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Spinosin Inhibits Aβ$_{1-42}$ Production and Aggregation via Activating Nrf2/HO-1 Pathway

Xiaoying Zhang[1], Jinyu Wang[1], Guowei Gong[2], Ruixin Ma[1], Fanxing Xu[3], Tingxu Yan[4], Bo Wu[4] and Ying Jia[4,*]

[1]Key Laboratory of Active Components of Chinese Medicine Screening and Evaluation, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang 110016,
[2]Department of Bioengineering, Zunyi Medical University, Zhuhai Campus, Zhuhai, Guangdong 519041,
[3]Wuya College of Innovation, Shenyang Pharmaceutical University, Shenyang 110016,
[4]Key Laboratory of Active Components of Chinese Medicine Screening and Evaluation, School of Functional Food and Wine, Shenyang Pharmaceutical University, Shenyang 110016, China

Abstract

The present research work primarily investigated whether spinosin has the potential of improving the pathogenesis of Alzheimer's disease (AD) driven by β-amyloid (Aβ) 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 Aβ$_{1-42}$ were determined by ELISA kit, and Thioflavin T (ThT) assay was used to detect the effect of spinosin on Aβ$_{1-42}$ 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 Aβ$_{1-42}$ production by impacting the procession of APP. In addition, spinosin inhibited the aggregation of Aβ$_{1-42}$. In conclusion, spinosin reduced Aβ$_{1-42}$ 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 β-amyloid (Aβ), 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 Aβ is β-site APP cleaving enzyme 1 (BACE1) (Das *et al*., 2016), while a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), a major α-secretase in non-amyloid APP processing, significantly contributes to the suppression of the production of Aβ (Postina *et al*., 2004; Wang *et al*., 2018). Accordingly, the inhibition of Aβ 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-

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***Corresponding Author**
E-mail: jiayingsyphu@126.com
Tel: +86-24-2398-6933, Fax: +86-24-2398-6259

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_2O_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-4*H*-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 $A\beta_{1-42}$ 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. Thioflavin T (ThT) was from Absin Bioscience Inc (Shanghai, China). Rabbit anti-APP antibody and anti-BACE1 antibody were obtained from Cell Signaling Technology Inc (Danvers, MA, USA). Anti-Nrf2, β-actin, HO-1 and ADAM10 antibodies were purchased from Proteintech Inc (Chicago, IL, USA). Other reagents were cultured grade purity.

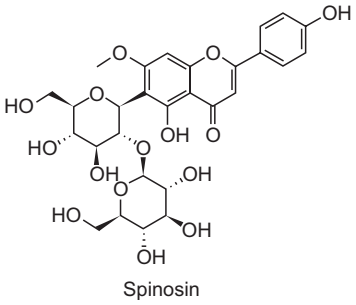

Fig. 1. The chemical structure of spinosin.

Cell culture and drug treatment

Wild type mouse Neuro-2a cells (N2a/WT), purchased from iCell Bioscience Inc., were derived from mouse neuroblastoma. N2a cells stably expressing human APP695 (N2a/APP695) were gifts kindly provided by Professor Huaxi Xu (Sanford-Burnham Medical Research Institute, La Jolla, CA, USA) and Professor Yunwu Zhang (Xiamen University, Fujian, China). The cells were cultured in medium consisting of an equivalent volume of DMEM and Opti-MEM with 5% fetal bovine serum in 5% CO_2 at 37°C. Stably transfected cells were screened in the presence of 0.2 g/L G418 disulfate salt. When growing up to 80% confluence, cells were incubated with varied doses of spinosin (0-400 μM) for 24 h, the effect of spinosin on cell viability was examined to determine the maximal concentration of spinosin that did not affect cell survival. To determine the impact of tretinoin on anti-oxidant activity of spinosin, cells were administrated with spinosin (6.25, 12.5, 25 μM) and tretinoin (1 μM) for 24 h, the conditioned medium and cells were collected separately for subsequent detection of various indicators.

Cell viability assay

Cell viability was measured by the MTT assay. N2a/WT and N2a/APP695 cells were placed in 96-well cell culture microplates (10^4 cell per well). They were treated with varied doses of spinosin (0-400 μM) and incubated for 24 h, the culture medium was then changed to the fresh medium containing 0.5 mg/ml MTT for 3 h. After that, the medium was removed and 100 μl of a solution containing 10% SDS, 5% isopropanol and 0.12 M HCl was added to each well, and the cells were further incubated at 37°C for overnight. The absorbance of the supernatant was measured at 570 nm (OD_{570}) by a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). For relative quantification, data were expressed as a relative percentage normalized to the control.

