Neuroprotective Effects of Spinosin on Recovery of Learning and Memory in a Mouse Model of Alzheimer’s Disease

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Abstract
Previous studies have shown that spinosin was implicated in the modulation of sedation and hypnosis, while its effects on learning and memory deficits were rarely reported. The aim of this study is to investigate the effects of spinosin on the improvement of cognitive impairment in model mice with Alzheimer’s disease (AD) induced by Aβ1-42 and determine the underlying mechanism. Spontaneous locomotion assessment and Morris water maze test were performed to investigate the impact of spinosin on behavioral activities, and the pathological changes were assayed by biochemical analyses and histological assay. After 7 days of intracerebroventricular (ICV) administration of spinosin (100 µg/kg/day), the cognitive impairment of mice induced by Aβ1-42 was significantly attenuated. Moreover, spinosin treatment effectively decreased the level of malondialdehyde (MDA) and Aβ1-42 accumulation in hippocampus. Aβ1-42 induced alterations in the expression of brain derived neurotrophic factor (BDNF) and B-cell lymphoma-2 (Bcl-2), as well as inflammatory response in brain were also reversed by spinosin treatment. These results indicated that the ameliorating effect of spinosin on cognitive impairment might be mediated through the regulation of oxidative stress, inflammatory process, apoptotic program and neurotrophic factor expression,suggesting that spinosin might be beneficial to treat learning and memory deficits in patients with AD via multi-targets.

Key Words: Spinosin, Semen Ziziphi spinosae, Alzheimer’s disease, Neuroprotection

INTRODUCTION
Alzheimer’s disease (AD) is an important age-related neurodegenerative disease typically leading to progressive cognitive decline, behavioral impairments, and finally dementia (Chu et al., 2012). It is assumed that the number of AD patients worldwide will quadruple from the current number of 36 million people by 2050 (Gomez-Ramirez and Wu, 2014). For the development of anti-AD drugs in the decade of 2002 through 2012, 244 compounds were assessed and the success rate for advancing agents for regulatory approval was only 0.4% (99.6% attrition) (Cummings et al., 2014). Thus, searching for effective agents for the treatment or alleviation of AD is urgently needed.

AD is characterized by the over-production of amyloid-beta (Aβ), which is regarded as one of the essential pathologic markers of AD (Yoon and Jo, 2012). Overproduction or lack of clearance of Aβ leads to an increased aggregation of Aβ, which is associated with the pathogenesis of AD (Hardy and Selkoe, 2002). Moreover, oxidative stress is involved in the development of AD (Butterfield and Stadtman, 1997) and is accompanied by the activation of microglial inflammation in the presence of Aβ-mediated inflammatory response (Zhang et al., 2013).
sedative and hypnotic effects of spinosin (Shin et al., 1978; Jiang et al., 2007; Wang et al., 2008), which may be related to the regulation of postsynaptic 5-HT receptor (Wang et al., 2010). Many studies suggested that sleep is linked with learning and memory function (Drummond and Brown, 2001; Graves et al., 2003), and spinosin was also reported to exert therapeutic potential in the treatment of cognitive impairment (Jung et al., 2014; Ko et al., 2015; Lee et al., 2016). Even so, the mechanism of neuroprotection by spinosin is still unclear.

Intracerebroventricular (ICV) injection of Aβ was reported to cause oxidative stress and inflammation, finally resulting in apoptosis of neuronal cells (Nagele et al., 2004); the neurotoxicity of Aβ ensured its effectiveness in producing animal models of AD (Palop and Mucke, 2010). In the current study, we investigated whether spinosin could ameliorate cognitive impairment in a mouse model of AD induced by ICV injection of Aβ, and further determined the underlying mechanisms - including the effect of spinosin on accommodating malondialdehyde (MDA) and brain-derived neurotrophic factor (BDNF) dysregulation, Aβ accumulation, neuronal apoptosis, and neuroinflammation.

MATERIALS AND METHODS

Animals and materials

Male specific pathogen-free Kun-Ming mice (35-40 g) were provided by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All the studies were performed in compliance with the Guideline for Animal Experimentation and the protocol was subject to approval by the Animal Ethics Committee of Shenyang Pharmaceutical University. All animals were housed under conventional conditions with appropriate temperature (22 ± 0.5°C), humidity (50-60%) control and a 12/12 h light/dark cycle with free access to food and water.

