Original Research Article

Effect of the plant flavonoid, rhoifolin, on memory and cognition in a rat model of Alzheimer's disease

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Abstract

Purpose: *To investigate the neuroprotective effect of the natural flavonoid rhoifolin in rats with streptozotocin (STZ)-induced (AD).*
Methods: *Morris water-maze and novel object recognition tests were carried out to estimate the effect of rhoifolin on memory and cognition. Histopathological analysis was made to observe thickness of hippocampal CA1 pyramidal layer. Analysis of oxidative stress markers was performed to estimate the effect of rhoifolin on oxidative stress in the hippocampus and frontal cortex.*
Results: *Morris water maze and novel object recognition tests showed a significant improvement in the memory, cognition and spatial learning in rhoifolin treated AD animals (p < 0.05). Moreover, rhoifolin treatment resulted in a significant increase in the CA1 pyramidal layer of AD animals indicating its neuroprotective properties (p < 0.05). The increase in the hippocampal CA1 area further validated the reversal of cognitive dysfunctions caused by STZ treatment. Furthermore, analysis of oxidative stress markers SOD, CAT, GPX, GRX, and MDA showed a significant improvement in the oxidative stress in the hippocampus and frontal cortex (p < 0.05).*
Conclusion: *The present study is the first report to demonstrate the effect of plant flavonoid, rhoifolin on STZ-induced AD in rat model. Rhoifolin improves spatial learning, cognition, and memory in STZ-treated rat model. Therefore, rhoifolin may be a promising therapeutic agent for the management of AD.*

Keywords: *hoifolin Flavonoids, Alzheimer's disease, Oxidative Stress, Memory disorders, Cognition disorders*

INTRODUCTION

Alzheimer's disease (AD) is a mental disorder caused by progressive degeneration of the central nervous system (CNS). Alzheimer's disease is the most common pathogenesis behind progressive senile dementia [1] which is marked by neuronal loss, deterioration of memory, cognition, and intellect [1]. The brain tissue in AD is distinguished by loss of synapses and cortical neurons, neuritic plaques and neurofibrillary tangles [2]. Amyloid precursor protein (APP) cleavage is mediated by β-site APP cleavage enzyme 1 and γ-secretase which causes generation of Amyloid β (Aβ) and leads to the generation of neuritic plaques [3]. The Aβ is also known to induce reactive oxygen species (ROS) leading to neuronal cell pathogenesis and

apoptosis in AD patients [3]. Several studies have highlighted the role of oxidative stress via increase in ROS as the key factor associated with pathogenesis of AD [4]. Therefore, anti-oxidant agents are believed inhibit the progression of AD [5].

Flavonoids are bioactive compounds of plant origin with a skeleton of 15-carbon atoms including two fused rings of 6-carbon atoms. Flavonoids are the polyphenolic compounds produced by the plants which constitute a part of human diet. Flavonoids contain phenolic hydroxyl groups that mediate their antioxidant activity and anti-inflammatory property [6,7]. Several previous studies have shown the beneficial role of a flavonoid-rich diet in learning and memory [6,7]. Moreover, it has been reported that almost 25 % of patients with AD also have flavonoid deficiency [8].

Rhoifolin is a flavanone which was first extracted from *Rhus succedanea and* it has been shown to play a key role in prevention of neurodegenerative disorders [9]. The antioxidative properties of rhoifolin may mediate its neuroprotective effect on AD. The present study investigated the effect of rhoifolin on rats with streptozotocin (STZ) induced AD. The effect of rhoifolin on thickness of CA1 pyramidal layer and spatial learning in the AD model was also studied. The ameliorative effect of rhoifolin on AD symptoms was studied in terms of Morris water maze test and novel object recognition test. Moreover, the ability of rhoifolin to scavenge free radicals was estimated by quantifying the levels of glutathione peroxidase (GPx) and glutathione reductase (GRX), and superoxide dismutase (SOD) and catalase (CAT) in the hippocampus and cerebral cortex. To our knowledge, this is the first report on the effect of rhoifolin on AD.

EXPERIMENTAL

Animals and evaluation of toxicity and dosage

Male Sprague-Dawley rats (3 months old, weighing 190-210 g) were provided by the institutional animal house. The experimental animals were kept under 12/12h light/dark cycle under controlled humidity and temperature. The rats were fed with the standard diet and water as required. The animal experiments were conducted according to guidelines issued by National Institute of Health, US [1]. The study was approved by Committee for Animal Care and Use, Lishui Medical University, China (approval no. MU/102/2018). Efforts were made to minimize the number of animals and their suffering.

