Osthole improves synaptic plasticity in the hippocampus and cognitive function of Alzheimer’s disease rats via regulating glutamate

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Abstract
Osthole, an effective monomer in Chinese medicinal herbs, can cross the blood-brain barrier and protect against brain injury, with few toxic effects. In this study, a rat model of Alzheimer’s disease was established after intracerebroventricular injection of β-amyloid peptide (25–35). Subsequently, the rats were intraperitoneally treated with osthole (12.5 or 25.0 mg/kg) for 14 successive days. Results showed that osthole treatment significantly improved cognitive impairment and protected hippocampal neurons of Alzheimer’s disease rats. Also, osthole treatment alleviated suppressed long-term potentiation in the hippocampus of Alzheimer’s disease rats. In these osthole-treated Alzheimer’s disease rats, the level of glutamate decreased, but there was no significant change in γ-aminobutyric acid. These experimental findings suggest that osthole can improve learning and memory impairment, and increase synaptic plasticity in Alzheimer’s disease rats. These effects of osthole may be because of its regulation of central glutamate and γ-aminobutyric acid levels.

Key Words
osthole; Alzheimer’s disease; learning and memory; long-term potentiation; glutamate; γ-aminobutyric acid; β-amyloid peptide; brain; hippocampus; neural regeneration

Research Highlights
(1) The learning and memory impairment of β-amyloid peptide (25–35)-induced Alzheimer’s disease rat models treated with intraperitoneal injection of osthole were significantly improved compared with controls.
(2) The hippocampal glutamate levels significantly decreased, while the γ-aminobutyric acid levels maintained unchanged in the Alzheimer’s disease rats after osthole treatment compared with controls.
(3) Osthole improves learning and memory impairment in Alzheimer’s disease rats via the central glutamate/γ-aminobutyric acid regulatory system.

Abbreviation
LTP, long-term potentiation
INTRODUCTION

Alzheimer’s disease is characterized by progressive cognitive function deficits because of the presence of numerous senile plaques and neurofibrillary tangles in brain regions\(^1\), β-amyloid peptide, the major component of these plaques\(^2\), triggers the release of excessive amounts of glutamate in the synaptic cleft by inhibiting the astroglial glutamate transporter, and by increasing the intracellular Ca\(^{2+}\) levels\(^3\) through enhancing the activity of N-methyl-D-aspartate receptors\(^4\). Overstimulation of the nerve cell by glutamate and intracellular Ca\(^{2+}\) accumulation underlie the pathogenic process, which eventually causes neuronal apoptosis, and disrupt synaptic plasticity\(^5\) and learning and memory functions. Similar to the whole β-amyloid peptide molecule (A\(\beta_{1-42}\)), the fragment A\(\beta_{25-35}\) is the active center of β-amyloid peptide\(^6\) and has the same neurotoxicity. Thus, the fragment A\(\beta_{25-35}\) has replaced full-length β-amyloid peptide when establishing animal models of Alzheimer’s disease to study the neurotoxic properties of β-amyloid peptide and evaluate anti-Alzheimer’s disease drugs\(^7\).

Osthole (7-methoxy-8-isopentenoxy-coumarin) is an ingredient of a traditional Chinese medicine from the natural product Cnidium monnieri (L.) Cusson, and has received considerable attention recently because of its diverse pharmacological functions, including anti-oxidative, anti-inflammatory, anti-tumor, anti-platelet and estrogen-like effects\(^8-12\). Our previous studies have shown that osthole can improve learning and memory impairment in dementia mouse models via inhibiting acetylcholinesterase activity, or by enhancing the activities of glutathione peroxidase and superoxide dismutase, and clearing oxygen free radicals in the brain\(^13-14\). Moreover, osthole can improve cognitive deficits and neuronal damage of vascular dementia induced by chronic cerebral hypoperfusion\(^15\). The central glutamate and γ-aminobutyric acid levels play an important role in the maintenance of normal brain functions, such as learning and memory. Under pathological and physiological conditions, glutamate and γ-aminobutyric acid have opposing regulatory effects\(^16\). The aims of our present study were to examine the influence of osthole on cognitive impairment in rats with senile dementia induced by A\(\beta_{25-35}\), and to discover osthole’s potential mechanism of action on the central glutamate/γ-aminobutyric acid regulation of learning and memory.

