

Myricetin protects hippocampal CA3 pyramidal neurons and improves learning and memory impairments in rats with Alzheimer's disease

Matin Ramezani^{1,2,*}, Niloufar Darbandi¹, Fariba Khodaghohi², Azam Hashemi¹

1 Department of Biology, Faculty of Science, Arak University, Arak, Iran

2 Neuroscience Research Center, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

How to cite this article: Ramezani M, Darbandi N, Khodaghohi F, Hashemi A (2016) Myricetin protects hippocampal CA3 pyramidal neurons and improves learning and memory impairments in rats with Alzheimer's disease. *Neural Regen Res* 11(12):1976-1980.

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Funding: The study was funded by the Department of Biology, Faculty of Science, Arak University, Iran, No. 38156-8-8349.

Abstract

There is currently no treatment for effectively slowing the progression of Alzheimer's disease, so early prevention is very important. Numerous studies have shown that flavonoids can improve memory impairment. The present study investigated the effects of myricetin, a member of the flavonoids, on intracerebroventricular streptozotocin induced neuronal loss and memory impairment in rat models of Alzheimer's disease. Myricetin at 5 or 10 mg/kg was intraperitoneally injected into rats over 21 days. Control rats were treated with 10 mL/kg saline. Behavioral test (the shuttle box test) was performed on day 22 to examine learning and memory in rats. Immediately after that, hematoxylin-eosin staining was performed to observe the morphological change in hippocampal CA3 pyramidal neurons. Myricetin greatly increased the number of hippocampal CA3 pyramidal neurons and improved learning and memory impairments in rats with Alzheimer's disease. These findings suggest that myricetin is beneficial for treatment of Alzheimer's disease.

Key Words: nerve regeneration; myricetin; Alzheimer's disease; streptozotocin; hippocampus; pyramidal neurons; CA3 region; behavioral test; neural regeneration

*Correspondence to:

Matin Ramezani, M.Sc.,
matinramezani89@gmail.com.

orcid:

0000-0003-0218-968X
(Matin Ramezani)

doi: 10.4103/1673-5374.197141

Accepted: 2016-11-29

Introduction

Alzheimer's disease is one of the most prevalent types of dementia. It usually occurs in people aged 65 years or older (Newman et al., 2007). Both environmental and genetic factors are relevant in the pathogenesis of AD. The unusual deposition of amyloid-beta (A β) plaques and the abnormal aggregation of tau proteins are neuropathological properties of AD (Vauzour, 2014). Neuronal degeneration and cognitive impairment are the most typical features of this disorder (Song et al., 2014), which can directly affect the patient's ability to recall or recognize new information processed in the hippocampus (Machado et al., 2009). The hippocampus is a part of the brain consisting of CA1, CA2, and CA3 fields and the subiculum. Information originating from dentate gyrus is transferred to CA3 and then integrated in CA1 (Mu et al., 2011).

Strong evidence has recently come to light to support the positive influences of flavonoids on the brain (Spencer, 2007; Macready et al., 2009; Rendeiro et al., 2009; Kumar et al., 2013; Vauzour, 2014; Rendeiro et al., 2015; Wang et al., 2016). Flavonoids are rich in compounds that reduce neurodegenerative efficacy (Vauzour et al., 2008). In addition, they strongly promote neural networks by stimulating neurogenesis (Vauzour et al., 2008; Oberbauer et al., 2013). They have

also been reported to enhance cognitive functions (Macready et al., 2009). Flavonoids exist in many vegetables and fruits and greatly influence different brain functions, such as cerebrovascular blood flow and synaptic plasticity, which are related to learning and memory (Rendeiro et al., 2015). Particularly, they are able to control and conserve neuronal survival, differentiation, and long-term potentiation in memory (Krishnaveni, 2012). The first and most important characteristics of flavonoids are their antioxidant activity and their ability to suppress reactive oxygen species formation (Kumar et al., 2013).

Numerous studies have confirmed neurogenesis in hippocampal neurons (Eriksson et al., 1998; Dong et al., 2003; Bruel-Jungerman et al., 2007; Liu et al., 2011; Mu et al., 2011). A large body of evidence has shown that flavonoids play a key role in neuronal improvement because of their high proportion of antioxidant features (Jung et al., 2010; Kim et al., 2010; Choi et al., 2012; Huang et al., 2012; Rendeiro et al., 2015; Wang et al., 2016). Some studies have also shown that flavonoids prevent and restrict neuronal damage in the hippocampal pyramidal layer (Rendeiro et al., 2009; Tongjaroenbuangam et al., 2011; Lee et al., 2012; Ashrafpour et al., 2015; Wang et al., 2016).

