Forsythoside B attenuates memory impairment and neuroinflammation via inhibition on NF- κ B signaling in Alzheimer's disease

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Research

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Abstract

Background

Alzheimer's disease (AD) is a type of progressive neurodegenerative disease related to neuroinflammation. Forsythoside B (FTS·B), a phenylethanoid glycoside isolated from plants, has been reported to exert various pharmacological effects. However, the neuroprotection of FTS·B related to neuroinflammation has not been systemically reported.

Methods

In amyloid precursor protein/presenilin 1 (APP/PS1) mice, behavioral tests including Morris water maze, Y maze and open field experiment were used to examine the cognitive function. Immunohistochemistry were used to analyze amyloid-beta ($A\beta$) deposition, Tau protein phosphorylation, the levels of 4-hydroxynonenal (4-HNE), glial fibrillary acidic protein (GFAP) and ionized calcium binding adapter molecule 1 (Iba1). Remarkably changed proteins related to neuroinflammation were filtered via proteomics and verified via enzyme-linked immunosorbent assay (ELISA) and western blot. Besides, BV-2 cells and HT22 cells were used to confirm the anti-neuroinflammation of FTS·B.

Results

FTS·B counteracted cognitive decline, ameliorated $A\beta$ deposition and Tau protein phosphorylation, attenuated microglia and astrocytes activation in the cortex and/or hippocampus. Furthermore, FTS·B affected vital signaling, particularly by decreasing the activation of JNK-interacting protein 3/C-Jun NH2-terminal kinase (JIP3/JNK) and suppressing WD-repeat and FYVE-domain-containing protein 1/toll-like receptor 3 (WDFY1/TLR3), further suppressing the activation of nuclear factor- κ B (NF- κ B) signaling. In BV-2 and HT22 cells, FTS·B prevented lipopolysaccharide (LPS)-induced neuroinflammation and reduced the microglia-mediated neurotoxicity.

Conclusions

FTS·B counteracted cognitive decline by triggering neurogenesis via signaling associated with inflammation, suggesting the promising therapeutic effects of FTS·B on AD treatment.

[#] The two authors contribute equally to the project

1. Background

In 2018, there were 50 million patients with dementia worldwide, which estimated as a new patient born every 3 seconds [1]. As one kind of neurodegenerative disease, the pathological features of Alzheimer's disease (AD) are the progressive cognitive decline along with extracellular accumulation of aggregated amyloid-beta ($A\beta$) plaques, neurofibrillary tangles composed of hyperphosphorylated Tau protein, serious encephalatrophy and neuroinflammation [2]. According to the previous research, inflammation is a

principal element in AD pathogenesis [3], characterizing by the releases of chemokines and proinflammatory cytokines and the activation of glial cells in central nervous system (CNS) [4]. The activated astrocytes and microglia could generate various proinflammatory factors like interleukin-6 (IL-6) and interleukin-1 beta (IL-1 β), which increased the deposition of A β and the levels of amyloid beta precursor protein (APP) in AD models [5]. Microglia mainly participate in the process of neuroinflammation, and its phagocytosis on surround hyperphosphorylated Tau aggregates and amyloid plaques restrain AD development [6]. Therefore, anti-inflammation has been presented as a potential therapeutic strategy in AD.

Current drugs for treating AD are mainly focused on symptomatic, but failed to aim at the underlying causes. Some new therapies are designed to lessen the generation of the modified protein Tau (or neurofibrillary tangles) or A β peptides (or plaques), which are hopeful in preventing neuronal loss and blocking disease progression. Unfortunately, the severe adverse effects are observed in AD patients [7]. Non-steroidal anti-inflammatory drugs (NSAIDs), the most important drugs for AD treatment widely, exhibits neuroprotection and antioxidation, suppress the production of free radical and the activation of nuclear factor- κ B (NF- κ B) and interleukins in the CNS [8, 9]. However, NSAIDs can only delay neuroinflammatory progression of AD patients, failed to prevent neurodegeneration [10]. Hence, new ways for stopping neuroinflammation are needed to be discovered.

Forsythiae Fructus (Chinese name: Lianqiao), the fruit of *Forsythia suspensa* (Thunb.) Vahl, is used as a traditional drug in Korea, China and Japan [11]. Forsythiae Fructus has been traditionally utilized in the treatment of inflammation and pyrexia [12]. The modern researches have confirmed the neuroprotective [13], anti-inflammatory [14], anti-virus [15], anti-oxidant [16] and anti-bacterial [17] effects of Forsythiae Fructus. The existence of nine phenylethanoid glycosides were found in Forsythiae Fructus in 2009 for the first time, namely Forsythoside B (FTS•B, C₃₄H₄₄O₁₉, chemical structure in Figure S1) [18]. FTS•B alleviates lipopolysaccharide (LPS)-induced acute lung injury via attenuating inflammatory cell infiltration and suppressing the activation of toll-like receptor 4 (TLR4)/NF- κ B signaling [19]. FTS•B increases the levels of IL-10 and suppresses the activation of NF- κ B, which ameliorated excessive inflammatory responses in septic patients [20]. FTS•B can rescue cardiac function via suppression the inflammation response due to its antioxidant properties [21]. Although the anti-inflammatory effect of FTS•B has been fully reported previously, its neuroprotection via modulation the neuroinflammation on AD has not been systematically studied.

In this study, the neuroprotection of FTS•B was confirmed in amyloid precursor protein/presenilin 1 (APP/PS1) mice, BV-2 microglial cells and hippocampal HT22 cells suggesting by its ameliorating AD-like behaviors, improving biochemical indicators, preventing histopathological alternations, and suppressing the inflammatory response by regulating NF- κ B signal pathway. Our study offered experimental evidence for FTS•B as a potential agent in clinical applications of AD treatment, especially or anti-neuroinflammation.