Spinosin (purity >99.0%) was provided by the National Institutes for Food and Drug Control of Pharmaceutical and Biological Products (Shenyang, China) and was suspended in physiological saline solution at concentrations of 0.2 and 2 µg/µL. Recombinant human Aβ (Sigma-Aldrich, St Louis, MO, USA) was dissolved and diluted in physiological saline to a stock concentration of 1.0 mg/mL, which was then sealed and incubated for 120 h at 37°C to allow the peptide to aggregate (Mao et al., 2015). Commercial kits for detection of MDA, BDNF, interleukin-6 (IL-6) and Bcl-2 were purchased from Nanjing Jiancheng Bio engineering Institute (Nanjing, China).

Treatment and experimental design

Mice were randomly divided into the following 4 groups (n=10 in each group): (I) control; (II) Aβ (10); (III) Aβ (100 µg/kg spinosin; (IV) Aβ (100 µg/kg spinosin). Each mouse in groups II-IV was anesthetized with intraperitoneal injection of chloral hydrate (4 g/kg), and 3 µL Aβ was injected into left hippocampus under the stereotaxic apparatus (AP: -0.5 mm; ML: -1.1 mm; DV: -3.0 mm). Aβ injection was performed over 3 min, and the needle was still in place for an additional 2 min before retracting. Then, animals were implanted with cannula (8.0 mm) located 5 mm above the right ventricle (AP: -0.2 mm; ML: -1.0 mm; DV: -3.0 mm). The cannula was fixed to the skull with dental cement. Mice were given ICV injection of spinosin (10 and 100 µg/kg) daily for 7 days after surgery. The timeline for animal experiments was provided in Fig. 1A.

Behavioral tests

Spontaneous locomotor activity: The spontaneous locomotor activity of mice was measured with a Multi-autonomous Activity Instrument (Huaibei Zhenghua Bioscience Technology Limited Company, Anhui, China) consisting nine chambers (25 cm diameter × 13 cm height) using a video-recorded analytical system (Shanghai Jiliang Software Technology Co. Ltd., Shanghai, China). The locomotor activity of each animal was determined by measuring the total distance of movements over a 5-min period.

Morris water maze test: Morris maze test was conducted according to the procedure established by Morris with slight modifications (Morris, 1984). The Morris water maze is a dark circular pool, 90 cm in diameter and 45 cm in height. It was filled with dark water (25-27°C) to a depth of 40 cm and the black platform (8 cm in diameter and 10 cm in height) was then placed in the same quadrant (Q4) on every trial with 1 cm below the water so that it was a featureless inner surface. On the first day of Morris maze test, mice were trained for two trials with a trial interval of 10 min to find the hidden escape platform. From the second day, mice received four trials per day for 4 consecutive days. They were randomly placed in the different quadrant and were allowed to swim for 90 s to seek the hidden platform. If the mice found the platform within 90 s and remained on it for at least 3 s, then the track was terminated. If the mice failed to find the platform within 90 s, the mice were guided to the platform and were allowed to stay on it for 15 s. On the 6th day, the platform was removed and the spatial
with control group; ** p<0.01 compared with A
say (ELISA) kits. In brief, protein samples from brain tissues
by commercial sandwich enzyme-linked immunosorbent as-
Biochemical analyses
The time spent by the animal in the target quadrant was
quadrant in probe trail sessions (C) of the Morris water maze test.

Four mice in each group were perfused transcardially with
saline followed by 4% paraformaldehyde (PFA) in phosphate
buffer saline (0.1 mol/L PBS, pH 7.2) after anesthesia. The
either brains were postfixed in 4% paraformaldehyde (PFA)
solution for 48 h, and then transferred to 30% sucrose in 0.1
mol/l PBS (pH 7.4) for at least 16 h until sectioning. Serial
(neighboring) sections of 10 µm thickness were sectioned and
stained with hematoxylin and eosin (H&E) staining (Ahmad et
al., 2005).

Statistical analyses
Results are expressed as mean ± SD. The significances
among different groups were determined using SPSS 19.0
(IBM Corp., New York, NY) where statistical significance were
assessed by one-way analysis of variance (ANOVA) followed
by Tukey’s multiple comparison test, p<0.05 was considered
statistically significant.

RESULTS
The spontaneous locomotor activity of mice
The influence of surgery and ICV administration of Aβ1-42 or
spinosin on spontaneous locomotor activity of the mice was
investigated. As shown in Fig. 1B, there was no significant
difference in the spontaneous locomotor activity indicated by
exploratory behavior among different groups after Aβ1-42 or
spinosin treatment (p>0.05), which suggested that surgeries
or ICV injection of Aβ1-42 or spinosin did not have impact on
locomotive activity of mice.

