Spin.osin Inhibits $A\beta_{1-42}$ Production and Aggregation via Activating Nrf2/HO-1 Pathway

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Abstract
The present research work primarily investigated whether spinosin has the potential of improving the pathogenesis of Alzheimer’s disease (AD) driven by $\beta$-amyloid ($A\beta$) overproduction through impacting the procession of amyloid precursor protein (APP). Wild type mouse Neuro-2a cells (N2a/WT) and N2a stably expressing human APP695 (N2a/APP695) cells were treated with spinosin for 24 h. The levels of APP protein and secreted enzymes closely related to APP procession were examined by western blot analysis. Oxidative stress related proteins, such as nuclear factor-erythroid 2-related factor 2 (Nrf2), and heme oxygenase-1 (HO-1) were detected by immunofluorescence assay and western blot analysis, respectively. The intracellular reactive oxygen species (ROS) level was analyzed by flow cytometry, the levels of $A\beta_{1-42}$ were determined by ELISA kit, and Thioflavin T (ThT) assay was used to detect the effect of spinosin on $A\beta_{1-42}$ aggregation. The results showed that ROS induced the expression of ADAM10 and reduced the expression of BACE1, while spinosin inhibited ROS production by activating Nrf2 and up-regulating the expression of HO-1. Additionally, spinosin reduced $A\beta_{1-42}$ production by impacting the procession of APP. In addition, spinosin inhibited the aggregation of $A\beta_{1-42}$. In conclusion, spinosin reduced $A\beta_{1-42}$ production by activating the Nrf2/HO-1 pathway in N2a/WT and N2a/APP695 cells. Therefore, spinosin is expected to be a promising treatment of AD.

Key Words: Alzheimer’s disease, Spinosin, Nrf2/HO-1, Neuroprotection

INTRODUCTION
Alzheimer’s disease (AD) is the most common neurodegenerative disease and the leading cause of dementia that mainly occurs in the elderly. It is estimated that 44 million people worldwide have dementia in 2018. As the population ages, the number of people with dementia will have more than tripled by 2050 (Lane et al., 2018). The major neuropathological hallmarks of AD include senile plaques, neurofibrillary tangles, synaptic dysfunction and neuronal loss, among which the deposition of brain $\beta$-amyloid (A$\beta$), derived from amyloid precursor protein (APP), contributes to the formation of senile plaques (Wang et al., 2017). The key enzyme in amyloidogenic APP processing for the production of A$\beta$ is $\beta$-site APP cleaving enzyme 1 (BACE1) (Das et al., 2016), while a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), a major $\alpha$-secretase in non-amyloid APP processing, significantly contributes to the suppression of the production of A$\beta$ (Postina et al., 2004; Wang et al., 2018). Accordingly, the inhibition of A$\beta$ by means of down-regulation of BACE1 and up-regulation of ADAM10 has emerged as apivotal therapeutic strategy for the treatment of AD.

Semen Ziziphi Spinosae (SZS), the seed of Ziziphus jujuba Mill. var. spinose (Bunge) Hu ex H. F. Chou, has been shown to possess sedative-hypnotic, anti-anxiety, and anti-depression effects (Fang et al., 2010; Liu et al., 2015). Flavonoids are the major bioactive components of SZS. Many studies have shown that flavonoids have antioxidant effects and can inhibit the production of reactive oxygen species (ROS) (Agati et al., 2012; Bao et al., 2016; Jung et al., 2017). Spinosin (desig-
nated as SPI, Fig. 1), the major active C-glycoside flavonoid in Szs, has been reported to be effective in the treatment of AD, which might be mediated through counteracting oxidative stress (Xu et al., 2019). However, the underlying mechanism by which spinosin confers an antioxidant effect is unclear.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. Under oxidative stress conditions, Nrf2 dissociates from Kelch-like ECH-associated protein-1 (Keap1) and undergoes nuclear translocation to activate the expression of antioxidant genes, such as heme oxygenase-1 (HO-1) (Loboda et al., 2016; Jeong et al., 2017; Bao et al., 2018).