ELISA assay

The concentrations of $A\beta_{1-42}$ in conditioned medium and cell lysates were quantified using ELISA kit following the manufacturer's protocol. Optical densities of each well at 450 nm were read by the microplate reader, and $A\beta_{1-42}$ concentration in each sample was determined by comparing with the $A\beta_{1-42}$ standard curves. All readings were in the linear range of the assay.

Thioflavin T (ThT) fluorescence assays

ThT (5 μM) was formulated into a working solution with 50 mM glycine-NaOH solution (pH 8.5). The final concentration of $A\beta_{1-42}$ and spinosin were 20 μM and 10 μM, respectively. $A\beta_{1-42}$ monomer and spinosin were incubated for 24 h at 37°C to examine the effect of spinosin on $A\beta_{1-42}$ oligomerization. In addition, in order to detect the effect of spinosin on $A\beta_{1-42}$ fibrosis, $A\beta_{1-42}$ monomer was incubated at 37°C for 48 h to be fully polymerized before incubation with spinosin for 24 h. After the above incubations completed, 50 μL of the sample was added to 150 μL of 5 μM glycine-NaOH working solution. The fluo-

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 lysate 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 (1×10^6/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. The results are expressed as mean ± SEM. The statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. $p<0.05$ was considered as statistically significant. Statistical analysis was performed with GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA).

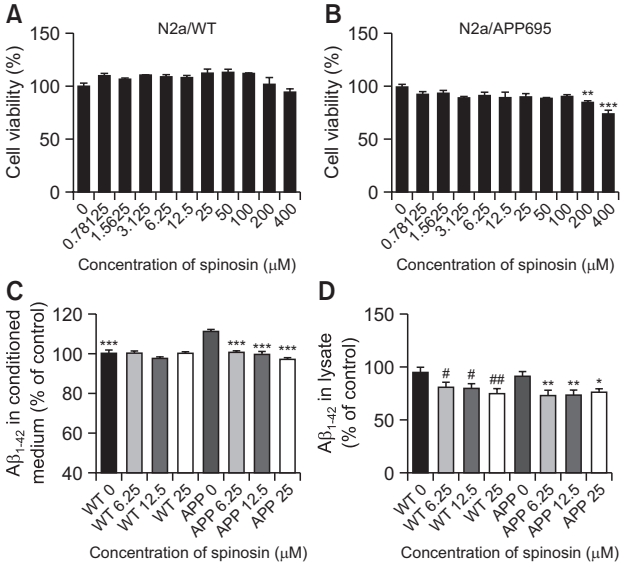

Fig. 2. Effects of spinosin on cell viability and the level of $A\beta_{1-42}$ of N2a/WT and N2a/APP695 cells. The cell viability of N2a/WT (A) and N2a/APP695 (B) cells was assessed by MTT reduction assay. $A\beta_{1-42}$ in conditioned medium (C) and intracellure (D) were measured by ELISA kit. Values are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus untreated N2a/APP695 cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, versus untreated N2a/WT cells.

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 $A\beta_{1-42}$ in N2a cells

The results of ELISA kit showed that the amount of $A\beta_{1-42}$ 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 $A\beta_{1-42}$ 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 $A\beta_{1-42}$ 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 0-6.25×10^3 nM spinosin on the levels of $A\beta_{1-42}$, 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 $A\beta_{1-42}$ 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

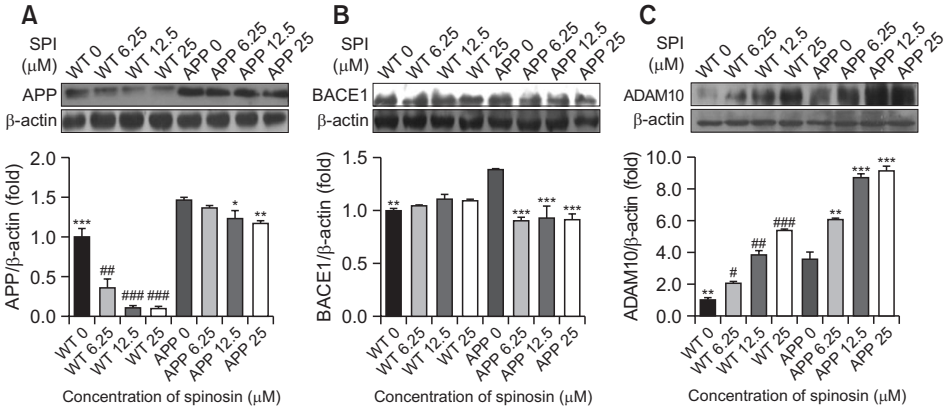

Fig. 3. Effects of spinosin on the expression of APP (A), BACE1 (B) and ADAM10 (C) proteins. Protein levels were determined by western blot. All western blot data were normalized by β-actin. Values are the mean ± SEM from experiments performed in triplicate. Significance was determined by Tukey's multiple comparisons test. *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.

