Tetrandrine Ameliorated Alzheimer's Disease through Suppressing the Inflammatory Activation of Microglia in 5XFAD Mouse

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Research

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Abstract

Background

Alzheimer's disease is a neurodegenerative disorder prevalent in aged population. Tetrandrine is a natural metabolite isolated from herbal medicine Stephania tetrandra with various activities. Our previous study showed that tetrandrine can ameliorate acute cognitive impairment in a rat model induced by Aβ 1-42 intra-hippocampus injection. In this study, we further investigated the therapeutic role of tetrandrine in the transgenic 5XFAD mouse, a chronic model of Alzheimer's disease.

Methods

High performance liquid chromatography (HPLC) was used to determine the distribution of tetrandrine in brain. 5XFAD mice were treated with intraperitoneal injection of saline or tetrandrine (10, 20 and 40 mg/kg per 2 days) for 2 months. Cognitive ability was measured by Morris water maze test. The distribution of amyloid plaque was determined by immunohistochemistry in the brains. The expression of TNFα, IL-1β, IL-6, COX-2, iNOS and NF-κB were quantified by RT-PCR. In vitro , the inflammatory activation of microglial BV2 cells was checked by secretion of TNFα and IL-1β determined by ESLIA. Proinflammatory factors including TLR4, NF-κB, iNOS and COX-2 were analyzed by Western blot. In PC12-derived neural cells, CCK8 assay was used to determine the cell viability. Flow cytometry was applied to quantify cell apoptosis. Immunofluorescence was used to study the expression of apoptosis-involved protein cleaved Caspase-3 and Bcl-2.

Results

Tetrandrine can be distributed in the brain after intraperitoneal injection. Injection of tetrandrine significantly improved the cognitive ability of 5XFAD mice in the Morris water maze test. 5XFAD mice receiving tetrandrine showed notably reduced deposition of amyloid plaque in the brain and decreased cell apoptosis in the hippocampus. Further, tetrandrine treatment suppressed the expression of pro-inflammatory cytokines (TNFα, IL-1β, IL-6) and inflammation modulator (COX-2, iNOS, NF-κB) in the brain tissue. In vitro , tetrandrine suppressed Aβ 1-42-induced inflammatory activation of microglial BV2 cells as revealed by decreased secretion of TNFα and IL-1β, and inhibited expression of TLR4, NF-κB, iNOS and COX-2. Treatment of PC12-derived neural cells with conditional medium from Aβ 1-42-stimulated BV2 cells remarkably impaired cell viability and promoted cell apoptosis, which was attenuated by tetrandrine pre-treatment of the BV2 cells.

Conclusions

Tetrandrine improves the Alzheimer's disease at least partially through suppressing microgrlia-mediated inflammation and neurotoxicity.

Background

Alzheimer's disease is the most common cause of dementia in aged people, which is characterized by progressive impairment of cognitive, learning and memory ability. The occurrence of Alzheimer's disease positively correlates with increasing age. Pathologically, Alzheimer's disease holds the features of chronic Aβ plaque deposition, neurofibillary tangle formation, synaptic degeneration and neuron loss [1]. Epidemiological study showed that about 3.9% of people with age older than 60 suffer from this disease. As the worldwide human society becomes aging, the incidence of Alzheimer's disease is anticipated to increase largely in future decades, casting a heavy burden on healthcare system [2].

Although the pathogenic mechanisms of Alzheimer's disease are complicated, studies during the past decades have given us considerable insights on this neurodegenerative disorder, among which chronic inflammation is regarded as an important feature in the brain of Alzheimer's disease and highly associated with the disease onset and progression [3]. It's known that microglia are the predominant cells responsible for the inflammation in the brain of Alzheimer's disease. In the central nervous system, microglia function as macrophage-like cells and can be activated into the pro-inflammatory status by Aβ plaque. The activated microglia produce proinflammatory factors, such as TNFα and IL-1β, inducing neurotoxicity and neuron loss. Furthermore, the inflammatory activation also abrogates the clearance of Aβ plaque by microglia themselves, which forms a vicious cycle resulting in accelerated accumulation of Aβ plaque [3]. Therefore, targeting the inflammation process is considered to be a promising strategy in the treatment of Alzheimer's disease. Indeed, appreciable evidences showed that various drugs with anti-inflammatory activity demonstrate therapeutic effects in animal models of Alzheimer's disease, for instance, cyclooxygenase-2 inhibitor rofecoxib [4], PPARgamma agonist pioglitazone, non-steroidal anti-inflammatory drug ibuprofen and the natural product extracted from plant Carcumalonga curcumin [5].