For surgery, the rats were anesthetized with intraperitoneal administration of ketamine (80 mg/kg) and xylazine (15 mg/kg). The rats head were immobilized using a stereotaxic instrument and two 23-gauge cannulas were bilaterally implanted in the lateral ventricles following rat brain atlas of Paxinos and Watson. The animals were allowed to recover for one-week post-surgery. Rhoifolin was administered in graded doses of 20, 40, 60, 80 and 100 mg/kg via intravenous route (described below) to five rats each. The animals were kept on a 10-day observation period. Health parameters such as diet, changes in weight, fluid intake and psyco-motor changes were measured. Rhoifolin did not show any toxicity at all the tested doses. Therefore, lowest two doses i.e. 20 and 40 mg/kg were selected for further experiments. The 3 mg/Kg dose of STZ was used based on previous reports [10].

Experimental groups

The animals were randomly assigned to five experimental groups of 10 rats each: control group, sham group, STZ group, 20 mg/kg rhoifolin group and 40 mg/kg rhoifolin group. Streptozotocin was injected on the 4th and 6th day of recovery, whereas rhoifolin (Sigma Aldrich USA) was injected on alternate days starting from 1st day up to 10th day.

All injections were administered through a cannula implanted in the lateral ventricles via the intracerebroventricular (ICV) route using a 27-gauge needle attached to a 10 uL syringe at the rate of 0.5 ul/min. The injections were administered into each lateral ventricle. The stereotaxic coordinates for ICV injection were: 0.9 mm posterior, 1.8 mm lateral and 3.8 mm ventral from the bregma level. Animals showing seizure-like symptoms during the recovery period were excluded from the study. All behavioral experiments were executed in the order mentioned below.

Morris water maze experiment

The Morris water maze test was performed to determine the spatial memory and learning of the animals. The test was performed in an empty room with no recognizable landmarks on the walls or the ceiling. The experimental tank was 180 cm in diameter and 90 cm in depth. A 10 cm acrylic platform was used as the escape platform. The tank was filled with tap water to a depth of 60 cm and temperature of water

maintained at 26 ± 1 °C. The tank was divided into four quadrants and the escape platform was randomly placed in the middle of one of the quadrants.

For training, the escape platform was kept 2 cm above the surface of the water. The animals were randomly released from one of the quadrants and were allowed to find their way to the platform for 1 min. If an animal was unable to locate the platform within the time, it was manually guided by the investigator. The animals were rescued from the escape platform after allowing them to stand for 30 s. Each animal was given four consecutive trials at 2 m intervals. The animals were trained for 5 days before the experiment. The experimental trials started on the 14th day of STZ infusion and lasted for four days with four trials per animal per day. The escape platform was placed 1cm below the surface and the water was mixed with non-fat dry milk to make it opaque. The swimming directions of animals were recorded using a video camera (Nikon, Japan) placed 1 m above the tank. The Morris water maze test data were analyzed for escape latency (time animal took to locate the platform), distance traveled (total path length of the distance traveled) and swimming speed. All data were analyzed using Open Control software [11].

Test of memory consolidation

Memory consolidation test was performed with probe trial. The escape platform was removed, and the animals were allowed to swim freely and try to locate the now-absent platform. Probe trials were performed 12 h after the Morris test. Memory consolidation was determined the amount of time spent in the quadrant in which the escape platform was formerly present.

Test for novel object recognition

This test was performed one month after completion of the memory consolidation test to nullify any effect of previous tests on the animals. The animals were habituated to a 120 x 60 x 40 cm empty metal box for 15 min for two days. On day 3, animals were placed in a box with two objects in one corner of the box. The animals were trained by allowing them to explore the objects for 15 min. On the fourth day, memory retention was tested by placing the animals in the same box with one new object. The exploration time for the familiar object (Tf) and the novel object (Tn) were recorded to calculate the proportion of Tf and Tn over the total times spent.

Preparation of tissues and histopathology

Nissl staining was performed to determine the thickness of the CA1 pyramidal layer. One animal from each group was randomly selected and deeply anesthetized using sodium pentobarbital (100 mg/kg i.p) 48 h after the commencement of the experiments. The sections were dehydrated, and the slides were rinsed in 95, 85 and 75 % gradient ethanol (v/v). Afterwards the slides were rinsed in water and then stained using crystal violet solution (0.5 %) for 15 min. The slides were prepared by covering the samples with a glass coverslip with paramount as the mounting medium.

