induction of matrix metalloproteinases causes inflammation and death of the neuron in the hippocampus and hinder of so enzymes could diminish the subsequent neuronal injury and it can be useful in proper therapies for related neurological syndromes (Jourquin et al., 2003). In addition, RA is able to give effect anti-inflammatory through decrease of the expression of nuclear factor-kappaB and tumor necrosis factor-α. Furthermore, RA decreases phosphorylated p53 and expression of caspase-3 in the kidney and thereby exhibits antiapoptotic activity (Domitrovic, Potocnjak, Crncevic-Orlic, & Skoda, 2014).
In consequence, this research approves that RA conforms neuroprotective effect versus kainic acid-induced damage which may be mediated through downregulation of nNOS and reduction of apoptosis.

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Conflicts of Interests

None to declare.

References


Tepe, B. (2008). Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of Salvia virgata (Jacq), Salvia staminea (Montbret & Aucher ex Bentham) and Salvia verbenaca (L.) from Turkey. *Bioresour Technol*, 99(6), 1584-1588.


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Legends:

Figure 1: The number of nNOS-positive neurons/0.1 mm$^2$ (A) and photomicrograph of CA3 region showing through immunohistochemical staining in sham, Kainic acid and Kainic acid + RA groups (B); RA: Rosmarinic acid. ** vs Sham; p<0.01 , *vs Sham; p<0.05 and # vs Kainic acid; p<0.05 (Means±SEM).

Figure 2: COX2 immunoreactivity intensity (A) and photomicrograph of CA3 region showing through immunohistochemical staining in sham, Kainic acid and Kainic acid + RA groups (B); RA: Rosmarinic acid. ** vs Sham; p<0.01 (Means±SEM).

Figure 3: MAPK immunoreactivity intensity (A) and photomicrograph of CA3 region showing through immunohistochemical staining in sham, Kainic acid and Kainic acid + RA groups (B); RA: Rosmarinic acid. *** vs Sham; p<0.001 (Means±SEM).

Figure 4: The number of TUNEL-positive cell/0.1 mm$^2$ (A) and photomicrograph of CA3 region through showing apoptosis assay in sham, Kainic acid and Kainic acid + RA groups (B); RA: Rosmarinic acid. # vs Kainic acid; p<0.05 (Means±SEM).