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Original Article

Fuzhisan, a chinese herbal medicine, suppresses beta-secretase gene transcription via upregulation of SIRT1 expression in N2a-APP695 cells

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Abstract: The accumulation of β-amyloid (Aβ) peptide plaques is the major pathogenic event in Alzheimer's disease (AD). Because β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) cleaves APP at the first amino acid of the Aβ domain and is the rate-limiting enzyme for Aβ peptide generation, the level of this aspartic protease is a focus of AD research. Fuzhisan (FZS), a Chinese herbal complex prescription that has been used for the treatment of AD for over 20 years, is known to enhance metabolic activity and cognitive ability in aged rats and AD patients. To confirm whether FZS's therapeutic effect related to BACE1 pathway, we investigated the intracellular molecules expression change after FZS treatment in N2a-APP695 cell line. In this study, we demonstrated that BACE1 transcription and translation were reduced, and SIRT1 expression was elevated in the N2a-APP695 cells treated with FZS. The therapeutic efficacy of FZS in AD may be derived from the downregulation of BACE1 expression.

Keywords: Fuzhisan, alzheimer's disease, BACE1, SIRT1

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is the most common cause of dementia in elderly individuals. Amyloid plaques and neurofibrillary tangles are regarded as the pathological hallmarks of AD. The major constituent of amyloid plaques is β-amyloid (Aβ) peptide, which is generated by the sequential cleavage of amyloid precursor protein (APP) via β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) and γ-secretase [1]. BACE1 cleaves APP at the first amino acid of the Aβ domain and is crucial for the production of Aβ peptides [2]. Thus, BACE1 is the rate-limiting enzyme for Aβ peptide generation and is a major drug target for AD [3]. Aβ peptide exists as two main species, Aβ1-40 and Aβ1-42 [4]. The generation of Aβ1-40 and Aβ1-42 triggers neuronal dysfunction, Tau phosphorylation, neurofibrillary tangles, inflammation, and neuronal loss, which results in cognitive impairment [5, 6]. In addition, phospho-Tau (Ser396) has been associated with AD [7].

BACE1 expression is regulated by a complex mechanism that comprises transcription, translation and post-translational regulation [8]. During the process of BACE1 transcriptional regulation, a number of transcription factors positively or negatively regulate BACE1 gene transcription, including peroxisome proliferator-activated receptor-γ (PPARγ) [9], nuclear factors (e.g., nuclear factor-kappa B; NF-κB) [10], cAMP response element binding protein (CREB) [11] and hypoxia-inducible factor (HIF1α) [12]. PPARγ is a negative transcription factor that binds to peroxisome proliferator response element (PPRE), which is present in the promoter region of the BACE1 gene [9]. The inhibitory effect of PPARγ depends on its coactivator, peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC-1α), and SIRT1-mediated deacetylation, which occurs in a ligand-independent manner [13]. In addition, SIRT1 can regulate the activities of other transcription factors regulating BACE1 [14].

Fuzhisan (FZS), which is a Chinese herbal complex prescription that contains ginseng root

(*Panax ginseng C. A. Mey*, Jilin, China), baikal skullcap root (*Scutellaria baicalensis Georgi*, Shanxi, China), the rhizome of Acorus calamus L. (*Acorus talarinowi Schotti*, Sichuan, China), and radix Glycyrrhizae (*Glycyrrhiza uralensis fisch*, Neimenggu, China) [15, 16], has been used for the treatment of dementia for over 20 years [15, 17, 18]. The chemical fingerprints of 12 batches of FZS have been established to quantify their components and similarities, which were above 0.95 [16]. *In vivo* studies have demonstrated that FZS improves spatial learning, increases hippocampal acetylcholine levels and enhances glucose metabolism in the brains of aged rats [15]. FZS has been shown to ameliorate the impaired cognitive ability of aged SAMP-8 mice and increase hippocampal neurogenesis and the long-term survival of Bromodeoxyuridine (BrdU)-labeled cells without affecting the proportions of BrdU-positive neurons and glial cells [18]. In addition, it has been demonstrated to improve blood flow in the frontal and temporal lobes and the callosal gyrus in AD patients and significantly improve the Alzheimer's Disease Assessment Scale-Cognitive subscale (ADAS-Cog) and Neuropsychiatric Index (NPI) scores at week 12 [17]. However, the underlying intracellular regulations of the neurotrophic and neuroprotective functions of FZS are unknown.