The occurrence of AD is often accompanied by oxidative stress and mitochondrial dysfunction in the brain (Cervellati et al., 2016; Ahmad et al., 2017; Nesi et al., 2017). It is well known that hydrogen peroxide (H$_2$O$_2$) is capable of inducing oxidative stress, resulting in a large production of ROS (Park, 2016; Lu et al., 2017; Jia et al., 2018). In addition, increasing evidence has shown that ROS can mediate APP cleavage by up-regulating the activity of BACE1 in SH-SY5Y cells or H4 human neuroglioma cells (Ko et al., 2010; Zhang et al., 2011). However, the uncertainty of the relationship between ROS and ADAM10 still remains.

In the current study, we investigated whether spinosin can exert an impact on the cleavage of APP through the Nrf2/HO-1 pathway, as a response, and influence the production of Aβ via an array of in vitro experiments with wild type mouse Neuro-2a cells (N2a/WT) and N2a stably expressing human APP695 (N2a/APP695) cells.

MATERIALS AND METHODS

Materials

Spinosin (6-(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)-5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4H-1-benzopyran-4-one) was purchased from Meilunbio (Dalian, China), with HPLC purity 98%. Dulbecco’s modified Eagle’s medium (DMEM, high glucose) was purchased from HyClone (Logan, UT, USA), fetal bovine serum was purchased from Clark (Richmond, VA, USA), Opti-MEM and G418 disulfate salt were from Gibco (Grand Island, NY, USA). Human Aβ$_{1-42}$ ELISA kit was purchased from Shanghai MLBIO Biotechnology (Shanghai, China). 2, 7-Dichlorofluorescein diacetate (DCFH-DA) and BCA protein assay kit were purchased from Meilunbio.

![Fig. 1. The chemical structure of spinosin.](https://doi.org/10.4062/biomolther.2019.123)
rescence intensity was detected with excitation wavelength of 448 nm and emission wavelength of 488 nm.

**Western blot analysis**

After 24 h of drug treatment, cells were lysed on ice using RIPA lysis supplemented with protease inhibitor for 15 min. The supernatant was collected by centrifuging the cell lysate at 13,000 rpm for 15 min at 4°C. Protein quantitative analysis was performed according to the instruction of the BCA protein quantification kit. Proteins (30 μg) were separated by 10% SDS-PAGE, then transferred to nitrocellulose membranes and blocked with 5% skim milk. After blocking, the membranes were incubated with primary antibodies against APP (1:1000), BACE1 (1:600), ADAM10 (1:500), Nrf2 (1:500), HO-1 (1:500), or β-actin (1:3000) overnight at 4°C. Blots were then washed with TBST buffer and incubated with the secondary antibodies at room temperature for 1.5 h before visualization with ECL. Band intensities were quantified using Image Pro 6.0 software (Media Cybernetics, Baltimore, MD, USA).

**Detection of intracellular ROS accumulation**

Intracellular ROS were detected by fluorescent probe 2, 7-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is converted by intracellular esterases, which is oxidized into the highly fluorescent dichlorofluorescein (DCF) in the presence of a proper oxidant. After incubation, cells (1×10⁶/mL) were incubated with DCFH-DA (10 μM, diluted with PBS) at 37°C in dark for 30 min. Then the cell suspension was loaded into a flow-specific tube and detected by flow cytometry at an excitation and emission wavelength of 485 and 538 nm.

**Statistical analysis**

The experiments were carried out at least in triplicate.

**RESULTS**

**Effects of spinosin on the survival of N2a cells**

We used MTT reduction assay to detect the effects of spinosin on the survival of N2a cells. The N2a/WT cells and N2a/APP695 cells were treated with spinosin (0-400 μM) for 24 h. The results showed that spinosin had no significant effects on N2a/WT cell viability in the range of 0-400 μM, but cytotoxicity was observed in N2a/APP695 cells with the stimulation of 200 and 400 μM spinosin (Fig. 2A, 2B, Supplementary Fig. 1A, 1B). Therefore, spinosin treatment within the range of 0-100 μM is safe for both N2a/WT and N2a/APP695 cells.