RESULTS

Quantitative analysis of experimental animals

A total of 96 Sprague-Dawley rats were initially included in the study and were randomly assigned to four groups: control, model (A\(\beta_{25-35}\) intracerebroventricular injection), low-dose osthole (A\(\beta_{25-35}\) intracerebroventricular injection, 12.5 mg/kg osthole; Ost 1), and high-dose osthole (A\(\beta_{25-35}\) intracerebroventricular injection, 25.0 mg/kg osthole; Ost 2) group, with 24 rats in each group. On day 1 of model establishment, rats were injected with A\(\beta_{25-35}\). During model establishment, one rat died because of excessive anesthesia. After that, no deaths occurred until the end of the experiments. The control and model rats were intraperitoneally injected with 1 mL/kg solvent, and osthole treated rats were intraperitoneally administered osthole (12.5 mg/kg or 25.0 mg/kg) once a day. The osthole doses were based on past experiments\(^13\) and conversion of the body surface area in mice. All animals were dosed for 14 consecutive days.

The effects of osthole on the learning and memory ability of Alzheimer’s disease rats

Learning and memory ability was detected using the Morris water maze. The Morris water maze trials consisted of a 4-day learning and memory training period with a probe trial on the fifth day. There was no difference between the groups on the first day of training (\(P > 0.05\)). With each successive training day, the escape latencies continuously were reduced in each experimental group. Compared with the control group, the model group displayed a longer escape latency (\(P < 0.05\) or \(P < 0.01\)), a lower percentage of target quadrant searching time and higher latency (\(P < 0.05\) or \(P < 0.01\); Figure 1), higher percentage of target quadrant searching time and higher numbers of crossing the platform in the probe trial (\(P < 0.01\)). These results indicated spatial learning and memory ability was impaired in the Alzheimer’s disease rats. Compared with the model group, osthole groups showed shorter escape latency (\(P < 0.05\) or \(P < 0.01\); Figure 1), higher percentage of target quadrant searching time and higher numbers of crossing the platform (\(P < 0.05\) or \(P < 0.01\); Table 1, supplementary Figure 1 online). These results indicated that osthole improved the learning and memory performance of Alzheimer’s disease rats.

The effects of osthole on pathological changes in the hippocampi of Alzheimer’s disease rats

On day 14 after model establishment, hippocampal sections were subjected to hematoxylin-eosin staining and were observed under a light microscope. No remarkable neuronal abnormalities in the hippocampi.
from the control rats were observed, while all brains of model rats showed degeneration and disorder in their hippocampal neurons. The bodies of the neurons were deeply stained with dye, and some neurons shrank and demonstrated vacuoles in the cytoplasm. In osthole treated groups, these phenomena were reduced or even disappeared. The neurons in the Ost 2 group (25.0 mg/kg) appeared more similar to normal neurons than those in the Ost 1 group (12.5 mg/kg). These changes indicated that osthole protected against the injuries of the hippocampus induced by Aβ25-35 (Figure 2).

The effects of osthole on long-term potentiation (LTP) induced by high frequency stimulus in the hippocampi of Alzheimer’s disease rats

In all groups, the population spike amplitude increased during the 60-minute observation and LTP was induced by high frequency stimulus (Figure 3), but the extent of the growth in population spike amplitude was different for each group. The population spike amplitude in the Alzheimer’s disease model group was lower than that in the control group ($P < 0.01$), while the population spike amplitude in osthole treated groups was higher than that in the model group at each time point after high frequency stimulus (Figure 4). Furthermore, compared with the control group, a significant reduction of the average population spike amplitude within 60 minutes after high frequency stimulus was observed in the model group ($P < 0.01$).

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**Table 1** The effects of osthole on the average escape latency of 4-day training trials and on probe trial in the Morris water maze in Alzheimer’s disease rats

| Group     | Average escape latency of 4-day training (second) | Number of times crossing the platform (in 120 seconds) | % time in target quadrant |
|-----------|--------------------------------------------------|------------------------------------------------------|---------------------------|
| Control   | 33.3±16.5                                        | 5.2±1.9                                              | 57.3±7.1                  |
| Model     | 55.8±11.8                                        | 2.1±0.7                                              | 31.5±4.4                  |
| Ost 1     | 41.3±15.8                                        | 3.3±1.4                                              | 38.2±5.6                  |
| Ost 2     | 34.7±13.5                                        | 4.1±1.5                                              | 43.4±6.1                  |

Ost 1: Osthole 12.5 mg/kg group; Ost 2: osthole 25.0 mg/kg group. The data are expressed as mean ± SEM of eight rats in each group. $^aP < 0.05$, $^bP < 0.01$, vs. control group; $^cP < 0.05$, $^dP < 0.01$, vs. model group (two-tailed t-test).

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Figure 1 The effects of osthole on the escape latency in Alzheimer’s disease rats.