2. Methods

2.1 Cell culture

BV-2 microglial cells (CL-0493) (Procell Life Science & Technology Co., Ltd., Wuhan, China) and HT22 cells (337709) (BeNa Culture Collection, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Procell Life Science & Technology Co., Ltd.), 1% 100 µg/mL streptomycin and 100 units/mL penicillin (Gibco, Grand Island, NY, USA) and maintained at 37 °C in a 5% humidified CO₂ incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

2.2 Cell viability and apoptosis assay

BV-2 cells seeded into 96-well plates were pretreated with 1 µM and 2.5 µM FTS•B or phosphate buffered saline (PBS) for 3 h, and subsequently co-incubated with 1 µg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) (dissolved in DMEM) for 24 h. The cell viability was analyzed using 3-(4, 5-dimethyl-2-thiazolyl) 2, 5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, USA) assay the same as our previous study [22].

In a separate experiment, BV-2 cells were pre-incubated with 1 µM and 2.5 µM FTS•B or PBS at 37 °C for 3 h following with 24-h co-incubation with 1 µg/mL LPS, and then cultured mediated were collected. HT22 cells seeded into 12-well plates were exposed to the collected medium for another 24 h. MTT assay was applied to analyze the HT22 cell viability, and Annexin V/propidium iodide (AV/PI) staining was applied to analyze the HT22 cell apoptosis the same as our previous study [23].

2.3 Experiments performed on AD mice and agent administration protocol

The experimental protocol was carried out under the Ethical Committee of Animal Research of Jilin University (20170301). Twenty-four B6C3-Tg (APP^{swe}PSEN1^{dE9})/Nju double transgenic male mice (Genotype: (App^{swe})T, (Psen1) T) (APP/PS1) (8 months, 40–45 g) and eight wild type male mice (Genotype: (App^{swe})W, (Psen1) W) (WT) (8 months, 40–45 g) were purchased from Nanjing Biomedical Research Institute of Nanjing University (NBRI), Jiangsu, China (SCXK (SU) 2015-0001), which were maintained at standard conditions (temperature 23 ± 1 °C and humidity 40–60%) with a 12 h light/dark cycle and food available *ad libitum*.

After a week of adjustable feeding, APP/PS1 mice were randomly divided into three groups (n = 8/group) and orally administered with 10 mg/kg and 40 mg/kg FTS•B and 5 mL/kg normal saline (model group) once a day for 4 weeks. A total of 8 WT mice orally administered with 5 mL/kg normal saline for 4 weeks served as the control group. After the last behavioral test, the mice were euthanized (by injection with 150 mg/kg 1.5% pentobarbital). The serum together with tissues including brain, liver, spleen and kidney were collected for biochemical and pathological analysis. The process of the drug administration and behavioral tests was shown in Figure S2.

2.4 Behavioral tests

2.4.1 Morris water maze test

Brain function related to spatial learning and memory of AD mice was assessed by Morris water maze (MT-200, Chengdu, China) through Water Labyrinth Video Tracking Analysis System (S7200, Chengdu Techman Software Co., Ltd., Chengdu, China). A circular pool filled with water containing milk with a platform placed 1 cm below the surface of water was used for training and experiment at 22–24 °C. On the 29th day, mice were trained 5 minutes/day for 4 days. On the 33rd day, mice were placed to the same quadrant of the pool, the escape latency of mice to locate the hidden platform was recorded within 60 s.

2.4.2 Y maze test

The spontaneous alternation test was assessed in Y maze with three symmetrical arms (300 × 200 mm) at 120° angles. The movements of mice were recorded by the video camera mounted above the maze. On the 34th day, mice were put into one distal end of arm to find the food on the other distal end of arm for 5 minutes (set as the maximum time by system). On the 35th day, the time for finding food and the movement locus of mice were recorded by the video camera.

2.4.3 Open field experiment Test

Open field experiment test was performed in a soundproof dark box (bottom area: 30 × 30 cm, central region: 15 × 15 cm) to observe autonomous behaviors of mice in the new environment. On the 36th day, mice were placed in fixed central location and allowed to start free environmental exploration for 5 min. The moving track and the latency time of mice in the center and surrounding areas were recorded by the instrument. The box was wiped off after completing the experiment to avoid the remaining odors and dirt interfere with the results of next test.

2.5 Biochemical criteria detection

BV-2 cells were pretreated with FTS•B at doses of 1 μM and 2.5 μM for 3 h, and then co-exposed to 1 μg/mL LPS for another 24 h. The levels of IL-6 (KT2163-A), tumor necrosis factor-α (TNF-α) (KT2132-A), inducible nitric oxide synthase (iNOS) (KT2454-A), nitric oxide (NO) (MM-0658M1) and IL-1β (KT2040-A) in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Kete Biotechnology Co., Ltd, Jiangsu, China) according to the manufacturer instructions.

Hypothalamus obtained from APP/PS1 mice were homogenized in ice-cold PBS, and the protein concentration was detected via the Pierce™ BCA protein assay kit (23227) (Thermo Fisher Scientific, USA). The levels of toll-like receptor 3 (TLR3; cat. no. KT9375-A), interferon regulating factor 3 (IRF3) (KT9374-A), p-IRF3 (MM-50143M1), interferon-beta (IFN-β) (KT2124-A), JNK-interacting protein 3 (JIP3) (KT9377-A), C-Jun NH2-terminal kinase (JNK) (KT9182-A), p-JNK (KT9372-A), APP (KT2824-A), p-APP (KT9378-A), Aβ (KT9256-A), TNF-α (KT2132-A), IL-1β (KT2040-A), IL-6 (KT2163-A), interleukin-8 (IL-8) (KT2123-A) and interleukin-12 (IL-12) (KT2105-A) in the serum and hypothalamus were detected by ELISA kits according to the manufacturer instructions (Jiangsu Kete Biotechnology Co., Ltd, Jiangsu, China).