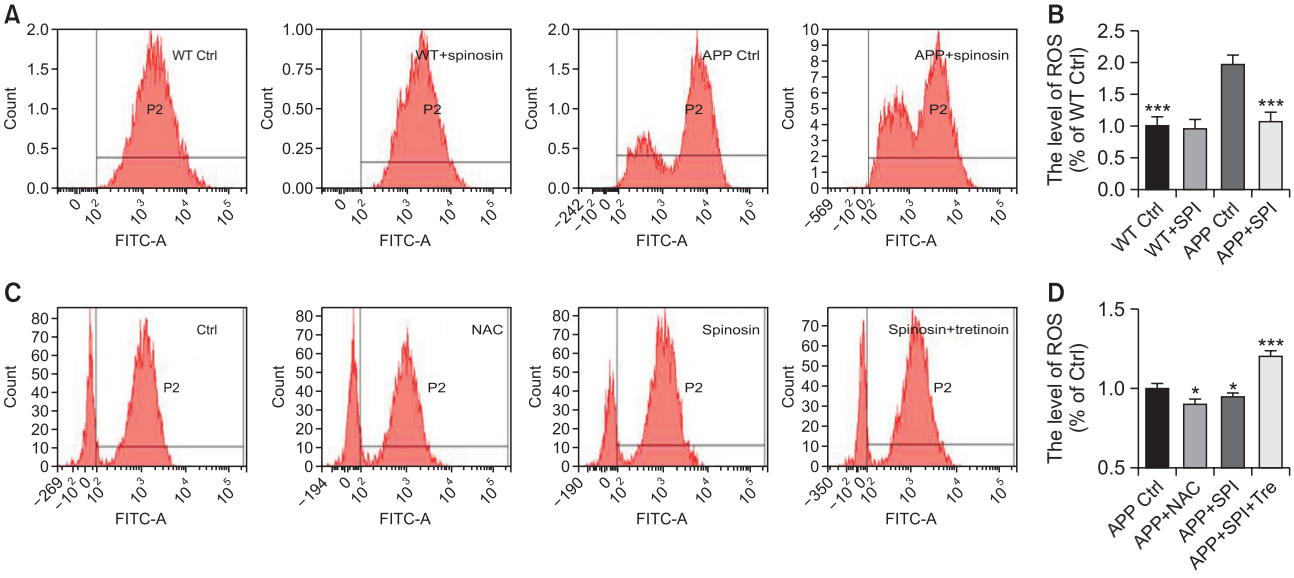

Fig. 4. Administration of spinosin down-regulates the level of ROS in N2a/APP695 cells. The ROS level of the N2a/APP695 control group was significantly higher than that of the N2a/WT control, and the treatment of spinosin significantly reduced the ROS in N2a/APP695 cells (A, B). Tretinoin reversed the decline in ROS levels caused by spinosin (C, D). Values are the mean ± SEM from experiments performed in triplicate. *p<0.05, ***p<0.001 versus untreated N2a/APP695 cells.

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 H_2O_2-induced changes of BACE1 and ADAM10 expression

H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 for 90 min can significantly up-regulate the expression of BACE1 by 67.68% (p<0.001) 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).

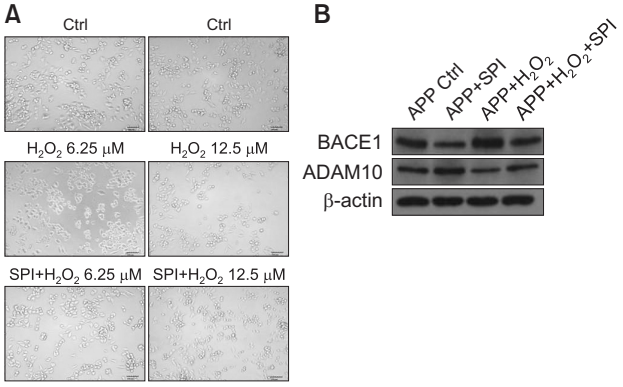

Fig. 5. Spinosin reversed H_2O_2-induced changes in BACE1 and ADAM10 of N2a/APP695 cells. Effects of H_2O_2 on cell morphology and cell survival (A). The levels of BACE1 and ADAM10 proteins were determined by western blot (B). All western blot data were normalized by β-actin. Values are the mean ± SEM 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 antioxidative 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 $A\beta_{1-42}$ production through the Nrf2/HO-1 signaling pathway.