Evaluation of oxidative stress

Hippocampus and frontal cortex are subjected to widespread oxidative stress during aging and memory loss. Therefore, the oxidative stress in these tissues was estimated by quantifying glutathione peroxidase (GPx) glutathione reductase (GRX), and superoxide dismutase (SOD) and catalase (CAT) and malondialdehyde (MDA). The hippocampus and frontal cortex were excised and stored at -80 °C and the tissue homogenates were sonicated and centrifuged at 12000 g for 30 min. The tissue supernatants were used for the estimation of oxidative stress using glutathione peroxidase estimation kit GRX estimation kit, SOD estimation kit and CAT Activity Assay Kit and lipid peroxidation (MDA) assay kit. All the kits were produced by Abcam, USA.

Statistical analysis

The data presented are the mean ± SEM of three experiments performed independently. Differences were determined between groups using One-Way Analysis of Variance (ANOVA) and Bonferroni's post-hoc analysis. All the statistical estimations were performed in GraphPad Prism software. Differences were taken statistically significant at $P < 0.05$.

RESULTS

Effect of rhoifolin on cognitive function under Morris test

The Morris water maze experiment was used to the cognitive function of the experimental groups. On the first day, the control, saline and sham-treated groups showed superior cognitive performance in the form of a low mean escape latency (Figure 1A). The STZ group showed a significantly high mean escape latency. The 20 and 40 mg/kg rhoifolin groups had a significantly

low mean escape latency when compared to the STZ group. This pattern of mean escape latency was observed on all four trial days and the performance of the rhoifolin treatment groups improved significantly in the latter days. However, there was no significant difference between the mean escape latency of 20 and 40 mg/kg rhoifolin treatment groups.
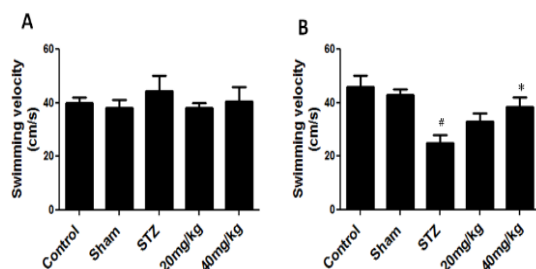

Figure 1: Effect of rhoifolin on swimming velocity (A) and time spent in the target quadrant (B) in streptozotocin (STZ)-induced Alzheimer's disease (AD) rat model. #*P* < 0.05 compared with the control group, *p* < 0.05 compared with the STZ-induced AD group

Effect of rhoifolin on the escape distance

The path length to the hidden escape platform was measured to assess the memory of experimental groups. Similar to the mean escape latency, the path length of the STZ group was significantly longer than that of the control and sham groups (Figure 1 B). However, the path length of the rhoifolin treatment groups showed a significant decrease compared to the STZ group on all four trial days. However, no significant change in path length was observed in control groups on all four days of testing. The path length experiment showed an improvement in the memory of STZ animals after treatment with rhoifolin.

Effect of rhoifolin on swimming velocity

There were no significant differences in swimming velocity among the groups (Figure 2 A). Therefore, STZ did not impact motor activity. Therefore, the improvement in the escape latency in the rhoifolin groups was caused by the shortened path length and not due to increased velocity.

Increased time spent in the target quadrants in rhoifolin groups

Learning and memory retention of experimental groups were evaluated by measuring the time spent in the target quadrant. The STZ group showed a significantly decreased time spent in

the target quadrant compared to the control and sham groups (Figure 1 B). The 20 and 40 mg/kg rhoifolin treatment groups showed a significant increase in time spent in the target quadrant when compared to the STZ group. There was no significant difference in time spent in the target quadrant between the 20 mg/kg and 40 mg/kg rhoifolin groups.