Ost 1: Osthole 12.5 mg/kg group; Ost 2: osthole 25.0 mg/kg group. The data are expressed as mean ± SEM of eight rats in each group. $^aP < 0.05$, $^bP < 0.01$, vs. control group; $^cP < 0.05$, $^dP < 0.01$, vs. model group (two-tailed t-test).

Figure 2 Pathological changes in the hippocampi of Alzheimer’s disease rats (hematoxylin-eosin staining, × 400).

No abnormalities appeared in the control group (A). However, disorder of the array, deeply stained nuclei and cytoplasm vacuolization of neurons appeared in the Alzheimer’s disease model group (B), a small number of deeply stained nuclei and neuronal vacuolization emerged in the osthole 12.5 mg/kg group (C), while neurons in the osthole 25.0 mg/kg group showed a similar staining pattern to normal neurons (D).

Figure 3 Long-term potentiation before (A) and after (B) induction by high frequency stimulus shown on the oscilloscope.

The population spike amplitude after high frequency stimulus is higher than it before high frequency stimulus and long-term potentiation was induced by high frequency stimulus. MN: Population spike range.
Compared with the model group, the average population spike amplitude significantly increased in osthole groups ($P < 0.01$; Figure 5). These findings indicate that osthole can reverse the inhibition of LTP in Alzheimer’s disease rats.

The effects of osthole on the amino acid levels in the hippocampi of Alzheimer’s disease rats
Compared with the control group, the levels of glutamate, γ-amino-butyric acid and the ratio of glutamate/γ-amino-butyric acid increased in the Alzheimer’s disease model group ($P < 0.05$). The extent of the increase of the glutamate level was greater than that of γ-amino-butyric acid, so the ratio of glutamate/γ-amino-butyric acid increased. Compared with the model group, the levels of glutamate and the ratio of glutamate/γ-amino-butyric acid in both Ost 1 and Ost 2 groups decreased ($P < 0.05$), while there was no significant difference in γ-amino-butyric acid levels ($P > 0.05$; Figure 6).
DISCUSSION

Currently intracerebroventricular injection of β-amyloid peptide can effectively create Alzheimer's disease animal models[17]. In the present study, a single icv injection of Aβ25-35 induced a learning and memory impairment in the Morris water maze test compared with the normal saline-injected control group, as previously shown in Um's studies[17]. In our previous studies, osthole showed a protective effect on learning and memory impairment in acute senile mouse models induced by AlCl3 via step-through and step-down tests[13]. Here we observed the effects of osthole on the cognitive disorder of Aβ25-35-induced Alzheimer's disease rats in the Morris water maze test. Our results indicate osthole dose-dependently improved the learning and memory impairments in Alzheimer's disease rats. From the second day of training in the Morris water maze experiments, the escape latencies shortened, and in the probe trial, the percentage of target quadrant searching time and the number of times the rat crossed the platform increased compared with the Alzheimer's disease model group. Furthermore, the pathological tests showed that osthole was neuroprotective for the hippocampal neurons of Alzheimer's disease rats.

In the past years, research on the neurophysiology of learning and memory has focused on LTP and neuronal synaptic plasticity. LTP is a long-lasting form of synaptic enhancement that has been postulated to underlie the mechanism of learning and memory in the mammalian hippocampus[18]. Therefore, hippocampal LTP has been widely used as a neuronal model of learning and memory[19]. We found that application of Aβ25-35 weakened the population spike amplitude of LTP induced by high frequency stimulation in the hippocampal dentate gyrus of rats, but did not completely inhibit the emergence of LTP. Moreover, our studies also showed that osthole was significantly protective for LTP impairment in Alzheimer's disease rats. These findings provide direct evidence that osthole is a potential neuroprotective agent for learning and memory impairment in Alzheimer's disease rats at the synaptic level. The mechanisms of the protective effect of osthole may be discovered by reducing the neurotoxicity of β-amyloid peptide, but future research is needed to determine the specific mechanisms.