Spinosin reduces oligomerization of $A\beta_{1-42}$

A number of evidence has shown that $A\beta_{1-42}$ oligomerization or fibrillization 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 $A\beta_{1-42}$ to produce fluorescence absorption at specific wavelengths, and the absorption intensity is positively correlated with the degree of

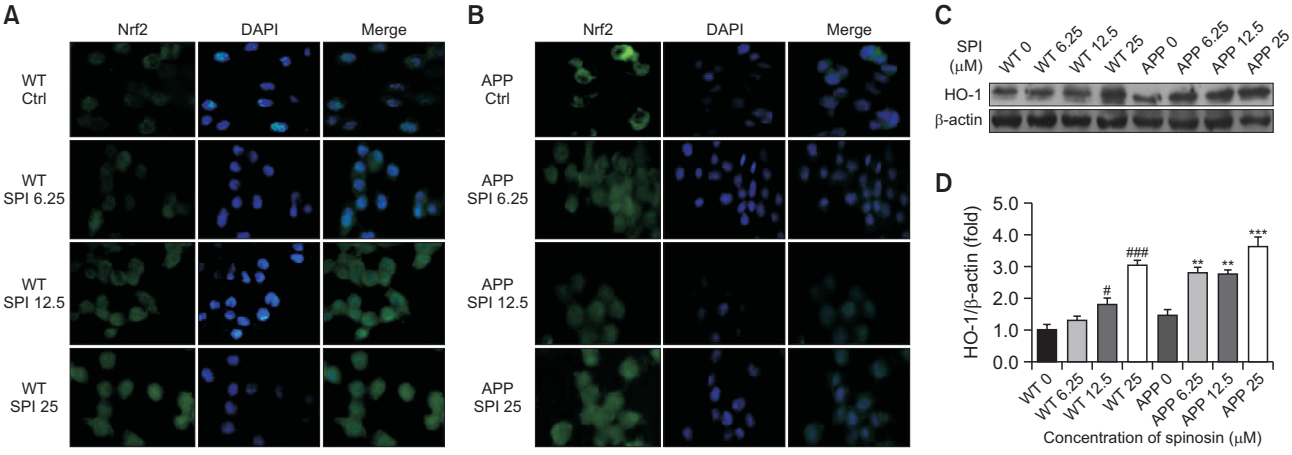

Fig. 6. The effect of spinosin on the nuclear translocation of Nrf2 (A, B) and the protein level of HO-1 (C) in N2a/WT and N2a/APP695 cells. The expression of HO-1 is normalized by β-actin (D). Values are the mean ± SEM from experiments performed in triplicate. **p<0.01, ***p<0.001 versus untreated N2a/APP695 cells; $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 versus untreated N2a/WT cells.