2.6 Hematoxylin and eosin (H&E) staining, Thioflavin S staining and Immunohistochemistry examination

The same as our previous study [24], 4% paraformaldehyde-fixed liver, spleen, kidney and brain tissues were dehydrated with alcohol, embedded with paraffin and cut into 5- μ m thick standard sections. Specimens were stained with hematoxylin and eosin (H&E) and assessed using inverted microscope CKX41 (Olympus, Japan).

Thioflavin S staining was used to visualize fibrillar A β deposits. After permeabilization in xylene for 10 min and rehydration in anhydrous ethanol for 5 min, brain slices were incubated with 1% thioflavin S in dark for 30 min, then differentiated in 70% ethanol and washed with distilled water. After fixing with 50% glycerin in PBS, slices were observed using fluorescent microscope (magnification $\times$ 100) (Olympus Corporation, Tokyo, Japan).

The brain sections were prepared as our previous study [25]. The sections were incubated with the primary antibody against A β (Dilution ratio 1:1000) (ab2539), p-Tau (phospho S396) (Dilution ratio 1:4000) (ab109390) and 4-hydroxynonenal (4-HNE) (Dilution ratio 1:200) (ab46545) (Abcam, Cambridge, MA, USA), glial fibrillary acidic protein (GFAP) (Dilution ratio 1:200) (bs-0199R) and ionized calcium binding adapter molecule 1 (Iba1) (Dilution ratio 1:200) (bs-1363R) (Bioss Inc., Beijing, China) at 4 °C overnight. The following day, sections were washed with PBS, incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (sc-3836) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 25 °C for 1 h, and incubated with streptavidin-organism horseradish peroxidase (HRP) complex (Shanghai BestBio Science, Shanghai, China) 25 °C for another 1 h. 5% diaminobenzidine tetrahydrochloride solution and hematoxylin were added to slices to counterstain (25 °C for 5 min). Photomicrographs were obtained via inverted fluorescent microscope (magnification $\times$ 40 and $\times$ 200) (Olympus Corporation, Tokyo, Japan).

2.7 Proteomics

2.7.1 Protein extraction and digestion

Samples were homogenized in radio immunoprecipitation assay (RIPA) buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 2% phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA) on ice for 30 min. After centrifugation, BCA protein assay kit was used to quantify protein concentrations of the supernate. Subsequent to acetone precipitation, re-suspend protein for tryptic digest, and cleaning up of sodium deoxycholate (SDC), peptide samples were obtained. Peptides were desalted by reversed phase chromatography (RPC-HPLC) using C₁₈ column. After desalination, peptides were dried and resuspended in buffer containing 0.1% formic acid (FA) and 2% acetonitrile (ACN).

2.7.2 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

1 µg of peptide were separated and analyzed via an Easy-nLC1200 nano-UPLC (Thermo Scientific, Waltham, MA) connected to a Q-Exactive mass spectrometry (Thermo Scientific, Waltham, MA). Separation was performed using a reversed phase column (100 µm, ID × 15 cm, Reprosil-Pur 120 C18-AQ, 1.9 µm, Dr. Math). A non-linear gradient was established using phase A (0.1% FA and 2% ACN in water) and phase B (0.1% FA and 80% ACN in water) to elute the peptides with a 120 min gradient at 300 nL/min flow rate. The gradient started with 8% phase B and increased to 35% during 92 min. In the next 20 min, phase B increased to 45% and finally, it augmented to 100% in 2 min and it was kept at 100% for 2 min. Data dependent acquisition was performed in profile and positive mode with Orbitrap analyzer at a resolution of 70,000 (@200 m/z) and m/z range of 350–1600 for MS1; For MS2, the resolution was set to 17,500 with a dynamic first mass. The automatic gain control (AGC) target for MS1 was set to 3.0×10^6 with max IT 50 ms, and for MS2 was set to 5.0×10^4 with max IT 100 ms. The top 20 most intense ions were fragmented by Higher Energy Collisional Dissociation (HCD) with normalized collision energy (NCE) of 27%, and isolation window of 2 m/z. The dynamic exclusion time window was 30 s.

2.7.3 MaxQuant analysis and LFQ quantification

Raw MS files generated on the mass spectrometer were processed with MaxQuant (Version 1.5.6.0). The protein sequence database (Uniprot_organism_2016_09) was downloaded from Uniprot. This database and its reverse decoy were then searched against via MaxQuant software. The quantification type was label-free quantification (LFQ) with match between run and intensity-based absolute quantification (iBAQ); Trypsin was set as specific enzyme with up to 3 miss cleavage; Oxidation [M] and Acetyl [protein N-term] were considered as variable modification (max number of modifications per peptide is 3), Carbamidomethyl [C] was set as fixed modification; Both peptide and protein FDR should be less than 0.01. Only unique and razor peptides were used for quantification. All the other parameters were reserved as default.

2.7.4 Bioinformatics and statistical analysis

Statistical analysis was performed on the standardized quantitative results to obtain the corresponding differentially expressed proteins. The experiment contained two biological replicates, and the arithmetic mean was used to calculate the fold change. The proteins with fold change expression (ratio A/B > 1.5 or ratio A/B < 0.66) were defined as significant differences, and subsequent GO, KEGG pathway, and protein interaction analysis were performed.