In the present study, we investigated Aβ peptide generation and the expression of BACE1 and its associated regulators after FZS treatment in N2a-APP695 cells to clarify the intracellular response induced by FZS to provide further understanding of its therapeutic potential.

Materials and methods

FZS preparation

Ginseng root, baikal skullcap root, the rhizome of Acorus calamus L and radix Glycyrrhizae were purchased from the Harbin Pharmaceutical Company (Harbin, China) and identified by Professor D.S. Wang, an expert in herbal medicine at the First Affiliated Hospital of Harbin Medical University; the materials were mixed at a ratio of 2:1:1:1, respectively. The aqueous extract of FZS was obtained according to Li *et al.* [15] as follows: the four ingredients were mixed in the designated proportions, macerated for 40 min in 8 volumes (v/w) of distilled water, and subsequently decocted for 1 h. The filtrate was collected, and the residue was decocted for an additional 1 h with 6 volumes (v/w) of distilled water. The filtrate was pooled and then lyophilized (crude extract). The crude extract was dissolved in water at 0.5 g/mL (crude drug) and stored at -20°C until use.

Cell culture

Mouse Neuro-2a (N2a) cells stably expressing human APP695 were obtained from Professor Huaxi Xu and maintained in DMEM/Opti-MEM (1:1, v/v) (Gibco, GrandIsland, NY, USA) with 200 μg/mL G418 (Gibco, GrandIsland, NY, USA), 10% fetal bovine serum (FBS; Gibco, GrandIsland, NY, USA), 100 units/mL penicillin-streptomycin, and 100 units/mL L-glutamine at 37°C and 5% CO_2 under humidified conditions. The cells were passaged every 3 days and grown to 80% confluence. The cells were incubated with varying doses of FZS (2-250 μg/mL) for 24 h to assess its effects on cell viability. To determine whether SIRT1 is involved in the effects of FZS to BACE1, SRT1720 (an SIRT1 agonist) (Merck Millipore, Darmstadt, Germany) (10 μg/mL) or EX527 (an SIRT1 antagonist) (Sigma-Aldrich, St. Louis, USA) (100 μg/mL) was exposed to N2a-APP695 cells.

Cell viability assay (CCK-8 assay)

Cell viability was detected using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). N2a and N2a-APP695 cells were incubated with FZS at doses of 0, 2, 7.5, 15, 30, 60, 125 or 250 (μg/mL) in 96-well plates for 24 h. Following FZS treatment, 20 μL of CCK-8 solution were added to each well containing 200 μL of medium, and the 96-well plate was continuously incubated at 37°C for 3 h. The absorbance for each well was measured at a wavelength of 450 nm to determine cell viability.

Immunofluorescence assay

To observe Tau phosphorylation in N2a and N2a-APP695 cells, the cells were first seeded in a 24-well plate at 2×10^5 cells per well. After 24 h of growth, the cell medium was replaced with fresh medium containing 125 μg/mL of FZS, and the cells were cultured for an additional 24 h. The cells were subsequently washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. The fixed