**Spinosin attenuates the secreted and intracellular Aβ₁-42 in N2a cells**

The results of ELISA kit showed that the amount of Aβ₁-42 secreted by N2a/APP695 cells is 11.12% (p<0.001) higher than that of N2a/WT cells. In addition, after the treatment of spinosin (25 μM), it decreased by 87.32% (p<0.001) in N2a/APP695 cell (Fig. 2C). While it had no significant changes in N2a/WT cells (p>0.05) (Fig. 2C). As can be seen from Fig. 2D, the intracellular levels of Aβ₁-42 in the untreated N2a/WT and N2a/APP695 cells were similar, and were significantly decreased following the spinosin treatment in both cell lines. Stimulation of 6.25, 12.5, and 25 μM spinosin down-regulated Aβ₁-42 levels by 14.48%, 16.26%, and 21.18%, respectively in N2a/WT cells, and by 19.97%, 19.59%, and 16.72%, respectively in N2a/APP695 cells. Meanwhile, we tested the effects of 0-25×10⁻⁵ nM spinosin on the levels of Aβ₁-42, and the results showed that there was no significant difference from control group in the range of 0-1.56 nM (p>0.05) (Supplementary Fig. 1C, 1D).

**Spinosin down-regulates the expression level of APP**

We have previously detected that Aβ₁-42 is inhibited by spinosin. Since APP is a precursor protein that produces Aβ, we next examined the level of APP in cells with different treatments. As shown in Fig. 3A, the protein level of APP in N2a/APP695 cells was 45% (p<0.001) higher than that in N2a/WT cells. When the concentration of spinosin was 25 μM, the inhibition rates of APP protein levels in N2a/WT and N2a/APP695 cells could reach 89% (p<0.001) and 21% (p<0.01), respectively.

**Spinosin affects the expression of BACE1 and ADAM10**

BACE1 and ADAM10 are two important enzymes in the processing of APP. The inhibition of BACE1 activity, promotion of ADAM10 activity, and ultimately the reduction of Aβ production contributes to the delay in the progression of AD. As shown in Fig. 3B, the protein level of BACE1 in N2a/APP695 cells was 38.43% higher (p<0.01) than that in N2a/WT cells. Spinosin (6.25-25 μM) down-regulated the level of BACE1 in N2a/APP695 cells (p<0.001), although it had no significant effect in N2a/WT cells (p>0.05). Significant upregulation of ADAM10 levels in N2a/WT and N2a/APP695 cells was observed in a
concentration-dependent manner when 6.25-25 μM spinosin was administered (Fig. 3C).

**Spinosin reduces the production of ROS**

Many studies have shown that the occurrence of neurodegenerative diseases is often accompanied by an increase in ROS production. Hence, we used a DCFH-DA fluorescent probe to determine the intracellular ROS content by flow cytometry. The results showed that spinosin (25 μM) significantly reduced the production of ROS (p<0.05) with antioxidant action in N2a/APP695 cells. However, it had no significant effect on ROS in N2a/WT cells (Fig. 4B). N-acetyl-L-cysteine (NAC) is a commonly used inhibitor of ROS. Our results suggested that NAC was capable of scavenging ROS, with action that was slightly stronger than spinosin, and the level of ROS was significantly increased following the treatment with tretinoin, which is a Nrf2 inhibitor (Fig. 4D). Additionally, the effects of lower concentration of spinosin (24.42, 97.66 and 390.63 nM) on intracellular ROS were detected in N2a/APP695 cells, while the results showed that there was no significant difference compared with the control group (p>0.05) (Supplementary Fig. 2).

**Spinosin reverses the H2O2-induced changes of BACE1 and ADAM10 expression**

H2O2 can induce oxidative stress that can subsequently cause cell apoptosis (Yang et al., 2017; Liu et al., 2019). Herein, we exposed the N2a/APP695 cells to 6.25 and 12.5
μM H2O2 for 90 min, followed by discarding the medium and adding spinosin (25 μM) prepared in fresh medium, and incubating for 24 h. As the concentration of H2O2 reached 12.5 μM, severe damage to N2a/APP695 cells was observed (Fig. 5A). Accordingly, we chose the concentration of 6.25 μM for further testing. The images showed that H2O2 treatment caused the cells to shrink, and the morphologies of the cells were improved after the addition of spinosin.