2.8 Western Blot

One part of the hippocampus tissues obtained from the experimental mice were lysed with RIPA buffer containing 1% protease inhibitor cocktail and 2% PMSF. BCA protein assay kit was used to quantify protein concentration. Subsequent to the separation of 40 µg lysates by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to polyvinylidene difluoride membrane (PVDF) (0.45 µm; Merck Millipore, Billerica, MA, USA). 5% bovine serum albumin (BSA) was used to block the membranes at 4 °C for 2 h. The membranes subsequently incubated with primary antibodies including JIP3 (Dilution ratio 1:500) (147 kDa) (bs-13648R), JNK (Dilution ratio 1:1000)

(48 kDa) (bs-2592R), Amyloid Precursor Protein (APP) (Dilution ratio 1:1000) (86 kDa) (bs-0112R), inhibitor of nuclear factor kappa-B kinase alpha + beta (IKK α + β) (Dilution ratio 1:500) (85 kDa) (bs-7557R), Interferon regulatory Factor 3 (IRF3) (Dilution ratio 1:1000) (47 kDa) (bs-52116R), p-IRF3 (Ser396) (Dilution ratio 1:500) (47 kDa) (bs-3195R), Iba1 (Dilution ratio 1:500) (16 kDa) (bs-1363R) and GFAP (Dilution ratio 1:500) (48 kDa) (bs-0199R) (Bioss Inc., Beijing, China), p-JNK1 + 2 + 3 (phosphor Y185 + Y185 + Y223) (Dilution ratio 1:10000) (48 kDa) (ab76572), p-IKK(α + β) (phosphor S176 + S177) (Dilution ratio 1:500) (85 kDa) (ab194528), p-APP (phosphor T743) (Dilution ratio 1:1000) (86 kDa) (ab206297), ELKS (Dilution ratio 1:5000) (128 kDa) (ab180507), inhibitor of nuclear factor kappa-B alpha (I κ B α) (Dilution ratio 1:10000) (35 kDa) (ab32518), p-I κ B α (phosphor S36) (Dilution ratio 1:10000) (35 kDa) (ab133462), nuclear factor kappa-B p65 (NF- κ B p65) (Dilution ratio 1:500) (65 kDa) (ab7970), and p-P65NF- κ B (phosphor S536) (Dilution ratio 1:10000) (65 kDa) (ab76302), WD-repeat and FYVE-domain-containing protein 1 (WDFY1) (Dilution ratio 1:1000) (46 kDa) (ab125329), and Toll-like receptor 3 (TLR3) (Dilution ratio 1:500) (108 kDa) (ab13915), and the reference protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Dilution ratio 1:500) (36 kDa) (ab181602) (Abcam, Cambridge, MA, USA) at 4 °C overnight. After washing with TBST (composes of 500 ml t-butyldimethylsilyl and 0.5 ml Tween-20), the membranes then incubated with HRP-conjugated secondary antibody (SH-0032; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) at a dilution of 1:2000 at 4 °C for 4 h. An enhanced chemiluminescence (ECL) kit (Merck Millipore, Billerica, MA) and imaging system (Bio Spectrum 600, UVP company, Upland, CA, USA) were used to visualize bands, and ImageJ software (National Institutes of Health, Bethesda, MD, USA) was applied to quantify the blots.

2.9 Statistical Analysis

All data were presented as the mean $\pm$ S.D. One-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons (Holm-Sidak test) was used to analyze differences and significance via SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The statistically significant difference was declared for $p < 0.05$.

3. Results

3.1 FTS·B counteracted cognitive decline and ameliorated pathologic alternations in APP/PS1 mice

Twenty-eight days' FTS·B administration showed no significant effects on the body weights (Table S1), organ structures including liver, spleen, kidney and brain of APP/PS1 mice (Figure S3), suggesting the safety using of FTS·B.

Morris water maze test has been widely used to evaluate learning and memory capabilities in animals [26]. Compared with vehicle-treated APP/PS1 mice, FTS·B-treated AD mice exhibited less chaotic movements (Fig. 1A), less escape latency time ($p < 0.05$) (Fig. 1B) and more times of crossing platform ($p < 0.05$) (Fig. 1C). Y maze test is usually used to examine the short-term memories of mice [27]. In Y maze test, the blue dot meant mice, the red dot meant food, and the purple line meant the movement tracks of

mouse. No visible difference on the training latency time among all experimental groups were noted ($p > 0.05$) (Fig. 1E). FTS-B suppressed the spontaneous alternation behaviors of the APP/PS1 mice (Fig. 1D), and helped the mice to find the food using less time in the formal test compared with vehicle-treated APP/PS1 mice ($p < 0.05$) (Fig. 1F). Autonomous exploration behaviors in new environment of mice were evaluated by open field experiment [28]. In the open field test, the blue dot meant the initial spot of mice, the red dot meant the terminal spot of mice, and the purple line meant the movement tracks of mouse. Compared with vehicle-treated APP/PS1 mice, FTS-B administration significantly reduced the aimless motions around the central field area (Fig. 1G), increased the distance in the surrounding area ($p < 0.01$) (Fig. 1H) and reduced the distance in the center area ($p < 0.05$) (Fig. 1I) of APP/PS1 mice. All data suggested that FTS-B strongly ameliorated cognitive decline in APP/PS1 mice.

The over-accumulation of fibrillary amyloid plaques of A β in the hippocampus and cerebral cortex were noted in vehicle-treated APP/PS1 mice, which were reduced in FTS-B-treated AD mice analyzing via Thioflavine S staining (Fig. 2A). In the immunohistochemistry detection, the area covered by A β positive plaques in the hippocampus of APP/PS1 mice were markedly higher than WT mice, which were strongly suppressed by FTS-B administration (Fig. 2B). As a microtubule-associated protein, Tau can keep the stabilization of the neuronal cytoskeleton and its hyperphosphorylation can lead to the formation of toxic neurofibrillary tangles in AD [29]. Excessive aggregations of p-Tau (Fig. 2C) and high expressions of 4-HNE (Fig. 2D) in hippocampus of vehicle-treated APP/PS1 mice were discovered compared to WT mice, which were all strongly attenuated after four-week FTS-B administration. The existence of activated microglia and astrocytes around A β plaques is a representative characteristic of the AD brain, and is conducive to the inflammatory process of brain damage [30]. Immunohistochemistry of Iba1 and GFAP in hippocampus were used to assess the activation of microglia and astrocytes. The increased GFAP positive cells noted in hippocampus of APP/PS1 mice were remarkably attenuated after FTS-B treatment (Fig. 2E). FTS-B also reduced the enrichment of Iba1 around blood vessels (Fig. 2F).