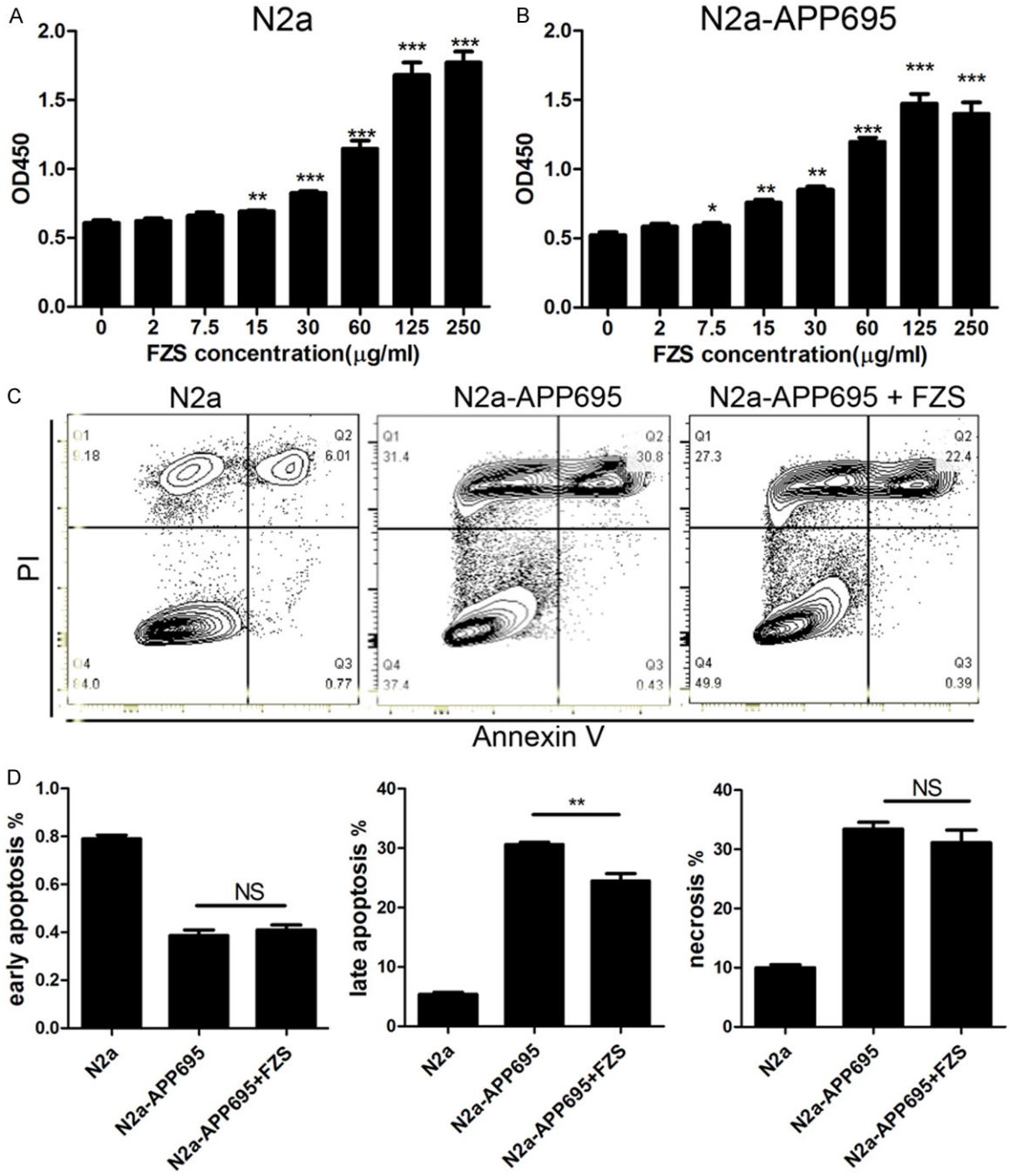

Figure 1. The influence of fuzhisan (FZS) on viability and apoptosis as induced by the serum starvation of N2a and N2a-APP695 cells. A: N2a cells were incubated with different concentrations of FZS. The viability of the N2a cells was concentration-dependent and was quantified by the CCK-8 assay (***P*<0.01 and ****P*<0.001). B: N2a-APP695 cells were incubated with different concentration of FZS. The viability of the N2a-APP695 cells was concentration-dependent and was quantified by the CCK-8 assay (**P*<0.05, ***P*<0.01 and ****P*<0.001). C and D: The effects of treatment with 125 μg/mL FZS on cell apoptosis induced by serum starvation (***P*<0.01). Cells were stained with Annexin-V and propidium iodide (*PI*). Three populations of cells were observed as follows: early apoptotic cells (Annexin-V+ PI-, lower right quadrant), cells in the late stages of apoptosis (Annexin-V+ PI+, upper right quadrants) and cells undergoing necrosis (Annexin-V- PI+, upper left quadrants).

cells were washed three times with PBS and permeabilized with 0.5% TritonX-100/PBS for 10 min. They were then washed with PBS three times and blocked with 3% FBS/PBS at 37°C