Spinosin improved Aβ1-42-induced cognitive impairment
in the Morris water maze test
For the long-term memory evaluated by Morris maze test, mice with Aβ1-42 injection spent longer time searching for the
hidden platform (latency time) than the control group during
the five days training sessions. On the other hand, mice treat-
ed with spinosin (10, 100 µg/kg) after Aβ1-42 injection showed
an improved learning and memory performance as compared to
mice in model group only received Aβ1-42 injection (Fig. 2A).
Accordantiy, the time spending in the target quadrant in Aβ1-42
group was lower than those in control group in the probe trial
on the 6th day of the test (p<0.01), while the treatment of high
dose of spinosin (100 µg/kg) significantly restored the time of
mice spending in the target quadrant (p<0.01) (Fig. 2B, 2C).
Spinosis decreased the level of MDA in hippocampus of mice with Aβ1-42 injection

Oxidative stress was evaluated by analyzing the level of MDA in hippocampus of mice. Compared with the control group, Aβ1-42 injected mice in model group showed a significant elevation of hippocampus level of MDA (p<0.01). After 5 days treatment with spinosin (10, 100 μg/kg), the MDA content was significantly reduced in the mice treated with both low and high doses of spinosin compared to model group (Fig. 3A).

Spinosis inhibited Aβ1-42 accumulation in the hippocampus of mice with Aβ1-42 injection

The Aβ1-42 levels in hippocampus homogenate were evaluated, and the results showed that ICV injection of Aβ1-42 significantly increased the Aβ1-42 level in the hippocampus of mice in model group (p<0.01). Compared with model group, the levels of Aβ1-42 were significantly decreased in the spinosin (10, 100 μg/kg) treatment groups (p<0.01) (Fig. 3B).

Spinosis restored the level of BDNF in the hippocampus and cerebral cortex of mice with Aβ1-42 injection

The level of BDNF was determined in the hippocampus and cerebral cortex of mice by ELISA. As shown in Fig. 4, the level of BDNF in both hippocampus and cortex of Aβ1-42 treated mice was lower than that of control mice (p<0.01), however, 100 μg/kg spinosin treatment significantly reversed BDNF level in both hippocampus and cortex (p<0.01), while ICV injection of low dose of spinosin (10 μg/kg) only increased BDNF expression in the cortex (p<0.01).

Spinosis reversed the level of Bcl-2 in the hippocampus and cerebral cortex of mice with Aβ1-42 injection

The anti-apoptotic effect of spinosin level of Bcl-2 was examined by ELISA. Compared with control group, a significant decrease in protein expression level of Bcl-2 was found in the hippocampus and cerebral cortex of the mice in model group with Aβ1-42 treatment (p<0.01). However, Bcl-2 was up-regulated significantly in both hippocampus and cerebral cortex following high dose rather than low dose of spinosin treatment compared with model group (Fig. 5), the results indicated that 100 μg/kg spinosin treatment could increase anti-apoptotic molecules level in the hippocampus and cerebral cortex area of Aβ1-42 injected mice.

Spinosis decreased the level of IL-6 in the hippocampus of mice with Aβ1-42 injection

To confirm the effect of spinosin on inflammatory response, the level of IL-6 in different groups was examined. IL-6 level in hippocampus was significantly higher in Aβ1-42 injected mice than control mice (p<0.01), whereas IL-6 level was significantly decreased with spinosin (100 μg/kg) treatment (p<0.01). However, no difference was found among different groups for the level of IL-6 in the cerebral cortex (Fig. 6).

Histopathology findings

In the control group, the neurons in CA1 region of hippocampus exhibited clear nuclei and intact neuronal bodies, while swollen and dispersed neuronal bodies were found in hippocampus of Aβ1-42 injected mice. After low or high dose spinosin treatment, the morphological aspects of neurons were recovered significantly compared with the model group (Fig. 7).

DISCUSSION

AD is characterized by cognitive impairment and neurodegeneration (De Strooper and Karran, 2016), and Aβ peptide deposition in the brain has been recognized as a major event during the neuropathogenesis of AD (Ianiski et al., 2012). Oral administration of spinosin was shown to improve memory in Aβ- or scopolamine-treated and normal naïve mice with cognitive dysfunction by inhibiting the 5-hydroxytryptamine 1A (5-HT1A) receptor, increasing choline acetyltransferase expression, reducing the number of glial cell, up-regulating adult hippocampal neurogenesis, and activating the ERK-CREB-BDNF signaling pathway (Jung et al., 2014; Ko et al., 2015; Lee et al., 2016). In our study, spinosin was directly administrated into the brains of Aβ1-42 injected mice to avoid the possible metabolic effect after oral administration, and the other potential targets of spinosin for the treatment of AD were identified.