The results indicated that pretreatment with 6.25 μM H2O2 for 90 min can significantly up-regulate the expression of BACE1 by 67.68% (p<0.01) and down-regulate the expression of ADAM10 by 26.52% (p<0.01) in N2a/APP695 cells. These effects were reversed by spinosin (Fig. 5B).

**Spinosin increases protein levels of Nrf2 and HO-1**

Nrf2/HO-1 is a classical antioxidant pathway that plays an important role in combating oxidative stress (Ren et al., 2019).

**Fig. 5.** Spinosin reversed H2O2-induced changes in BACE1 and ADAM10 of N2a/APP695 cells. Effects of H2O2 on cell morphology and cell survival (A). The levels of BACE1 and ADAM10 proteins were determined by western blot (B). All western blot data were normalized by β-actin. Values are the mean ± SEM from experiments performed in triplicate. Significance was determined by Tukey’s multiple comparisons test.

Under oxidative stress, Nrf2 localizes to the nucleus where it binds to a DNA promoter and initiates transcription of antioxidant genes, of which, HO-1 is a target gene of Nrf2 (Kim et al., 2018). To demonstrate the exact mechanism by which spinosin exerts neuroprotection, we examined Nrf2 nuclear translocation by immunofluorescence assay and the expression of HO-1 by western blot analysis. The results shown in Fig. 6 revealed that spinosin treatment significantly up-regulated the expression of HO-1 and the level of nuclear translocated Nrf2 in N2a/WT and N2a/APP695 cells.

**Spinosin regulates APP processing via the Nrf2/HO-1 signaling pathway**

To determine whether the Nrf2/HO-1 pathway is involved in the inhibitory effect of spinosin on Aβ production, we exposed N2a cells to tretinoin, an inhibitor of Nrf2 (Meng et al., 2016), to detect the downstream related indicators. It has been reported that tretinoin inhibits Nrf2 activity through its physical interaction with Nrf2, thus preventing Nrf2 from binding to the antioxidant response element (ARE) and activating its target gene (Wang et al., 2007; Suzuki et al., 2013). It was found that treatment with tretinoin (1 μM) for 24 h was able to reverse a series of changes brought about by spinosin. Tretinoin treatment increased the levels of APP and BACE1 proteins, and decreased the levels of ADAM10 and HO-1 proteins in N2a/WT and N2a/APP695 cells (Fig. 7). We also treated cells with ML385, a specific inhibitor of Nrf2 (Singh et al., 2016), to detect the expression of these proteins. ML385 was shown to exert effects similar to those of tretinoin (Supplementary Fig. 3, 4). In conclusion, spinosin inhibited Aβ1-42 production through the Nrf2/HO-1 signaling pathway.

**Spinosin reduces oligomerization of Aβ1-42**

A number of evidence has shown that Aβ1-42 oligomerization or fibrillation is critical for neurodegeneration (Bloom et al., 2005), suggesting that the prevention of this process might be an effective approach for the treatment of AD (Jiang et al., 2019). ThT can be inserted into oligomerized Aβ1-42 to produce fluorescence absorption at specific wavelengths, and the absorption intensity is positively correlated with the degree of

**Fig. 6.** The effect of spinosin on the nuclear translocation of Nrf2 (A, B) and the protein level of HO-1 (C) in N2a/WT and N2a/APP695 cells. The expression of HO-1 is normalized by β-actin (D). Values are the mean ± SEM from experiments performed in triplicate. **p<0.01, ***p<0.001 versus untreated N2a/APP695 cells; *p<0.05, **p<0.001 versus untreated N2a/WT cells.
The impacts spinosin on fibril disaggregation of Aβ1-42 cells. The expressions of APP (A, B), BACE1 (C, D), ADAM10 (E, F) and HO-1 (G, H) were detected by western blot. And they were normalized by β-actin. Values are the mean ± SEM from experiments performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 versus untreated N2a/APP695 cells; *p<0.05, **p<0.01, ***p<0.001 versus untreated N2a/WT cells.

Fig. 7. Nrf2 inhibitor treatment reversed the role of spinosin in N2a cells. The expressions of APP (A, B), BACE1 (C, D), ADAM10 (E, F) and HO-1 (G, H) were detected by western blot. And they were normalized by β-actin. Values are the mean ± SEM from experiments performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 versus untreated N2a/APP695 cells; *p<0.05, **p<0.01, ***p<0.001 versus untreated N2a/WT cells.