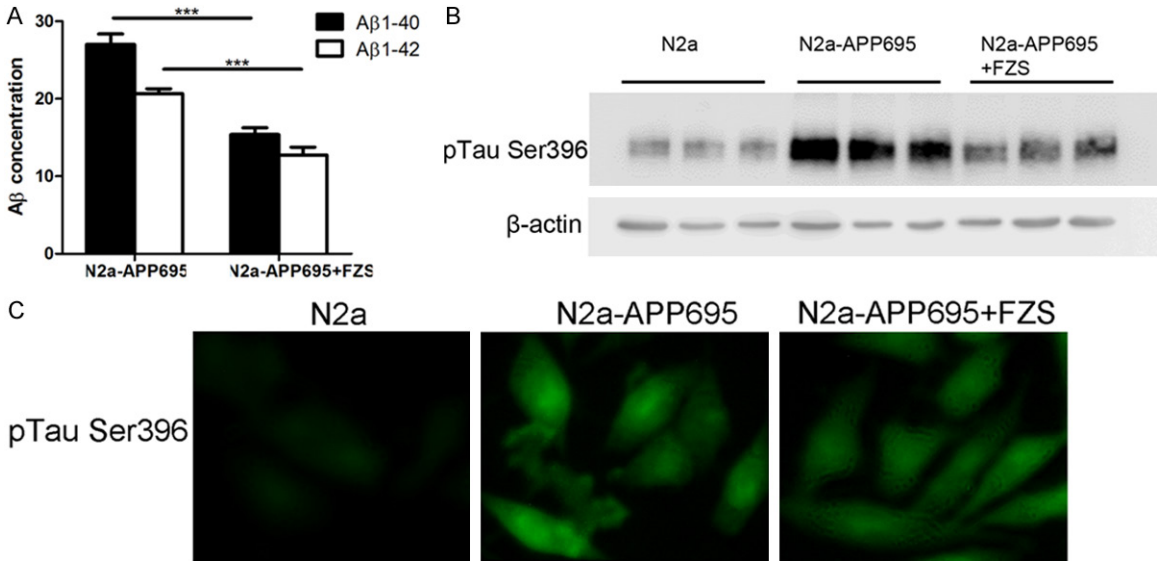

Figure 2. The secretion of Aβ1-40 and Aβ1-42 and the expression of phosphorylated Tau (Ser396) in cells that were both treated and not treated with 125 μg/mL FZS. A: The levels of Aβ1-40 and Aβ1-42 in the N2a-APP695 cells that were both treated and not treated with 125 μg/mL FZS (****P*<0.001). B: Western blot results of the phospho-Tau (Ser396) levels in the N2a and N2a-APP695 cells that were both treated and not treated with 125 μg/mL FZS. C: Immunofluorescence results of Tau phosphorylation at the Ser396 sites in the N2a and N2a-APP695 cells that were both treated and not treated with 125 μg/mL FZS.

for 1 h. After three additional washes with PBS, the cells were incubated overnight at 4°C with a rabbit anti-phospho-Tau (Ser396) antibody (1:100) (Sigma-Aldrich, St. Louis, MO, USA). The cells were then washed three times with PBS and incubated with a FITC-labeled goat anti-rabbit secondary antibody (1:100) (ZSGB-BIO, China) at room temperature for 2 h. Next, the cells were washed three times with PBS and stained with Hoechst 33258 (Beyotime, China) for 5 min. The stained cells were subsequently washed twice with PBS and observed under a fluorescence microscope (Axiovert 200, Zeiss, Germany).

Flow cytometric detection of apoptotic cells

Apoptotic cells were detected using an Annexin-V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences, Bedford, MA, USA). Briefly, FZS-treated and untreated cells were subjected to 24 h of serum starvation and collected in a 6-well plate at a seeding density of 5×10^5 cells per well as previously described [19]. The cells were subsequently washed with PBS, digested with trypsin, and harvested by centrifugation at 1000 rpm for 5 min at room temperature. The cells were then washed four times with PBS. The samples were prepared according to the manufacturer's protocol. Cell apoptosis was detected using a BD Fortessa (BD Biosciences).

Aβ enzyme-linked immunosorbent assay (ELISA)

Media were collected from FZS-treated and FZS-untreated cell cultures. The concentrations of Aβ1-40 and Aβ1-42 were quantified using ELISA kits (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocols. The optical densities in each well were measured using a plate reader at 450 nm.

Immunoblotting

The protein concentrations were determined by the Bradford method. The protein sample and loading buffer mixture (10 μL) were separated using a 4-15% precast polyacrylamide gel (Bio-Rad Laboratories, USA) and transferred to PVDF membranes. The membranes were incubated with the appropriate antibodies, which included a rabbit anti-APP antibody (Cell Signal Technology, USA) (1:1000), rabbit anti-BACE1 antibody (Cell Signal Technology, USA) (1:1000), rabbit anti-phospho-Tau (Ser396) (Sigma-Aldrich, St. Louis, MO, USA) (1:500), rabbit anti-PPARγ antibody (Sigma-Aldrich, St. Louis, MO,