Myricetin (3,3',4',5',7'-hexahydroxyflavone) is a com-

mon natural flavonoid found in many fruits, vegetables, and herbs. It is well-known for its antioxidant activity and anti-apoptotic effects (Kang et al., 2010). Myricetin plays a substantial role in the treatment and prevention of some diseases due to its potent iron-chelating capability, antioxidant and free-radical scavenging activities (Yao et al., 2014). Myricetin, as a well-known flavonoid with anti-oxidative, anti-inflammatory, anti-apoptotic, and neuroprotective properties, reportedly plays an important role in the hippocampus of stressed mice (Ma et al., 2015).

According to these studies, we assumed that myricetin as a potential flavonoid is beneficial for learning and memory in experimental models of Alzheimer's disease. In addition, it can improve neurons in hippocampal CA3 pyramidal layers in rats with Alzheimer's disease. The present study examined the possible effects of myricetin on learning and memory impairments and hippocampal CA3 pyramidal neurons in Wistar rat models of Alzheimer's disease induced by intracerebroventricular (i.c.v.) streptozotocin (STZ).

Materials and Methods

Animals

Fifty adult male Wistar rats, weighing 220–250 g, were provided by Pasteur Institute, Tehran, Iran. The rats were housed two per cage in an animal house, kept at $22 \pm 2^\circ\text{C}$ and a 12 hour dark/light cycle, and were given free access to food and water.

Rats were anesthetized by intraperitoneal (i.p.) injection of 10% ketamine (50 mg/kg) and 2% xylazine (5 mg/kg) (Struck et al., 2011) and all efforts were made to minimize the pain and distress of experimental animals. All procedures for the treatment of animals were reviewed and approved by the Research and Ethics Committee of the Biology School, University of Arak, Iran and performed according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Treatment

All rats were equally and randomly divided into five groups. In the control group, rats daily received i.c.v. (10 μL for each lateral ventricle) and i.p. (10 mL/kg) injections of normal saline. In the STZ group, rats received injection of STZ (3 mg/kg, i.c.v., 10 μL on each side, on first and third days) under the stereotaxic surgery and normal saline (10 mL/kg, i.p.). In the STZ + 5 mg/kg myricetin and STZ + 10 mg/kg myricetin groups, rats received injection of STZ (3 mg/kg, i.c.v.) and myricetin (5 and 10 mg/kg respectively, i.p.). In the saline + myricetin group, rats received injection of normal saline (10 μL for each lateral ventricle, i.c.v.) and myricetin (10 mg/kg, i.p.). Myricetin/saline i.p. administration started 1 day before stereotaxic surgery and was performed in the morning between 9:00 and 12:00 over 21 days. After 21 days, the passive avoidance test (the shuttle box test) was performed on the 22nd day. Immediately after that, rat brains were removed and stained with hematoxylin-eosin to examine the effects of myricetin on hippocampal CA3 pyramidal neurons.

Drugs

The drugs used in the study were STZ (Sigma-Aldrich, St. Louis, MO, USA) and myricetin (Sigma-Aldrich). They were dissolved in sterile 0.9% saline just before the experiment. STZ was administered into the lateral ventricle, and myricetin or saline was i.p. injected.

Stereotaxic surgery

After anesthesia induction, the animal's head was shaved and then placed into a stereotaxic instrument (Stoelting, Chicago, IL, USA). 10 μL of STZ (ICV) was injected into each lateral ventricle *via* a Hamilton syringe. The stereotaxic coordinates were chosen based on the rat brain atlas of Paxinos and Watson (1998): anteriorposterior = -0.8 mm; mediolateral = ± 1.4 mm; dorso-ventral = -3.5 mm from bregma.

Behavioral test

At the end of the treatment, the passive avoidance test (the shuttle box test) was performed. It consisted of two equally-sized compartments, a bright room (white compartment, 20 cm \times 20 cm \times 30 cm), and a dark one (black compartment, 20 cm \times 20 cm \times 30 cm). The chambers were separated by a guillotine-type door.