3.2 FTS-B regulated cytokines levels in serum and brain of APP/PS1 mice

In order to analyze the specific effects of FTS-B in AD, proteomic analysis was performed on the hippocampus collected from each group. Among all the detected proteins, 23 obvious changed proteins related to AD process were successfully identified (Table S2). Clustering heatmap of the significant proteins showed that FTS-B up-regulated 16 proteins and down-regulated 7 proteins in hippocampus of APP/PS1 mice (Fig. 3A). According to the protein-protein interaction analysis, 116 interactions and 49 expected interactions were found (Fig. 3B).

Based on the screening data of proteomic analysis, the remarkably changed cytokines were confirmed using ELISA. The over-expression of WDFY1, a potentiator of the TLR3 signaling, can lead to the elevation on phosphorylation of IRF3, as well as IFN- β gene expression [31]. Compared with vehicle-treated APP/PS1 mice, FTS-B treatment, especially at 40 mg/kg, resulted in 37.3% ($p < 0.01$), 12.5% ($p < 0.05$) and 76.1% ($p < 0.001$) increment on serum levels of TLR3, p-IRF3/IRF3 and IFN- β (Table 1), and 24.7% ($p <$

0.05), 150% ($p < 0.001$) and 52.6% ($p < 0.05$) increment on cerebral levels of TLR3, p-IRF3/IRF3 and IFN- β (Table 2). JNK takes charge of the phosphorylation of APP at Thr668 in differentiated neurons, which can generate A β peptide after two endoproteolytic cleavages [32]. The overproduction of JIP3, p-JNK/JNK, p-APP/APP and A β noted in the serum and brain lysis of APP/PS1 mice ($p < 0.05$) were significantly reversed by 4-week FTS-B administration ($p < 0.05$) (Tables 1 and 2). The activated neuroglia stimulated by A β can increase expressions of proinflammatory chemokines such as TNF- α and ILs, which might lead to increasing A β production and neuronal death [33, 34]. Compared with vehicle-treated APP/PS1 mice, FTS-B strongly reduced the hyper-levels of f TNF- α ($p < 0.01$), IL-1 β ($p < 0.05$), IL-6 ($p < 0.01$), IL-8 ($p < 0.05$) and IL-12 ($p < 0.05$) in the serum and brain of the APP/PS1 mice (Tables 1 and 2).

Table 1
The effects of FTS-B on cytokines levels in the serum of APP / PS1 mice.

	WT	APP/PS1		
		FTS-B (mg/kg)		
	10	40		
TLR3 (ng/mL)	29.6 $\pm$ 4.5	21.7 $\pm$ 5.3##	27.3 $\pm$ 4.8*	29.8 $\pm$ 3.3**
p-IRF3/IRF3	1.2 $\pm$ 0.2	0.8 $\pm$ 0.1##	0.9 $\pm$ 0.1*	0.9 $\pm$ 0.1*
IFN- β (pg/mL)	95.4 $\pm$ 19.7	51.5 $\pm$ 20.3###	89.8 $\pm$ 43.7*	90.7 $\pm$ 17.9***
JIP3 (ng/L)	233.9 $\pm$ 117.9	379 $\pm$ 101.7##	274.7 $\pm$ 161.2	219.9 $\pm$ 115.6**
p-JNK/JNK	5.3 $\pm$ 1.7	8.4 $\pm$ 2.8##	5.3 $\pm$ 2.3*	5.4 $\pm$ 1.8*
p-APP/APP	0.2 $\pm$ 0.1	0.4 $\pm$ 0.1##	0.2 $\pm$ 0.2**	0.3 $\pm$ 0.1**
A β (mg/L)	2.5 $\pm$ 0.5	3 $\pm$ 0.4#	2.4 $\pm$ 0.6**	2.3 $\pm$ 0.5**
TNF- α (ng/L)	132.6 $\pm$ 37.3	189.7 $\pm$ 64.1#	101.5 $\pm$ 71.6**	103 $\pm$ 50.2**
IL-1 β (ng/L)	91 $\pm$ 17.1	124.9 $\pm$ 25.1#	95.9 $\pm$ 13.8**	87.8 $\pm$ 21.4**
IL-6 (pg/mL)	193.1 $\pm$ 25.3	223.7 $\pm$ 22.2#	190.2 $\pm$ 21.3**	177.9 $\pm$ 33.7**
IL-8 (pg/mL)	367.3 $\pm$ 65.6	471.8 $\pm$ 64.8##	364.1 $\pm$ 107*	310.3 $\pm$ 64.9***
IL-12 (ng/L)	58.6 $\pm$ 18.4	82.3 $\pm$ 11.2##	69.5 $\pm$ 10*	67.2 $\pm$ 8.2*
Data are expressed as the mean $\pm$ S.D. (n = 8). # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. WT mice. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. APP/PS1 mice.				

Table 2
The effects of FTS·B on cytokines levels in the brain of APP / PS1 mice.