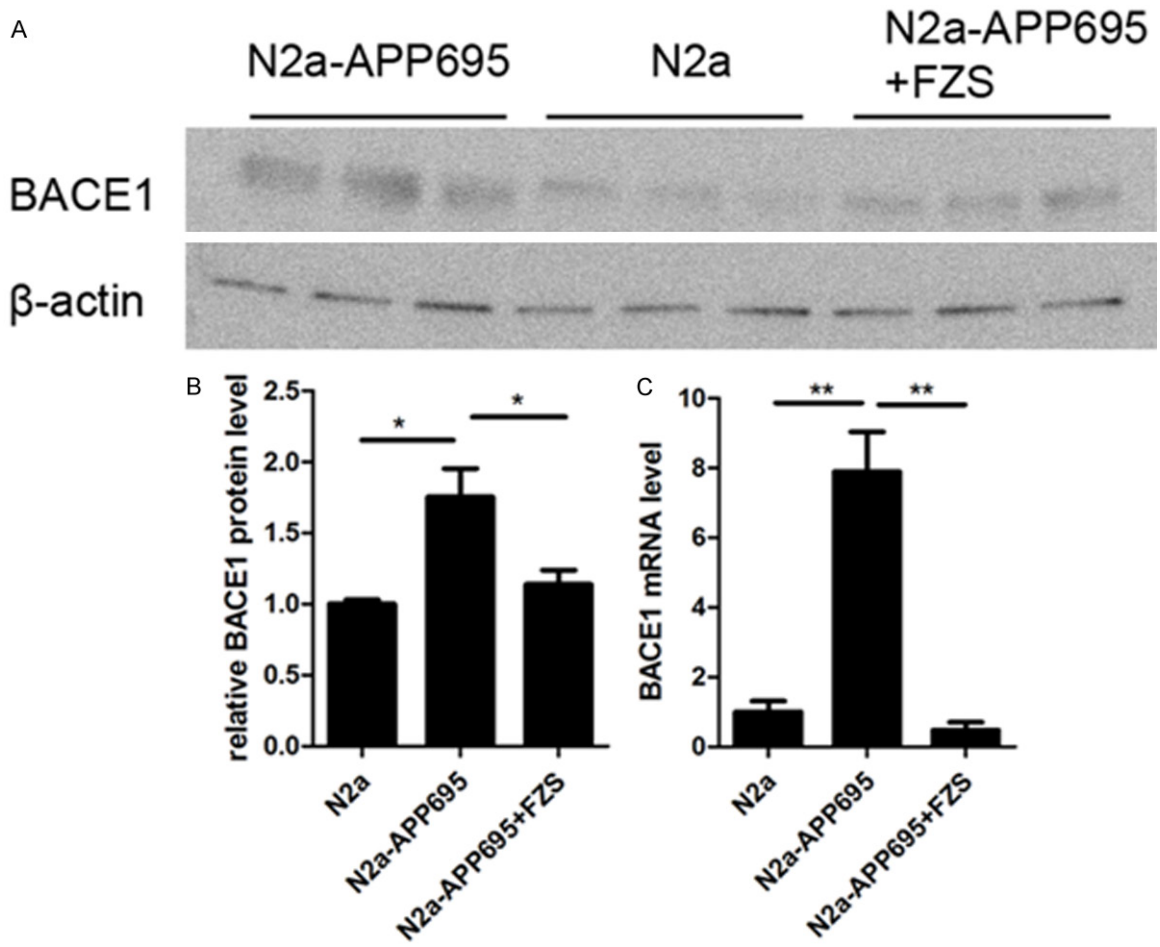

Figure 3. The mRNA and protein levels of BACE1 in the N2a and N2a-APP695 cells that were both treated and not treated with 125 μg/mL FZS. A: The protein level of BACE1 in the N2a and N2a-APP695 cells that were treated or not treated with 125 μg/mL FZS. B: Semi-quantification of the Western blotting data, which are presented as densitometry values normalized to β-actin. The data are expressed as the mean ± SEM (*P<0.05). C: The mRNA level of BACE1 in the N2a and N2a-APP695 cells that were treated or not treated with 125 μg/mL of FZS (**P<0.01).