Tetrandrine is a bisbenzylisoquinoline alkaloid purified from the root of herbal medicine Stephania tetrandra. Previous studies showed that tetrandrine demonstrates anti-inflammatory activity in a wide range of cell types, including T cells [6], monocytes[7], pancreatic acinar cells [8], astrocytes [9] and so on. Tetrandrine protects against sevoflurane anesthesia-induced cognitive impairment by attenuating inflammation and cell apoptosis in aged rats [10]. In addition, Yang et al. reported that tetrandrine suppresses lipopolysaccharide (LPS)-induced inflammatory activation of cultured microglia [11]. In our previous work with an acute Aβ 1-42-induced rat model of Alzheimer's disease, we demonstrated that tetrandrine can ameliorate cognitive impairment through inhibiting NF-κB activity and inflammation [12]. To further confirm the cognitive protective role of tetrandrine and investigate the underlying mechanism in a model more resembling clinical Alzheimer's disease, in this study, we tried to validate the therapeutic effects of tetrandrine in the transgenic 5XFAD mice which show chronic deposition of amyloid plaques in the brain accompanied with cognitive impairment. Given the important role of microglia in the pathology of Alzheimer's disease, we further attempted to investigate the effects of tetrandrine on Aβ-induced microglial inflammatory activation and the resulted neurotoxicity in neurons.

Methods

Animals

The double transgenic 5XFAD mouse strain (Tg6799), which co-expresses mutant human APP and PS1 in neural cells, was previously described [13] and purchased from Nanjing Biomedical Research Institute of Nanjing University. The 5XFAD mice were maintained as hemizygotes in B6/SJL background. Mice were kept in SPF facility at a 12-hour light/dark cycle with free access to food and water. The primers used for genotyping the human-derived double transgen fragments are hAPP-F 5'-AGGACTGACCACTCGACCAG-3', hAPP-R 5'- CGGGGGTCTAGTTCTGCA T-3'; hPS1-F 5'- AATAGAGAACGGCAGGAGCA-3' and hPS1-R 5'-GCCATGAGGGCACTAATCAT-3'. All animal experiments were approved and complied with corresponding regulations issued by the Animal Experimental Ethic Committee of Southwest Medical University.

Tetrandrine Treatment

To generate hemizygous 5XFAD mice for downstream experiments, transgenic mice were crossed with wild type B6/SJL mice. In the offspring, the double transgene-positive mice which were supposed to be hemizygous and the non-transgenic littermates were enrolled for in vivo animal experiment. For tetrandrine treatment, the heterozygous 5XFAD mice were allocated into 4 groups. 3 groups of 5XFAD mice were intraperitoneally injected with 10 (n = 9), 20 (n = 12), 40 (n = 6) mg/kg of tetrandrine (tetrandrine hydrochloride, cat# H20053840, Yintao Pharma. China) respectively every 2 days from 5-month old to 7-month old. The left one group (n = 6) of age-matched 5XFAD mice received vehicle (0.9% NaCl) injection. Additional group (n = 8) of age-matched non-transgenic littermates were also injected with saline and serve as healthy control. At the end of the experiment, cognitive behavior of the mice was assessed by Morris water maze test as described below. Next day, mice were sacrificed by cervical dislocation. The brain was harvested and cut into halves along the sagittal axis. Half of the brain was fixed with 4% paraformaldehyde (in PBS) for 24 h followed by standard paraffin embedding. The left half of brain was snap frozen in liquid nitrogen and stored at -80 ^{O}C for downstream RNA analysis.