	WT	APP/PS1		
		FTS·B (mg/kg)		
10	40			
TLR3 (ug/g prot)	30.7 ± 8.2	22.7 ± 5.4###	27.6 ± 7.9*	28.3 ± 6.5*
p-IRF3/IRF3	0.2 ± 0.1	0.2 ± 0.0#	0.5 ± 0.3*	0.5 ± 0.3***
IFN-β (ng/g prot)	30.7 ± 10.4	17.3 ± 7.1#	23.4 ± 11.5	26.4 ± 5.5*
JIP3 (ng/g prot)	81.4 ± 48	171.8 ± 99.5#	59.6 ± 60.4*	53.8 ± 29.9**
p-JNK/JNK	7.1 ± 1.8	10 ± 2.8#	5.6 ± 3*	5.7 ± 2.1**
p-APP/APP	0.3 ± 0.1	0.6 ± 0.3#	0.2 ± 0.2**	0.2 ± 0.1**
Aβ (mg/g prot)	1.1 ± 0.2	1.5 ± 0.2###	0.9 ± 0.3***	1.2 ± 0.2**
TNF-α (ng/g prot)	2.1 ± 0.2	2.3 ± 0.2#	1.7 ± 0.4***	1.8 ± 0.3**
IL-1β (ng/g prot)	68.4 ± 22.9	113.6 ± 37.1##	82.5 ± 30.5*	75.4 ± 18.1**
IL-6 (ng/g prot)	176.7 ± 12.3	215.7 ± 23.3###	171.2 ± 23.8***	170.4 ± 27.6**
IL-8 (ng/g prot)	96 ± 42.7	177.5 ± 79.5#	96.2 ± 52.3*	105.5 ± 25.9
IL-12 (ng/g prot)	37.6 ± 8.5	56 ± 14.9#	29.7 ± 15.8**	29 ± 11.8**
Data are expressed as the mean ± S.D. (n = 8). # <i>p</i> < 0.05, ## <i>p</i> < 0.01 and ### <i>p</i> < 0.001 vs. WT mice. * <i>p</i> < 0.05, ** <i>p</i> < 0.01 and *** <i>p</i> < 0.001 vs. APP/PS1 mice.				

3.3 FTS·B regulated the proteins expression and activation related to neuroinflammation in the hippocampus of APP/PS1 mice

Compared with the APP/PS1 mice, FTS·B visibly downregulated the expressions of JIP3, p-JNK(1 + 2 + 3) and p-APP in the hippocampus (Fig. 4A). ELKS can combine with IKK complex and involve in the activation of NF-κB [35]. FTS·B administration suppressed the levels of ELKS, p-IKK(α + β), p-IκBa and p-NFκB (Ser536) (Fig. 4B), and enhanced the levels of WDFY1, TLR3 and p-IRF3 in the hippocampus of APP/PS1 mice (Fig. 4C). Furthermore, compared with the APP/PS1 mice, FTS·B downregulated over 51.4% and over 24.6% levels of Iba1 and GFAP in hippocampus, respectively (Fig. 4D).

3.4 FTS·B prevented LPS-induced neuroinflammation in BV-2 cells

FTS·B alone (1 μ M and 2.5 μ M) or co-incubation with LPS (1 μ g/mL) showed no significant effects on cell viabilities of BV-2 cells (Fig. 5A). The LPS-caused enhancement on the levels of IL-6 ($p < 0.01$) (Fig. 5B), TNF- α ($p < 0.01$) (Fig. 5C), iNOs ($p < 0.05$) (Fig. 5D), NO ($p < 0.05$) (Fig. 5E) and IL-1 β ($p < 0.01$) (Fig. 5F) in BV-2 cells were strongly suppressed by FTS·B pre-incubation ($p < 0.05$) (Fig. 5B-F).

3.5 FTS·B reduced the microglia-mediated neurotoxicity

The activated microglia exerts neurotoxicity effects by releasing proinflammatory enzymes and mediators [36]. When conditioned media from LPS-stimulated microglia was added to cultured HT22 cells, the apoptosis rate of HT22 cells was significantly increased ($p < 0.001$) (Fig. 6A and B) and cell viability was significantly reduced ($p < 0.001$) (Fig. 6C). However, pretreatment of BV2 cells with FTS·B prior to LPS stimulation significantly suppressed the apoptosis rate of HT22 cells ($p < 0.001$) (Fig. 6A and B) and enhanced cell viability ($p < 0.05$) (Fig. 6C), demonstrating the neuroprotective effects of FTS·B.

4. Discussion

APP/PS1 double transgenic mice with AD imitate human progressive cognitive deficits and neuropathological characteristics genetically such as chronic A β accumulation, inflammatory development and deteriorate with age [37]. Tauopathy in the form of neuritic plaque and cerebral β -amyloidosis in the form of A β plaques are two pathological features of AD [38]. Neurofibrillary tangles consist of intracellular bundles of self-assembled p-tau, induced by A β deposition, thus leading to the neuronal degeneration [39]. Neurofibrillary tangles are conducive to neuroinflammation due to the activated NF- κ B signaling and increased pro-inflammatory cytokines levels via misfolded tau [40]. 4-HNE, a marker of oxidative stress, exists with a higher concentration in AD patients [41], which triggers A β aggregation via accelerating A β protofibrils formation and inhibiting straight and mature fibrils production. Studies about A β protofibrils and oligomers in the process of neurotoxicity which eventually results in AD indicate that A β aggregation induced by 4-HNE is crucial to AD pathogenesis [42]. A β aggregation active microglia and astrocytes and lead to central neuroinflammation response via the production of neurotoxic mediators and pro-inflammatory cytokines. Neuroinflammation is relevant with increased incidence of AD and cognitive dysfunction, leading to the behavioral defects and neuron loss [43]. In this study, FTS·B significantly enhanced the learning and memory capabilities of APP/PS1 mice evaluating via behaviors tests; additionally, FTS·B obviously suppressed the deposition of A β , the formation of neurofibrillary tangles composed of p-Tau protein and the levels of 4-HNE. Represent as the brain's defenders, astrocytes and microglia are conducive to the clearance of A β toxic clusters in the absence of the external invader situation. Microglia secretes various protective factors and benefits to cell survival. Astrocytes confer protective effect on the formation of a barrier between neurons and A β [43]. The chronic neuroinflammation in the AD microenvironment results in neuronal compromise via inhibition of microglial A β clearance and increased neurotoxic factors [44]. Upregulated levels of inflammatory cytokines lead to the deterioration of brain injury [45], and pro-inflammatory factors and TNF- α secreted by activated microglia can increase tauopathy and A β deposition over short periods [46]. Iba1 and GFAP are recognized as specific biomarkers of microglia and astrocytes, respectively [47]. FTS·B

suppressed the expression of GFAP and reduced the enrichment of Iba1 around blood vessels in the hippocampus, and reduced the levels pro-inflammatory cytokines in serum and hippocampus of APP/PS1 mice. Based on the data obtained in APP/PS1 mice, FTS-B-mediated neuroprotection against AD-like behaviors may be related to its anti-neuroinflammation.