Table 1: Effect of rhoifolin treatment on escape latency and traveled distance in streptozotocin (STZ)-induced Alzheimer's disease (AD) rat model

Treatment	Day/days	Escape latency	Distance travelled
Control	1	35± 6.1	600± 54.3
	2	33± 5.6	590± 44.2
	3	29± 3.8	588± 33.6
	4	28± 3.2	587± 43.1
Sham	1	37± 7.2	602± 51.3
	2	35± 6.9	603± 51.3
	3	34± 4.7	602± 61.2
	4	33± 3.9	601± 56.2
STZ	1	69± 11.1	1500± 68.1
	2	67± 10.7	1450± 66.4
	3	68± 9.3	1420±70.1
	4	67± 10.3	1400±69.2
20mg/kg	1	52± 8.2	1200± 59.1
	2	50± 7.1	1170±66.2
	3	48± 6.7	1150±71.3
	4	47± 5.2	1140±56.6
40mg/kg	1	45± 5.1	1080±55.3
	2	44± 4.9	1030±61.3
	3	44± 3.7	1000± 59.2
	4	43± 2.9	950±66.1

Effect of rhoifolin on recognition memory

Familiar object recognition memory was tested using the novel object recognition test. In this test, if the animal recognizes the familiar object easily during the trial, then the recognition index is high. The recognition index of the STZ group was significantly lower than that of the control, sham, and treatment (Figure 2). However, 20 and 40 mg/kg treatment groups showed a significantly higher recognition index in comparison to the STZ group.

Effect of rhoifolin on thickness of CA1 pyramidal layer

The CA1 pyramidal layer thickness was estimated using Nissl staining. The mean thickness of the CA1 pyramidal layer was significantly larger than the STZ infusion group (Figure 3). The 20 mg/kg and 40 mg/kg rhoifolin treatment groups showed significant recovery in the mean CA1 pyramidal layer thickness over the STZ group.
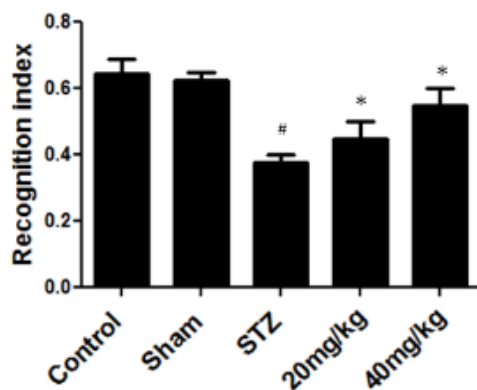
Figure 2: Effect of rhoifolin on recognition memory in streptozotocin (STZ)-induced Alzheimer's disease (AD) rat model. Recognition index was estimated by calculating the time spent with familiar (Tf) and novel (Tn) objects over the total time spent. #P < 0.05 compared with the control group, *p < 0.05 compared with the STZ induced AD group
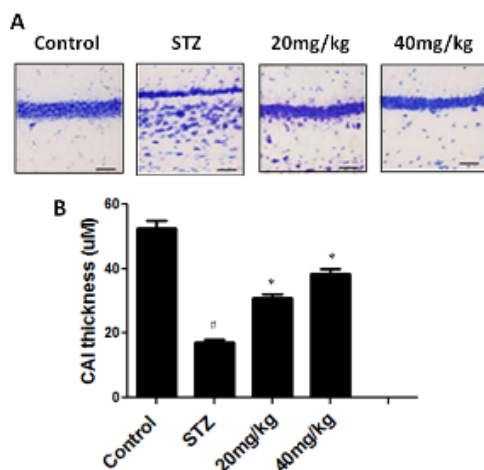

Figure 3: Effect of rhoifolin on CA1 pyramidal layer thickness in streptozotocin (STZ) induced Alzheimer's disease (AD). Nissl staining analysis (A) and the thickness of the CA1 pyramidal layer was quantified (B). The scale bar indicates 50 μM. #P < 0.05 compared with the control group; *p < 0.05 compared with the STZ induced AD group

Effect of rhoifolin on oxidative stress

Oxidative stress was estimated in the tissue supernatants of the hippocampus and cerebral cortex of STZ-induced AD rats. The activities of oxidative stress markers SOD, CAT, GPX, and GRX showed a significant decrease while the levels of MDA were significantly upregulated in the tissue of STZ group (Figure 4). Rhoifolin resulted in a significant change in the levels of these markers, indicating a reduction in oxidative stress in the brain of STZ rats with AD.
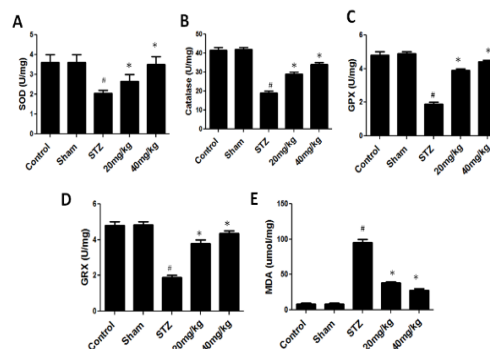