Prior to the experiment, all animals were placed into the apparatus for approximately 30 minutes (habituation stage). Afterward, each rat was individually placed in the bright chamber and the guillotine door was opened. As soon as the rat entered the dark chamber, the door was closed, and an electric shock was delivered by an isolated stimulator (1Hz, 3 seconds, 1/5 MA) through the grid floor. Twenty-four hours later, each rat was placed in the bright chamber for the retention test. The step-through latency (STL) and the total time spent in the dark chamber (TDC) were measured.

Tissue preparation and hematoxylin-eosin staining

Rats were deeply anesthetized with 3.5% chloral hydrate (35 mg/100 g, i.p.), and vessels were perfusion fixed by intracardiac infusion with phosphate buffer solution (0.1 M, pH 7.4) followed by 200 mL of 4% paraformaldehyde fixative in phosphate buffer solution (0.1 M, pH 7.4) for 15 minutes. Rat brains were removed, isolated, post fixed in 4% para formaldehyde (24 hours), and embedded in paraffin. Coronal sections, 10 μm in thickness, were taken from the dorsal hippocampus and stained with hematoxylin and eosin. Finally, the numbers of damaged and intact neurons in the hippocampal CA3 pyramidal layer were counted (Koike et al., 2008) using a light microscope (BX40, Olympus, New York, USA) connected to a camera (Olympus, DP12), and quantitatively analyzed by ImageJ software (Neuroscience Center of Shahid Beheshti University, Iran).

Statistical analysis

All results are expressed as the mean \pm SEM. For the behavioral test, STL and TDC were analyzed by one-way analysis of variance (ANOVA) and Tukey's *post-hoc* test. Statistical results were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences between groups in the average

number of intact and damaged neurons in the hippocampal CA3 pyramidal layer were analyzed using GraphPad Prism Software (version 5.00) (GraphPad Software Inc., San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Effect of myricetin on cognitive function of rats with Alzheimer's disease

Passive avoidance test results showed that STL was decreased and TDC was increased in the STZ group, indicating memory impairment, than in the control group ($P < 0.05$). STL was significantly increased and TDC was significantly decreased in the STZ + 10 mg/kg myricetin group than in the STZ group ($P < 0.01$). There were no significant differences in STL and TDC between STZ + 5 mg/kg myricetin and STZ groups ($P > 0.05$). STL was increased and TDC was decreased in the saline + 10 mg/kg myricetin group than in the STZ group (Figure 1).

Effect of myricetin on hippocampal CA3 pyramidal neuron density in rats with Alzheimer's disease

Histological studies revealed striking changes in neuron morphology in each group. The number of intact neurons in the hippocampal CA3 pyramidal layer was markedly decreased in the STZ group, and there were some irregularities in the layer; however, the number of intact neurons was significantly increased in the control group and saline + myricetin group. Despite the fact that STZ greatly damaged neurons, myricetin (10 mg/kg/d, i.p.) significantly increased the number of intact neurons containing large, round, and regular neurons (Figure 2A–E).

Although i.c.v. injection of STZ resulted in severe hippocampal CA3 pyramidal neuronal damage, saline did not lead to any severe neuronal damage in this area in control rats; the number of damaged neurons in the STZ group was significantly increased than that in the control group ($P < 0.05$). However, the number of damaged neurons was significantly decreased in the STZ + 10 mg/kg myricetin group than in the STZ group ($P < 0.01$) (Figure 2F). These results suggest that myricetin (10 mg/kg/d) exhibited significant protective effects on STZ-induced neuronal damage.

Discussion

The hippocampus plays a vital role in learning and memory. Moreover, hippocampal CA3 pyramidal neurons are significantly vulnerable to the negative effects of STZ. Therefore, hippocampal CA3 neurons located near the DG were the target of the current study. Hematoxylin-eosin staining showed that the number of intact neurons was greatly decreased in the STZ group; however, myricetin (10 mg/kg) alleviated neuronal damage in hippocampal CA3 region of rats receiving i.p. injection of STZ. This result indicates that myricetin at 10 mg/kg/d can promote neuronal recovery and at the same time improve neurogenesis in the hippocampus. i.c.v. injection of STZ in rats lead to learning, memory and

cognitive impairments and worsened inflammatory response in the central nervous system (Baluchnejadmojarad., 2009; Khalili et al., 2010). The underlying mechanism remains unclear; however, it is likely attributed to the peripheral performance in the body (Grunblatt et al., 2007).