The activation of microglia cells induced by LPS, categorized as an endotoxin, increased neurotoxicity by generating proinflammatory and cytotoxic factors such as NF- κ B [48]. LPS-induced BV-2 microglial cell is used as a classic inflammatory model to evaluate anti-neuroinflammatory effects of potential therapeutic candidates [49]. FTS-B significantly suppressed the production of pro-inflammation cytokines in BV-2 cells stimulated by LPS, confirming its anti-inflammatory properties. For revealing the neuroinflammation of microglial response is responsible for the neuron damage, HT22 cells were incubated with secondary metabolite obtained from BV-2 cells stimulated by LPS and/or FTS-B. Our data support that FTS-B can inhibit the neuroinflammation caused by LPS in BV-2 cells, and further reduce damage on HT22 cells. These data confirmed our *in vivo* findings that FTS-B shows anti-AD property via its regulation neuroinflammation.

In hippocampus of APP/PS1 mice, FTS-B regulated the expression and activation of proteins related to neuroinflammation. Upregulated expression of JNK exists in AD patients, which promotes APP amyloidogenic cleavage and amyloid plaques formation [50]. Transport activated JNK3 is known to be retrograded by JIP3, a peripheral membrane protein of JIP family, from the nerve damage site [51, 52]. MAPK pathways, represented by JNK and p38, can induce transcription factor NF- κ B [53], which is a transcription factor controlling expressions of various cytokines involved in inflammatory process, which usually recognized as a main regulator of inflammation [54]. NF- κ B complex combines with inhibitor proteins like I κ B α and exists in the cytoplasm. ELKS, a vital IKK regulatory subunit, recruits I κ B α to the IKK complex and in turn regulates IKK activation [35]. Plenty of extracellular signals such as LPS and TNF α can induce the activation of NF- κ B via cytoplasmic IKK complex. Activated IKK cause I κ B phosphorylation and subsequently degradation mediated by ubiquitin-proteasome, which releases NF- κ B to the nucleus [55]. After translocating into the nucleus, the activated NF- κ B dimer binds to specific DNA sequences and mediates transcription of inflammatory genes [56]. Microglial NF- κ B is activated by a series of inflammatory conditions to control the production of pro-inflammatory cytokines such as IL-6, IL-1 β and TNF α [57]. FTS-B decreased the releases of pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β) and reduced the levels of iNO $_2$ and NO in LPS-exposed BV-2 cells, indicating its protective effect on neuroinflammation. Furthermore, WDFY1 overexpression enhances TLR3-mediated NF- κ B activation, and IRF3 phosphorylation, which induces productions of IFNs and various proinflammatory cytokines [58]. Altogether, NF- κ B signaling may be involved in FTS-B-mediated neuroprotection via anti-neuroinflammation in APP/PS1 mice.

5. Conclusions

In summary, we first systematically reported that FTS-B counteracted cognitive decline by triggering neurogenesis via ameliorating A β deposition and Tau protein phosphorylation, and attenuating microglia

and astrocytes activation. These effects were related to anti-neuroinflammation via modulation NF- κ B signaling pathway.

Abbreviations

3-(4, 5-dimethyl-2-thiazolyl) 2, 5-diphenyl-2H-tetrazolium bromide assay (MTT); 4-hydroxynonenal (4-HNE); Acetonitrile (ACN); Alzheimer's disease (AD); Amyloid-beta ($A\beta$); Amyloid-beta precursor protein (APP); Amyloid precursor protein/presenilin 1 (APP/PS1); Automatic gain control (AGC); Bovine serum albumin (BSA); Central nervous system (CNS); C-Jun NH2-terminal kinase (JNK); Conditioned media (CM); Dulbecco's modified Eagle's medium (DMEM); Enhanced chemiluminescence (ECL); Enzyme-linked immunosorbent assay (ELISA); Fetal bovine serum (FBS); Formic acid (FA); Forsythoside B (FTS•B); Glial fibrillary acidic protein (GFAP); Hematoxylin and eosin (H&E); Higher Energy Collisional Dissociation (HCD); Horseradish peroxidase (HRP); Inhibitor of nuclear factor kappa-B alpha ($I\kappa B\alpha$); Inducible nitric oxide synthase (iNO_s); Inhibitor of nuclear factor kappa-B kinase alpha ($IKK\alpha$); Inhibitor of nuclear factor kappa-B kinase beta ($IKK\beta$); Intensity-based absolute quantification (iBAQ); Interferon-beta ($IFN-\beta$); Interferon regulating factor 3 (IRF3); Interleukin-1 beta ($IL-1\beta$); Interleukin-6 (IL-6); Interleukin-8 (IL-8); Interleukin-12 (IL-12); Ionized calcium binding adapter molecule 1 ($Iba1$); JNK-interacting protein 3 (JIP3); Label-free quantification (LFQ); Lipopolysaccharide (LPS); Nitric oxide (NO); Non-steroidal anti-inflammatory drugs (NSAIDs); Normalized collision energy (NCE); Nuclear factor- κ B (NF- κ B); One-way analysis of variance (ANOVA); Phenylmethanesulfonyl fluoride (PMSF); Polyvinylidene difluoride membrane (PVDF); Phosphate buffered saline (PBS); Radio immunoprecipitation assay (RIPA); Reference protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Reversed phase chromatography (RPC-HPLC); Sodium deoxycholate (SDC); Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); Toll-like receptor 3 (TLR3); Toll-like receptor 4 (TLR4); Tumor necrosis factor-alpha ($TNF-\alpha$); WD-repeat and FYVE-domain-containing protein 1 (WDFY1).