Figure 4: Effect of rhoifolin on the level of oxidative stress markers; SOD (A), CAT (B), GPX (C), GRX (D), and MDA (E) in STZ induced Alzheimer's disease (AD). The activity was measured in units/mg of protein. #P < 0.05 compared with the control group, *p < 0.05 compared with STZ-induced AD group

DISCUSSION

The extant management regimen for AD includes acetylcholinesterase inhibitors and memantine. However, none of the medications has been effective in halting or delaying AD. Moreover, these treatment regimens have several significant side effects [12]. Therefore, a natural alternative to the present AD management regimens is urgently required.

ICV-STZ infusion is well known to produce cognitive symptoms similar to sporadic dementia of the AD type [13]. Streptozotocin administration through ICV induces Aβ plaques, oxidative stress, neuroinflammation and apoptosis [13]. Moreover, it causes a reduction in energy metabolism leading to dysfunction of cognitive abilities. It has been shown that 3 mg/kg dose of STZ is appropriate to evaluate compounds that modulate oxidative stress and inflammation. Moreover, a negligible difference in lesions between one week and three months of AD induction has been reported. Glucose repair and loss of energy metabolism are a source of oxidative stress in the brain [14]. Moreover, the antagonistic effects of Aβ plaques aggregation and oxidative stress cause further loss of cognitive neurological functions [7]. In the present study, STZ infusion showed significant loss of spatial learning, cognition and memory consolidation.

The Morris water maze test is a well-known experimental protocol to access the spatial learning and memory consolidation [15]. In this study, rhoifolin treatment showed significant improvement in the mean escape latency, path length and time spent in the target quadrant. In contrast, the swimming velocity showed no significant improvement in rhoifolin treatment

groups. Therefore, the time to acquisition of the escape platform was not an effect of the difference in motor activity but was resulted from an improvement in spatial memory. The novel object recognition test is a common behavioral experiment for the estimation of learning and memory [15]. Habituation plays a key role in the conduct of this test where the experimental animals are expected to spend variable time with familiar objects. However, loss of memory may interfere with the familiarization process as all objects may appear to be novel to the animal. The rhoifolin treated rats showed improved performance in familiarization and thus showed a significantly improved recognition index compared to the AD rats. The higher recognition index indicated improved cognitive function and memory in the rhoifolin groups.

The hippocampus of the temporal lobe is known to be primarily affected in the pathophysiology of AD and has been shown in several AD animal models and clinical studies [16]. Histopathological analysis indicated the shrinking of the CA1 pyramidal layer in the STZ group indicating a contraction of neurons in the CA1 pyramidal layer. Furthermore, the increased thickness in the rhoifolin treatment groups indicated alleviation of cognitive dysfunction. The shrinkage of neurons is caused by ROS, generated during the generation of Aβ plaques, which leads to toxicity and cell death in hippocampal neurons [17]. Oxidative stress plays a key role in the pathophysiology of AD [17]. In the present study activity of oxidative stress markers such as SOD, CAT, GPx, and GRX in rhoifolin treated STZ rats was significantly elevated. However, the level of lipid peroxidation marker malondialdehyde was significantly reduced in rhoifolin treated STZ rats. Their results indicated that the rhoifolin treatment resulted in a significant improvement in the oxidation state of the hippocampus and frontal cortex tissues. Previously, plant flavonoid quercetin has been shown to reduce the oxidative stress in STZ mice [18]. Therefore, these results indicate that the neuroprotective effect of rhoifolin in STZ induced AD may be mediated by inhibition of free radicals leading to the disappearance of the plaques.

CONCLUSION

The findings of this study show that treatment with the plant flavonoid, rhoifolin, improves spatial learning, cognition, and memory in STZ-treated rat model. Moreover, a significant improvement in the CA1 pyramidal layer has also been observed in the rhoifolin-treated AD rats.

Thus, rhoifolin may be a potential therapeutic agent for the management of AD.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaocheng Huang, Shaochang Wu, Yanbin Wei and Shuiyun Meng performed the experimental work, carried out the literature survey, analysed and compiled the data. Ruilai Jiang designed the study and wrote the manuscript. All the authors read the paper thoroughly and approved it for publication.

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