The proposed pathogenic mechanisms of Alzheimer's disease generally include the loss of cholinergic function, oxidative stress, amyloid cascade, inflammatory mediators, steroid hormone deficiencies and excitotoxicity[16]. The hypothesis that glutamate-mediated excitotoxicity is involved in the pathogenesis of Alzheimer’s disease is finding increasing acceptance in the scientific community. Previously, the cholinergic system was noted to regulate learning and memory, but now the central glutamate/γ-aminobutyric acid regulatory system is becoming more prominent. The balance of glutamate and γ-aminobutyric acid levels are crucial to maintain the normal functions of the brain, including learning and memory[20]. Some studies have found elevated glutamate levels in Alzheimer's disease brains[21], which means reducing glutamate levels might be beneficial in Alzheimer's disease. High performance liquid chromatography results showed that constructing the rat Alzheimer's disease models by icv injection of Aβ25-35 can disturb the metabolism of amino acid transmitters in the hippocampus. Increasing glutamate and γ-aminobutyric acid levels and altering the glutamate/γ-aminobutyric acid ratio results in excitotoxicity and injury to central neurons. The toxic role of glutamate was related to the neurotoxicity of Aβ25-35. After the intervention of osthole, the levels of glutamate and the ratio of glutamate/γ-aminobutyric acid were decreased, while the level of γ-aminobutyric acid was not affected in the hippocampus. These results demonstrate that osthole can decrease the excess release of glutamate, reduce the glutamate/γ-aminobutyric acid ratio, inhibit the excitotoxicity of glutamate by reversing the imbalance of the ratio of glutamate/γ-aminobutyric acid, and thus, improve the learning and memory disturbance of Alzheimer’s disease rats.

Therefore, osthole can improve spatial learning and memory impairment, protect damaged neurons and mediate hippocampal suppression of LTP in Alzheimer’s disease rats. Moreover osthole can decrease the excess glutamate, an excitatory amino acid resulting in excitotoxicity, and the ratio of glutamate/γ-aminobutyric acid in the hippocampi of Alzheimer's disease rats. To our knowledge, this is the first report demonstrating the mechanism of the glutamate/γ-aminobutyric acid regulatory system and its involvement in improving learning and memory. Moreover, as an effective component of traditional Chinese medicine, osthole is safe with very low toxicity. Also, osthole can pass through the blood-brain barrier[22], which forms the pharmacokinetic foundation for treating central nervous system diseases. Therefore, osthole shows great potential as a novel drug for the treatment of Alzheimer’s disease.
MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
This experiment was performed at the Laboratory of Neuropharmacology, Department of Pharmacology, School of Medicine, Hebei North University, China in 2010.

Materials
Animals
A total of 64 healthy male Sprague-Dawley rats (aged 6–8 weeks, weighing 200 ± 20 g, specific pathogen free grade) were obtained from the Experimental Animal Breeding Center of Chinese Medical Science Academy (No. SCKK (J ing) 2009-0008). All rats were allowed food and water ad libitum during the experiments and were housed at 25 ± 1°C, with a humidity of 40–70%, under natural illumination. All animal experiments and care were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[23].

Drugs
Osthole was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, Jiangsu Province, China). Osthole was dissolved in N,N-dimethylformamide with the assistance of tween-80 (N,N-dimethylformamide: tween-80: normal saline = 1:1:8) and stored at 4°C. Aβ25-35 was purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA). Aβ25-35 was dissolved in 0.9% sterile normal saline to 2.0 mM and incubated at 37°C for 96 hours to form aggregated Aβ25-35 before use.

Methods
Alzheimer’s disease model establishment
According to the methods in the literature[24], the rats were placed onto a stereotaxic device (Huaibei Zhenghua Apparatus and Equipments Co., Ltd., Huaibei, Anhui Province, China), were anesthetized with 10% chloral hydrate (300 mg/kg) and maintained at 37 ± 0.5°C with a heating pad. The correct placement was obtained by positioning the rat so that the lateral ventricle[25] was 0.8 mm posterior to bregma, 1.8 mm lateral to midline, and 3.5 mm underneath the skull surface. Aβ25-35 (2 mM) in one intracerebroventricular injection was administered slowly in a 5 µL volume over a 10-minute period. After injection, the needle was kept in place for 30 minutes to prevent liquid leakage. Control group rats were administered isometric normal saline (5 µL). After the operations, the rats were returned to their cages and constantly monitored during recovery (supplementary Figure 2 online).

Drug intervention
After intracerebroventricular injection, the rats in the control and model groups were intraperitoneally injected with 1 mL/kg solvent (N,N-dimethylformamide, tween-80 and normal saline at 1:1:8 in volume) and osthole treated rats were intraperitoneally administered with osthole (12.5 mg/kg or 25.0 mg/kg) once a day. The dose levels were based on past experiments[13] and the conversion of the body surface area. All animals were dosed consecutively for 14 days.