Declarations

Ethics approval and consent to participate

Institution Animal Ethics Committee of Jilin University approved the experimental protocol (No. SY201904011).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

DW and YZ designed the experiments. FK, XJ, RW and SZ performed the experiments. FK and XJ processed data. FK and XJ wrote the paper. DW and YZ revised the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

FTS·B counteracted cognitive decline in APP/PS1 mice. (A) In Morris water maze test, the path tracings on the testing day were automatically recorded. FTS·B reduced (B) the escape latency for four testing days, and (C) the times of crossing platform on the testing day of APP/PS1 mice in Morris water maze test. (D) The path tracings were automatically recorded in Y maze test. (E) FTS·B failed to influence the training latency, (F) but strongly reduced the testing latency in the Y maze test. (G) The path tracings in the open field test were automatically recorded. (H) FTS·B increased the distance in the surrounding area, (I) and reduced the distance in the center area of APP/PS1 mice in the open field test. Data are expressed as the mean $\pm$ S.D. (n=8). #p<0.05, ##p<0.01 and ###p<0.001 vs. WT mice. *p<0.05 and **p<0.01 vs. APP/PS1 mice. FTS·B: Forsythoside B; WT: Wild type.

Figure 2

FTS•B ameliorated pathologic alternations in brains of APP/PS1 mice. FTS•B alleviated the deposition of A β in brains of APP/PS1 mice detecting via (A) Thioflavine S staining (in hippocampus and cortex) (100 $\times$; Scale bar: 100 μ m) (n=3) and (B) immunohistochemistry (in hippocampus) (40 $\times$; Scale bar: 200 μ m) (200 $\times$; Scale bar: 50 μ m) (n=3). FTS•B reduced the levels of (C) phosphor-Tau protein (n=3), (D) 4-HNE (n=3), (E) GFAP (n=3) and (F) Iba1 (n=3) in the hippocampus of APP/PS1 mice. (40 $\times$; Scale bar: 200

μm) (200×; Scale bar: 50 μm). FTS•B: Forsythoside B; 4-HNE: 4-hydroxynonenal; GFAP: Glial fibrillary acidic protein; Iba1: Ionized calcium binding adapter molecule 1.

Figure 3

Label-free proteomics performed to evaluate the protein changes caused by FTS•B administration in hippocampus of APP/PS1 mice. (A) The heatmap showed alternations on the cellular protein profiles in the hippocampus between WT and APP/PS1 mice with or without FTS•B administration. (B) The protein interaction image showed that significantly up- or down-regulated proteins may be closely related to inflammation. FTS•B: Forsythoside B; WT: Wild type.

Figure 4

The regulation of FTS·B on proteins related to neuroinflammation in hippocampus of APP/PS1 mice. (A) FTS·B reduced the expression levels of JIP3, and the phosphorylation levels of JNK and APP. (B) FTS·B suppressed the expression levels of ELKS, and the phosphorylation levels of IKK(α+β), IκBα and NF-κB p65. (C) FTS·B enhanced the expression levels of WDFY1 and TLR3, and the phosphorylation levels of IRF3. (D) FTS·B reduced the expression levels of Iba1 and GFAP. Quantification data were normalized by GAPDH and corresponding total proteins (n=3), and reported as the folds of those from corresponding WT group. FTS·B: Forsythoside B; JIP3: JNK-interacting protein 3; JNK: C-Jun NH2-terminal kinase; APP: Amyloid-beta precursor protein; IKK: Inhibitor of nuclear factor kappa-B kinase; IκBα: Inhibitor of nuclear factor kappa-B alpha; NF-κB: Nuclear factor-κB; WDFY1: WD-repeat and FYVE-domain-containing protein 1; TLR3: Toll-like receptor 3; IRF3: Interferon regulating factor 3; Iba1: Ionized calcium binding adapter molecule 1; GFAP: Glial fibrillary acidic protein.

Figure 5

FTS•B incubation regulated the levels of inflammatory cytokines in LPS-exposed BV-2 cells. (A) FTS•B alone or co-incubation with LPS showed no effects on cell viability of BV-2 cells. Compared with LPS-alone exposed cells, FTS•B co-incubation strongly suppressed the release of (B) IL-6, (C) TNF-α, (D) iNOs, (E) NO and (F) IL-1β in BV-2 cells determined by ELISA. Data are expressed as the mean ± S.D. (n=3). #p<0.05 and ##p<0.01 vs. non-treated cells. *p<0.05, **p<0.01 and ***p<0.001 vs. LPS alone exposed cells. FTS•B: Forsythoside B; LPS: Lipopolysaccharide; IL-6: Interleukin 6; TNF-α: Tumor necrosis factor α; iNOs: Inducible nitric oxide synthase; NO: Nitric oxide; IL-1β: Interleukin 1β; ELISA: Enzyme linked immunosorbent assay.

Figure 6

FTS·B reduced the microglia-mediated neurotoxicity induced by LPS. Mouse HT22 cells were treated with CM from BV-2 microglia exposed to LPS for 24 h with or without FTS·B. After 24 h incubation, the (A and B) apoptosis rate and (C) cell viability were assessed. Data are expressed as the mean $\pm$ S.D. (n=3). ###p<0.001 vs. control group. *p<0.05, **p<0.01 and ***p<0.001 vs. group treated with LPS. FTS·B: Forsythoside B; CM: Conditioned media; LPS: Lipopolysaccharide.

Supplementary Files

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