USA) (1:500), mouse anti-PGC-1α antibody (Merck Millipore, Ohio, USA) (1:1000), and rabbit anti-SIRT1 antibody (Merck Millipore, Ohio, USA) (1:1000) and HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Santa Cruz, NY, USA) (1:2000). Western blot chemiluminescence detection was subsequently used to quantify band densities.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was prepared by reverse transcription (PrimeScript RT Reagent Kit, Takara) and amplified by qRT-PCR (Premix EX Taq II Perfect Real Time, Takara) with the following primers: BACE1: forward, 5'-GCATGATCATTGGTGGTATC-3', reverse, 5'-CCATCTTGAGATCTTGACCA-3' [20]; SIRT1: forward, 5'-TTGTGAAGCTGTTCGTGGAG-3', reverse, 5'-GGCGTGGAGGTTTTTCAGTA-3' [21]; PGC-1α: forward, 5'-TAGGCCCAGGTACGACAGC-3', reverse, 5'-GCTCTTTGCGGTATTCATCC-3' [22]; PPARγ: forward, 5'-CCACCAACTTCGGAATCA-3', reverse, 5'-TTTGTGGATCCGGCAGTTA-3' [22]; and GAPDH: forward, 5'-ACAGCCGCATCTTCTTGTGC-3', reverse, 5'-CACTTTGCCACTGCAAATGG-3' [21].

Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). The group differences were estimated using analysis of vari-

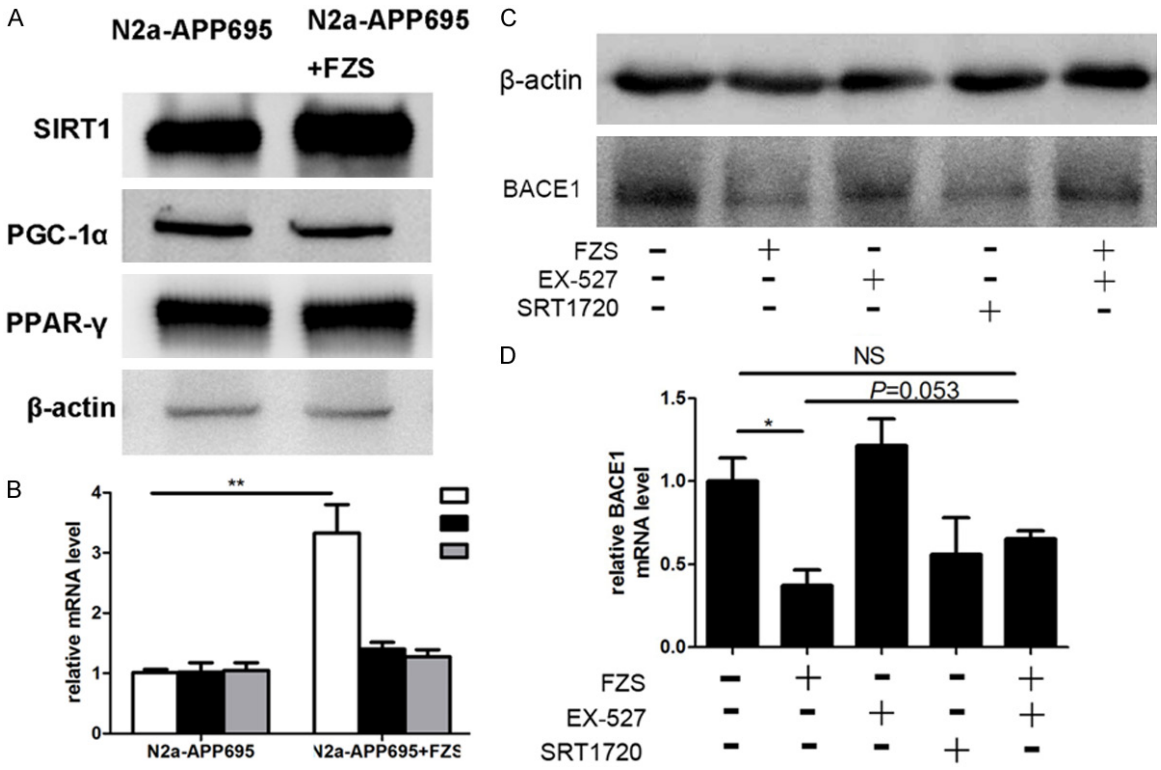

Figure 4. The expression levels of SIRT1, PGC-1α, PPARγ and BACE1 in N2a-APP695 cells. A: The protein expression levels of SIRT1, PGC-1α and PPARγ in the N2a-APP695 cells that were treated with 125 μg/mL FZS compared with the untreated cells. B: The mRNA levels of SIRT1, PGC-1α and PPARγ in the N2a-APP695 cells that were treated with 125 μg/mL FZS (***P*<0.01). C: The expression of BACE1 protein in the N2a-APP695 cells that were treated with 125 μg/mL FZS, 10 μg/mL SRT1720 or 100 μg/mL EX527. D: The expression of BACE1 mRNA in the N2a-APP695 cells that were treated with 125 μg/mL FZS, 10 μg/mL SRT1720 or 100 μg/mL EX527 (**P*<0.05).

ance (ANOVA) followed by Duncan's multiple range test. A *P* value of <0.05 was considered statistically significant, including **P*<0.05, ***P*<0.01 and ****P*<0.001.