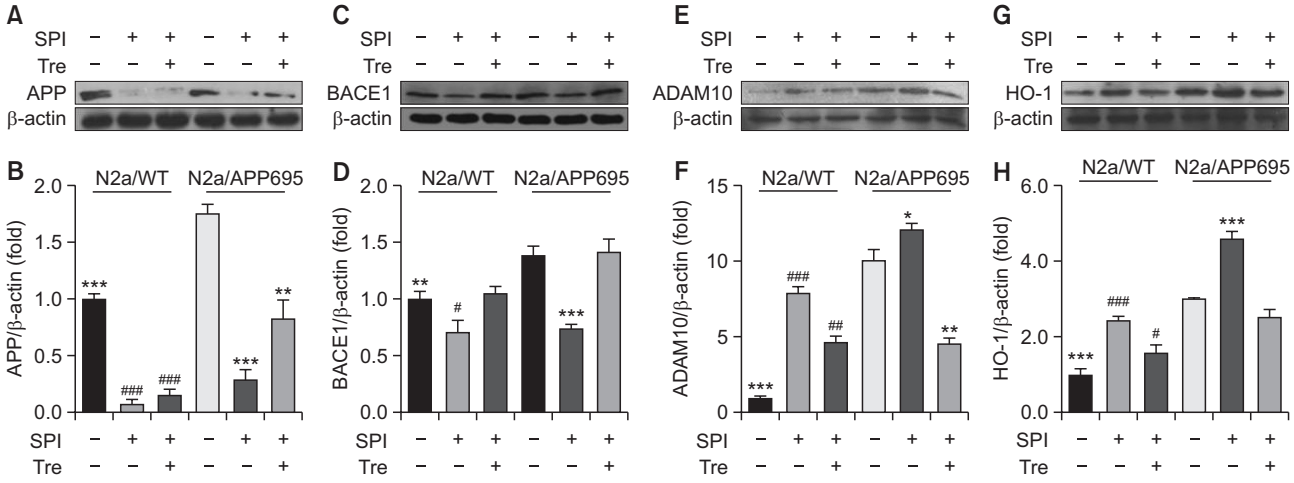

Fig. 7. Nrf2 inhibitor treatment reversed the role of spinosin in N2a cells. The expressions of APP (A, B), BACE1 (C, D), ADAM10 (E, F) and HO-1 (G, H) were detected by western blot. And they were normalized by β-actin. Values are the mean ± SEM from experiments performed in triplicate. *p<0.05, **p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

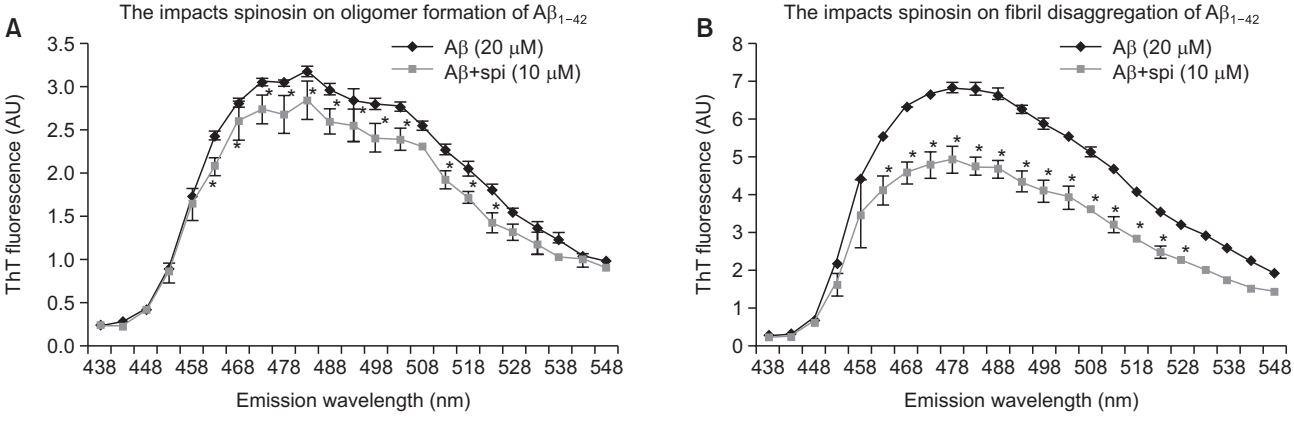

Fig. 8. Representative emission spectra of the thioflavin T (ThT) for the effect of spinosin on the oligomerization (A) and fibrillation (B) of $A\beta_{1-42}$. *p<0.05 versus Aβ model group.

oligomerization of $A\beta_{1-42}$ (Li *et al*., 2019). Our results indicated that spinosin inhibited the oligomerization of $A\beta_{1-42}$ (Fig. 8A) and reduced its toxicity. Meanwhile, the degree of aggregation of fibril $A\beta_{1-42}$ was reduced by spinosin treatment (Fig. 8B).

DISCUSSION

Flavonoids have neuroprotective effects and other biological activities (Takekoshi *et al*., 2014; Guan and Liu, 2016), as they are potent antioxidantsthat 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 $A\beta_{1-42}$ 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 $A\beta_{1-42}$ of N2a/APP695 cells are higher than those of N2a/WT cells. Spinosin down-regulated the level of $A\beta_{1-42}$ and inhibited the oligomerization of $A\beta_{1-42}$ 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.

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 $A\beta_{1-40}$ and $A\beta_{1-42}$ fragments (Postina *et al*., 2004; Corbett *et al*., 2015). The accumulation of long-chain $A\beta_{1-42}$ is the main cause of senile plaques in AD patients, and the oligomeric form of $A\beta_{1-42}$ 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 Aβ 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 $A\beta_{1-42}$ production through the activation of the Nrf2/HO-1 signaling pathway, and decreased the formation of toxic $A\beta_{1-42}$ 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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