Morris water maze test was conducted to confirm the effectiveness of spinosin in control of cognitive impairment of mice
with AD induced by Aβ1-42. The ICV administration of spinosin was shown to improve cognitive deficits in Aβ1-42 injected mice as evidenced by shorter escape latency and increased time spent in the target quadrant (Fig. 2). The results in the behavioral task indicated a neuroprotective effect of spinosin in the brain. In addition, the spontaneous locomotor activity reflected as evidenced by shorter escape latency and increased time spent in the target quadrant (Fig. 2). The results in the behavior of mice was not affected by Aβ1-42 or spinosin injection (Fig. 1).

Pathological conditions of AD are evidenced to link with oxidative stress, and the level of lipid peroxidation is considered as an important factor in AD (Gao et al., 2012). The level of MDA, a marker of lipid peroxidation index, was evaluated in hippocampus rather than cortex since a high level of MDA is significantly increased in hippocampus after ICV injection of Aβ1-42 (Ziech et al., 2010; Wu et al., 2017). Our results showed that spinosin significantly attenuated the up-regulation of MDA in hippocampus induced by ICV injection of Aβ1-42. The evidence indicated that cognitive impairments improved by spinosin might be associated with its anti-oxidative effects (Fig. 3A).

As the increase of Aβ1-42 was considered as an index of neurotoxicity (Pauwels et al., 2012), and the accumulation of deposits of Aβ is one of the classical neuropathological hallmarks (Kowalska, 2004). The level of Aβ1-42 in hippocampus, the region received Aβ1-42 injection, was evaluated in the current study. Our study showed that ICV injection of Aβ1-42 significantly increased Aβ1-42 level in the hippocampus of mice in model group, while spinosin significantly decreased the Aβ1-42 level compared with model mice. These results indicated a potential improving effect of spinosin in AD via inhibition of Aβ1-42 accumulation (Fig. 3B).

The reports of association of neuroinflammation with AD pathology have led to increased interest in pursuing anti-inflammatory therapy for AD (Tuppo and Arias, 2005). Singh and Guthikonda (1997) compared plasma concentrations of IL-6, IL-12, IFN-γ, and IFN-γ-τ in AD patients with control subjects, and IL-6 was found to be selectively elevated in AD patients. Our results showed an increased level of IL-6 in hippocampus of mice after Aβ1-42 injection, while the administration of spinosin decreased IL-6 level in the hippocampus (Fig. 6). Accordingly, Ko et al. (2015) reported an anti-inflammatory activity of spinosin by reducing the number of activated microglia and astrocytes in mice with Aβ1-42 injection. Thus, the anti-amnesic activity of spinosin was, in part, mediated by inhibiting inflammation.

BDNF, a key molecule in the maintenance of synaptic plasticity and memory storage in the hippocampus, has been shown to protect neurons from a variety of brain diseases (Numakawa et al., 2010). Additionally, the neuroprotection by BDNF is partly mediated by the anti-apoptotic effect through the up-regulation of Bcl-2 (Almeida et al., 2005). In our study, spinosin was found to exert anti-apoptotic effect through the regulation of Bcl-2 (Fig. 5), and the results also demonstrated a neuroprotective effect of spinosin associated with the up-regulation of BDNF in both hippocampus and cortex (Fig. 4), which is consistent with a previous study showing the activation of ERK-CREB-BDNF signaling pathway in the hippocampus after spinosin stimulation (Lee et al., 2016). Therefore, spinosin might protect the neurons by regulating the expres-

**Fig. 6.** Effect of spinosin on the protein level of IL-6 in hippocampus (A) and cortex (B) of Aβ1-42 injected mice. Values are mean ± SD (n=6). **p<0.01 compared with control group; *p<0.01 compared with Aβ1-42 group.

**Fig. 7.** Histopathological changes of neurons in hippocampus of mice after Aβ1-42 injection. Representative images of neurons in hippocampal CA1 region of mice from control, Aβ1-42, Aβ1-42+10 µg/kg spinosin, and Aβ1-42+100 µg/kg spinosin groups. The arrows indicated neuronal degeneration of hippocampal CA1 region.
sion of BDNF as its potential target.

In conclusion, in addition to previously reported targets of spinosin, neuroprotective effects of spinosin were found through the regulation of oxidative stress, Aβ$_{1-42}$ accumulation, as well as apoptotic program in brain. These findings suggest that spinosin may represent a potential therapeutic agent to improve cognitive function in patients with AD through the regulation of multi-targets.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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