STZ in rat brain promoted inflammatory response after 3 weeks (Chu et al., 2005; Lester-Coll et al., 2006). Following 3 weeks of i.c.v. injection of STZ, great changes were observed in the rat brain (Agrawal et al., 2009). Free radicals induce neurotoxicity. Moreover, they lead to the symptoms of Alzheimer's disease (Khalili et al., 2010). There is evidence that STZ injections into the brain induce insulin resistance and lead to an increase in amyloid precursor protein processing to produce beta-amyloid and tau pathology (Kamat, 2015). Tau protein and A β are the most important pathological properties of AD. They are responsible for upcoming pathological events that result in a lack of cognitive performance, neuropsychiatric alterations, and eventually lead to neuronal death (Kamat, 2015).

Flavonoids can reportedly affect different kinds of synaptic plasticity, regulate receptor activation, signaling pathways and gene expression, and promote long-term potentiation (Rendeiro et al., 2012). In spite of many studies reporting flavonoid-mediated neuroprotection, there is no enough information regarding the interaction of flavonoids or their circulating metabolites with the blood-brain barrier (Youdim et al., 2004). Yao et al. (2014) demonstrated that myricetin is a lipophilic compound with low water solubility but high solubility in organic solvents or with solubilizers. It can pass through biological membranes such as the blood-brain barrier. Moreover, they show strong neuroprotective effects under *in vivo* conditions (Das et al., 2008; Hamaguchi et al., 2009), and their metabolites have been found in the brain tissue of rodents after oral administration (Paulke et al., 2006). These results display their ability to cross the blood-brain barrier (Paulke et al., 2006; Franco et al., 2010). In some age-related diseases, oxidative stress may contribute to the breakdown of blood-brain barrier, so flavonoids can enter the brain more easily than under the healthy state (Youdim et al., 2004).

It is strongly believed that Alzheimer's disease and other neurodegenerative disorders are triggered by multifactorial processes, including neuroinflammation, glutamatergic excitotoxicity, increases in iron, and/or the depletion of endogenous antioxidants (Jellinger, 2001). Flavonoids can inhibit apoptosis and other damage in neurons through neurotoxic species, but not directly. They modulate a number of protein kinase and lipid kinase signaling cascades, like PI3 kinase (PI3K)/Akt, tyrosine kinase, protein kinase C (PKC), and MAPK signaling pathways (Spencer, 2009). Spencer (2009) reported that flavonoids induce angiogenesis and new nerve cell growth in the hippocampus. Under the destructive effects of STZ, microglia and astrocytes produce cytokines (interleukin-1 β , tumor necrosis factor- α) and nitric oxide (NO \bullet) that lead to glial-induced neuronal death. Flavonoids play a truly significant role in the inhibition of NO \bullet , interleukin-1 β , and TNF- α in activated microglial cells (Spencer, 2009). Flavones have been shown

to significantly inhibit 6-hydroxydopamine-induced JNK activation and neuronal cell death, and they may suppress JNK activity and apoptosis induced by hydrogen peroxide, 4-hydroxy-2-nonenal, and tumor necrosis factor- α (Kobuchi et al., 1999).

The present study clearly demonstrated that myricetin (10 mg/kg/d; i.p.) significantly inhibited cognitive impairments caused by i.c.v. injection of STZ. It can also increase the survival of hippocampal CA3 pyramidal neurons. The present findings add evidence to treatment of Alzheimer's disease using myricetin as a flavonoid.

Author contributions: All authors contributed to conception of this study, participated in data analysis and paper writing and approved the final version of this paper for publication. ND supervised the study.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using CrossCheck to verify originality before publication.

Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

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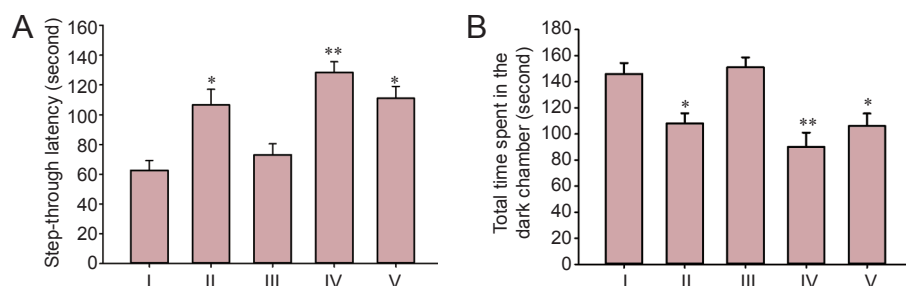


Figure 1 Effect of myricetin (5 and 10 mg/kg) on learning and memory of Alzheimer's disease rats measured by passive avoidance test (the shuttle box test).