Fig. 8. Representative emission spectra of the thioflavin T (ThT) for the effect of spinosin on the oligomerization (A) and fibrillation (B) of Aβ1-42. *p<0.05 versus Aβ model group.

Discussion

Flavonoids have neuroprotective effects and other biological activities (Takekoshi et al., 2014; Guan and Liu, 2016), as they are potent antioxidants that scavenge the oxygen free radicals in the body. As a natural flavonoid, spinosin has low cytotoxicity and can easily pass through the blood-brain barrier (BBB) to protect neurons from oxidative damage (Lee et al., 2016b). Spinosin has been used to counteract sedation and hypnosis (Li et al., 2007). Our group has found that it also has neuroprotective effects and is beneficial for improving learning and memory (Xu et al., 2019). In the present study, we demonstrated that spinosin inhibited Aβ1-42 production by activating the Nrf2/HO-1 signaling pathway in N2a/WT and N2a/APP695 cells.

N2a cells stably expressing human APP695 are model cells commonly used to investigate the pathogenesis of AD. As a precursor protein of Aβ, APP is cleaved to produce Aβ. Our previous study showed that spinosin reversed Aβ-induced neurological damage in vivo (Xu et al., 2019). The current study indicated that the levels of APP and secreted Aβ1-42 of N2a/APP695 cells are higher than those of N2a/WT cells. Spinosin down-regulated the level of Aβ1-42 and inhibited the oligomerization of Aβ1-42 through the ThT assay, which is consistent with a previous in vivo study (Ko et al., 2015).

There has been little research on the antioxidant effects of spinosin. Previous in vivo studies in our group found that spinosin can regulate lipid peroxidation and inhibit oxidative stress (Xu et al., 2019). The results of the current study indicate that spinosin can activate the Nrf2/HO-1 pathway, inhibit the production of intracellular ROS, and exert antioxidant effects. It was also found that the level of ROS in N2a/APP695 cells was significantly higher than that in N2a/WT cells, which indicated that excessive APP could cause oxidative stress.

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The processing of APP mainly involves three hydrolases: ADAM10, BACE1 and γ-secretase (Zhang and Song, 2013; Dawkins and Small, 2014). The non-amyloid pathway mainly produces soluble sAPPα fragments by ADAM10 and γ-secretase. In the amyloid pathway, APP is sequentially hydrolyzed by BACE1 and γ-secretase to obtain Aβ1-42 and Aβ1-40 fragments (Postina et al., 2004; Corbett et al., 2015). The accumulation of long-chain Aβ1-42 is the main cause of senile plaques in AD patients, and the oligomeric form of Aβ1-42 is the most toxic. Therefore, inhibition of the amyloid pathway of APP or promotion of the non-amyloid pathway is beneficial for the prevention of AD pathogenesis.

It has been reported that ROS can induce an increase in BACE1 levels in SK-N-MC cells (Lee et al., 2016a), but the effect on ADAM10 is unknown. Our study indicated that Nrf2 inhibitor, ML385 or tretinoin, effectively inhibited the expression of Nrf2 and further inhibited the expression of HO-1 (Supplementary Fig. 3, 4). Moreover, spinosin inhibited the expression of BACE1 and promoted the expression of ADAM10 in N2a/APP695 cells, and these effects were reversed by the administration of Nrf2 inhibitors. Herein, the reason for the large increase in ADAM10 levels in N2a/APP695 cells may be the activation of a negative feedback regulation mechanism to down-regulate the sharply elevated Aβ1 levels by the non-amyloid pathway. The present study indicates for the first time that spinosin differentially mediates the expression of BACE1 and ADAM10 with the activation of the Nrf2/HO-1 pathway.

To summarize, spinosin inhibited ROS and Aβ1-42 production through the activation of the Nrf2/HO-1 signaling pathway, and decreased the formation of toxic Aβ1-42 oligomers. Therefore, spinosin is likely to be a promising drug for the treatment of AD.

CONFLICT OF INTEREST

All the authors declare that they have no conflicts of interest.

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