Results

FZS increases N2a-APP695 cell viability and reduces apoptosis induced by serum starvation

N2a and N2a-APP695 cells were incubated with FZS at different doses for 24 h. Cell viability was measured using the CCK-8 assay. FZS increased cell survival in a concentration-dependent manner. For the N2a cells, cell survival increased following exposure to 15 μg/mL FZS (*P*<0.01) and plateaued at a concentration of 30 μg/mL (*P*<0.001), and viability peaked at 125 μg/mL (*P*<0.001) (**Figure 1A**). For the N2a-APP695 cells, cell survival increased following exposure to 7.5 μg/mL FZS (*P*<0.05) and plateaued at 15 μg/mL (*P*<0.01), and viability peaked at 125 μg/mL (*P*<0.001) (**Figure 1B**). Therefore, 125 μg/mL of FZS was selected as the optimal concentration for subsequent experiments. To investigate the influence of FZS on apoptosis induced by serum starvation, apoptosis was further assessed in N2a and N2a-APP695 cells using flow cytometry (**Figure 1C**, **1D**). As expected, compared with the control group, treatment with 125 μg/mL FZS reduced the apoptosis induced by serum starvation in the N2a-APP695 cells, and it particularly reduced late apoptosis (Annexin-V^+ PI^+) (*P*<0.01).

Secretion of Aβ1-40 and Aβ1-42 and expression of phosphorylated tau (Ser396) decrease following FZS treatment in N2a-APP695 cells

N2a-APP695 cells were incubated with 125 μg/mL FZS, and the levels of secreted Aβ1-40 and Aβ1-42 were detected using an ELISA assay (**Figure 2A**). Compared with the control group, the extracellular levels of Aβ1-40

(P<0.001) and Aβ1-42 (P<0.001) were significantly decreased following the FZS treatment. Western blotting analysis and immunofluorescence were applied to detect the level of phospho-Tau (Ser396) after treatment with 125 μg/mL FZS. The results (**Figure 2B**, **2C**) indicated that the expression of phospho-Tau (Ser396) was significantly attenuated following the FZS treatment in the N2a-APP695 cells.

Transcription and translation of BACE1 decrease after FZS treatment in N2a-APP695 cells

Western blot analysis was used to examine the expression of BACE1 (**Figure 3A**, **3B**). BACE1 protein expression was increased in N2a-APP695 cells compared with the N2a cells (P<0.05) and was decreased following treatment with 125 μg/mL FZS in the N2a-APP695 cells compared with the FZS-untreated group (P<0.05). We subsequently used qRT-PCR to detect the expression of BACE1 mRNA in N2a-APP695 cells. The result, which was consistent with the Western blot assay, demonstrated that the expression of BACE1 mRNA was reduced after treatment with 125 μg/mL FZS (P<0.01).

SIRT1 is upregulated and is involved in BACE1 expression regulation following FZS treatment in N2a-APP695 cells

We detected the protein expression levels of SIRT1, PGC-1α and PPARγ in N2a-APP695 cells treated with 125 μg/mL FZS. Compared with the control group, protein expression of SIRT1 increased, but those of PGC-1α and PPARγ did not markedly increase (**Figure 4A**). Consistent with the Western blotting results, qRT-PCR indicated that the mRNA level of SIRT1 was upregulated, but those of PGC-1α and PPARγ did not markedly increase following treatment with 125 μg/mL FZS (**Figure 4B**) (P<0.01).

To determine whether SIRT1 is involved in the regulation of BACE1 expression by FZS in N2a-APP695 cells, the cells were exposed to SRT1720 (a SIRT1 agonist) or EX527 (a SIRT1 antagonist) [23]. The Western blotting and qRT-PCR results demonstrated that the mRNA and protein levels of BACE1 decreased after FZS (P<0.05) or SRT1720 pretreatment (**Figure 4C**, **4D**). In contrast, its mRNA and protein levels increased (not significant) after EX527 pretreatment. Moreover, compared with the FZS treatment, the combined application of EX527 and FZS led to antagonistic effects, in which EX527 suppressed the impact of FZS on SIRT1 and increased the expression of BACE1 mRNA (P=0.053) and protein.