Morris water maze test
The Morris water maze (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China) consisted of a circular tank (120 cm in diameter, 50 cm in height) filled with 25 ± 0.5°C water and a colorless plexiglass escape platform positioned 1 cm below the water surface in one of the four quadrants. A video camera was mounted above the center of the pool and all trials were recorded for subsequent analyses. External maze cues consisting of laboratory furniture and lights were constant throughout the experiments. Rats were trained four times every day from day 10 to day 13 after injection of Aβ25-35. Each time the rats were placed in the water facing the pool wall from different starting quadrants, and the time required for the rats to find the hidden platform (escape latency) was recorded. The rats were allowed to swim 120 seconds to find the hidden platform each time. If the rats could not find the platform within 120 seconds, the rats were given a score of 120 seconds and then physically placed on the platform for 20 seconds. A 3-minute interval was allowed between training and the escape latency was recorded[26-27]. Twenty-four hours after the last training day, a probe trial was made by removing the platform from the tank and rats were allowed to swim freely for 120 seconds[28]. The time that rats spent in the target quadrant (where the platform was once positioned) and the number of times the rats crossed the platform sites were recorded (supplementary Figure 3, Video 1 online).

Pathological examination of hippocampi
After Morris water maze testing, rats were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.) and the brains
were transectionally perfused with heparinized saline (40 U/mL, 4°C), followed by 4% paraformaldehyde (4°C). The brains were removed and fixed overnight in 4% paraformaldehyde at 4°C, and embedded in paraffin. Brain slices at 5 μm thickness were stained with hematoxylin and eosin. Hippocampal lesions were observed under the light microscope (Olympus BX60, Tokyo, Japan) and the images were collected by a digital photomicrography device (Nikon F15, Tokyo, Japan).

**Electrophysiological testing**

Electrophysiological tests were conducted on day 14 after intracerebroventricular injection of Aβ25-35. Small holes (1.5 mm in diameter) were drilled on one side of the skull where stimulating and recording electrodes were placed according to the stereotaxic atlas of the rat brain. The recording electrode was positioned in the granule cells of the hippocampal dentate gyrus (3.8 mm posterior to bregma, 2.5 mm lateral to midline, and 3.5 mm underneath to skull surface) and the bipolar stimulating electrode was implanted in the front perforant path of the entorhinal area (7.5 mm posterior to bregma, 4.2 mm lateral to midline, and 3.0 mm underneath to skull surface). The positions of stimulating and recording electrodes were adjusted until the maximum population spike stimulated by fixed stimulus intensity was found. The stimulus intensity was decreased until obtaining 50% of the maximum population spike and was kept constant during the entire experiment. The test stimuli (1/30 Hz, 0.1 ms square wave) produced by an electronic stimulator was delivered to the perforant path through the stimulation isolator and stimulating electrode. The evoked action potential was displayed in the oscilloscope and analyzed by the computer software. Before LTP was induced by one serial train of high frequency stimulus, the population strike amplitude was recorded for 30 minutes. The average of the population strike amplitude in 30 minutes before the high frequency stimulation was regarded as the basic synaptic transmission level. The synaptic transmission level of each time point after high frequency stimulation was shown in the form of the relative value (%) compared with the basic synaptic transmission level. If the population strike amplitude increased more than 30% and lasted longer than 30 minutes, LTP had formed (supplementary Figure 4 online).

**Amino acid content determination**

Behavioral tests were conducted on day 14 after intracerebroventricular injection of Aβ25-35. Rats were sacrificed by decapitation under anesthesia and hippocampi were immediately isolated on ice and made into 10% brain homogenates with methanol and water (v/v=1:1). The homogenates were centrifuged at 3000 × g for 10 minutes at 4°C, and the supernatants were saved in the -80°C freezer until testing for glutamate and γ-amino-butyric acid levels. The levels of glutamate and γ-amino-butyric acid were determined by pre-column derivatization high performance liquid chromatography. The wavelength of the ultraviolet detection was 360 nm.

**Statistical analysis**

All data are expressed as mean ± SEM and analyzed by one-way analysis of variance and then by two-tailed t-tests for different groups using the SPSS 12.0 software package (SPSS, Chicago, IL, USA). P values less than 0.05 were deemed to be statistically significant.

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**Author contributions:** Xiaohua Dong performed the experiments, analyzed the data and wrote the manuscript. Danshen Zhang guided this study and was in charge of funding. Li Zhang revised manuscript and approved the final version. Wei Li provided statistical support. Xianyong Meng provided technical help.

**Conflicts of interest:** None declared.

**Ethical approval:** All animal experiments and care were performed in accordance with China Animal Welfare Legislation and were approved by the Hebei North University Committee on Ethics in the Care and Use of Laboratory Animals.

**Supplementary information:** Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

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