(A, B) Step-through latency (A) and total time spent in the dark chamber (B) in passive avoidance test were measured after intracerebroventricular injection of STZ followed by 21-day treatment of myricetin. Values are expressed as the mean \pm SEM with 10 rats in each group. * $P < 0.05$, ** $P < 0.01$, vs. STZ group (one-way analysis of variance followed by Tukey's *post-hoc* test). I-V: STZ (intracerebroventricular injection of STZ + intraperitoneal injection of normal saline), control (intracerebroventricular and intraperitoneal injections of normal saline), STZ + 5 mg/kg myricetin (intracerebroventricular injection of STZ + intraperitoneal injection of 5 mg/kg myricetin), STZ + 10 mg/kg myricetin (intracerebroventricular injection of STZ + intraperitoneal injection of 10 mg/kg myricetin), saline + 10 mg/kg myricetin (intracerebroventricular injection of normal saline + intraperitoneal injection of 10 mg/kg myricetin) groups, respectively. STZ: Streptozotocin.

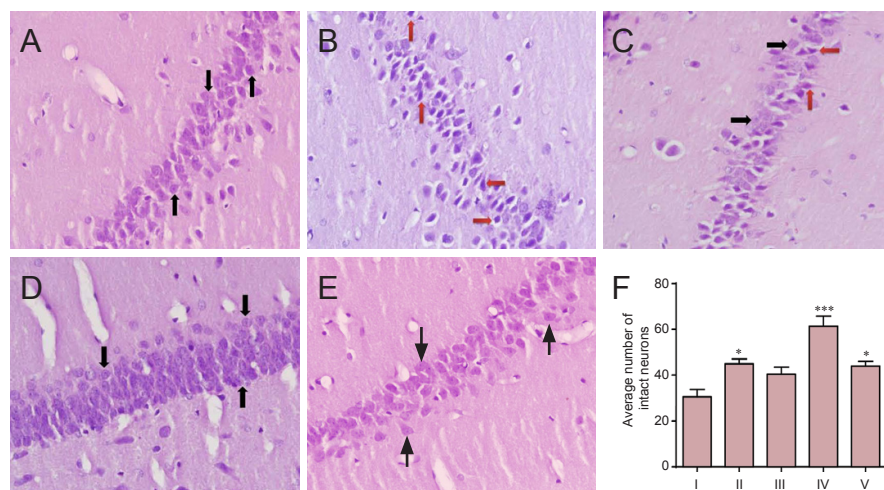


Figure 2 Effect of myricetin (5 and 10 mg/kg) on morphology of hippocampal CA3 pyramidal neurons of rats with Alzheimer's disease (hematoxylin-eosin staining, original magnification $\times 40$).

(A) Control group (natural and intact neurons with arranged CA3 layer); (B) STZ group (significant neural loss and an irregular layer); (C) STZ + 5 mg/kg myricetin (most of the cells were damaged; however, there were some intact neurons); (D) STZ + 10 mg/kg myricetin (neurons were neatly arranged with a lot of intact and round cells); (E) saline + 10 mg/kg myricetin (regular layer and significant increases in intact neuron). Black arrows indicate intact neurons and red arrows represent damaged neurons (F) The average number of intact neurons in hippocampal CA3 pyramidal layer. All data are represented as the mean \pm SEM. * $P < 0.05$, *** $P < 0.001$, vs. STZ group (one-way analysis of variance followed by Tukey's *post-hoc* test). I-V: STZ (intracerebroventricular injection of STZ + intraperitoneal injection of normal saline), control (intracerebroventricular and intraperitoneal injections of normal saline), STZ + 5 mg/kg myricetin (intracerebroventricular injection of STZ + intraperitoneal injection of 5 mg/kg myricetin), STZ + 10 mg/kg myricetin (intracerebroventricular injection of STZ + intraperitoneal injection of 10 mg/kg myricetin), saline + 10 mg/kg myricetin (intracerebroventricular injection of normal saline + intraperitoneal injection of 10 mg/kg myricetin) groups, respectively. STZ: Streptozotocin.

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