Discussion

Some active ingredients of FZS have been used in Chinese traditional medicine for thousands of years to enhance the general wellbeing of individuals and to treat a variety of disorders, especially cerebrovascular diseases [24-26]. Ginsenoside has been shown to decrease neurofibrillary tangle accumulation via the regulation of neprilysin and PKA activities in an AD mouse model and decrease Tau phosphorylation at Thr231, Thr205, and Ser396 in Rg1-feeding APP/PS1 mice [26]. Quan Q *et al*. also have reported that ginsenoside decreases the Aβ1-42 level via the upregulation of PPARγ and IDE expression in the hippocampus in an AD rat model [24]. Moreover, *Scutellaria baicalensis* can mitigate chronic cerebral hypoperfusion and lipopolysaccharide infusion-induced memory impairments [25]. These findings indicate that certain ingredients of FZS act as neuroprotective drugs, especially for the prevention and treatment of AD. Our study has also proven that FZS treatment can significantly increase cell viability and decrease late apoptosis induced by serum starvation to provide neuroprotection.

The metabolic pathways of APP include physiological processing (non-amyloidogenic pathway) and pathological processing (amyloidogenic pathway) [27]. In the non-amyloidogenic pathway, APP is sequentially cleaved by α-secretase and γ-secretase generating the secreted APP derivative sAPPα and a non-toxic 3-kDa peptide. In contrast, in the amyloidogenic pathway, APP is sequentially cleaved by BACE1 and γ-secretase, which generates the secreted APP derivative sAPPβ and Aβ peptides of different lengths [8]. The non-amyloidogenic pathway is believed to play a role in increasing neuronal plasticity, enhancing synaptic signaling and reducing neuronal susceptibility to cellular stress [27]. Thus, increasing the expression of α-secretase could induce the increased generation of neuroprotective sAPPα, whereas increasing β-secretase expression could lead to the increased generation of neurotoxic Aβ

peptide and trigger neuronal dysfunction and Tau phosphorylation [5, 28]. According to previous reports, α-secretase and β-secretase have opposite effects on Aβ generation and are assumed to compete for APP as a substrate [29]. In our study, the expression of BACE1 and phosphorylated Tau (Ser396) and the secretion of Aβ1-40 and Aβ1-42 were decreased after the FZS treatment in the N2a-APP695 cells. According to these results, we assumed that FZS might attenuate the amyloidogenic pathway by decreasing the expression of BACE1.

SIRT1 is a new histone deacetylase that is dependent on the coenzyme nicotinamide adenine dinucleotide (NAD+) and is primarily expressed in the nucleus [30]. It has been shown to function in conjunction with PGC-1α to compensate for neuronal mitochondrial loss [31]. PGC-1α activity is dependent on SIRT1 deacetylation [32], and the Sirt1-PGC-1α transcriptional complex is able to modulate several pathological events in AD, including the reduction of Aβ peptide aggregation and inflammation, the regulation of mitochondrial dynamics and the increase of neuronal energy availability [33]. Wang *et al*. have demonstrated that BACE1 transcription is modulated by PGC-1α *in vitro* and *in vivo*, and the inhibitory effect of PGC-1α is dependent on the activation of PPARγ via SIRT1-mediated deacetylation [9]. In addition, Marwarha *et al*. have demonstrated that the transcription factor NF-κB positively regulates BACE1 transcription, and its activity is tightly regulated by the mammalian sirtuin SIRT1 [14]. In our study, the transcription and translation of BACE1 were decreased after FZS treatment in the N2a-APP695 cells. The expression of SIRT1 protein increased, but the expression of PGC-1α and PPARγ did not markedly increase after FZS treatment in the N2a-APP695 cells. In addition, EX527 suppressed the impact of FZS on SIRT1 and increased the expression of BACE1 mRNA and protein. Thus, the influence of FZS on BACE1 via the upregulation of SIRT1 expression may be not related to the SIRT1-PPARγ-PGC-1α pathway. The detailed molecular mechanism of the effects of FZS on BACE1 need further research.

In summary, the present study is the first to report that FZS may attenuate the amyloidogenic pathway by increasing SIRT1 expression as well as decreasing BACE1 transcription, ultimately conferring neuroprotection. These findings provide new insights on the neuroprotective role of FZS, which represents a potential and highly effective therapeutic agent for AD treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

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