Morris Water Maze Test

The Morris water maze test was performed with 5 consecutive days of memory training before the probe trial in a circular swimming pool (120 cm in diameter, 50 cm in depth, Shanghai Xinxin Information Tech. Inc. China) filled with water mixed with milk powder. The pool was divided into 4 equal fan-shaped quadrants with one quadrant containing a circular translucent platform (8 cm in diameter) submerged 1.5 cm below the water surface. Four plates with different geometric shape of different color were hanged around to serve as spatial cues. For training, the mice were placed into the water at the edge of one quadrant with face towards the wall. The mice were allowed to swim to find the platform until 90 seconds elapsed. Mice which found the platform were allowed to stay on it for 10 s. Mice which failed to find the platform in 90 seconds were guided to the platform by experimenter and also allowed to stay for 10 s. The time for mice to find the platform was recorded as escape latent time. Each mouse was trained 4 times with the entrance to the water from different quadrants per day. For the probe trial session on the 6th day, the platform was removed. Mice were placed into the water from the opposite quadrant to the

quadrant previously containing the platform and allowed to swim for 90 s. The length of time that the mice spent in target quadrant (previously with platform) and number of the times the that mice crossed the site of removed platform were recorded for downstream analysis.

High Performance Liquid Chromatography (HPLC)

C57BL/6 mice were injected with 40 mg/ml tetrandrine or saline. Mice were sacrificed at indicated time points by cervical dislocation. The brains were harvested and homogenized in 1 ml of methanol. After clearing by centrifugation, the supernatant was dried under nitrogen and reconstituted in 0.2 ml of methanol/0.3% triethylamine (98/2, v/v). 20 μl of the solution was loaded onto the LC1260 HPLC system equipped with an Innoval ODS-2 column (4.6 × 250 mm, 5 μm) settled at 30 ^{o}C. The mobile phase is methanol/0.3% triethylamine (98/2, v/v, 1 ml/min). The detection wavelength is 282 nm.

Immunohistochemistry (IHC)

The paraffin embedded brain tissue was sectioned at 4 μm. The sections were dewaxed and rehydrated in gradual ethanol. Antigen retrieval was performed by boiling in 0.01M citrate buffer (pH 6.0) for 10 min. The tissue sections were treated with 3% H_2O_2 for 15 min to inactivate endogenous peroxidase and blocked by 10% goat serum for 1 h. Primary antibodies were applied overnight at 4 ^{o}C with indicated dilution ratio. After washing with PBS, the sections were incubated with HRP-conjugated secondary antibody solution for 30 min at 37 ^{o}C. Again, the sections were washed with PBS. The signal was developed in DAB solution and monitored under microscope. The chromogenesis was stopped by washing in distilled H_2O. Then, the sections were counter-stained with hematoxylin and dehydrated. At last, the sections were cleared in xylene and mounted with neutral balsam mounting medium. Photos were captured with a light microscope (Nikon Eclipse 80i, Japan). Antibodies used in IHC are rabbit anti Aβ 1–42 antibody (Abcam, cat# ab10148, USA) and HRP-conjugated goat anti rabbit antibody (Abcam, cat# ab6721, USA).

TUNEL

TUNEL was performed with the peroxidase (POD)-based In Situ Cell Death Detection Kit following the manufacturer's instruction (Roche, cat# 11684817910, Switzerland). DAB was used for signal development. The section was counter-stained with hematoxylin before mounting. Images were captured as described above. The percentage of apoptotic cell in hippocampus was calculated with Image J software (NIH, USA).

RT-PCR

The brain tissue was milled into powder in liquid nitrogen. About 0.05 g of powder was used for RNA isolation with Trizol (Beyotime, cat# R0016, China) according to the standard instruction. 1 μg of RNA

was reverse-transcripted into cDNA with PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, cat# 6210B, Japan). RT-PCR was performed with UltraSYBR Mixture (CwBio, cat# CW0957, China). Primers used in this study included TNFα forward 5'- CCCTCCAGAAAAGACACCATG-3', reverse 5'-CACCCCGAAGTTCAGTAGACAG-3'; IL-1β forward 5'- GCTTCAGGCAGGCAGTATCA-3', reverse 5'-TGCAGTTGTCTAATGGGAACG-3'; IL6 forward 5'- GGGACTGATGCTGGTGACAAC-3', reverse 5'-CAACTCTTTTCTCATTTCCACGA-3'; COX-2 forward 5'- GGGGTGATGAGCAACTATTCC-3', reverse 5'-GAGGCAATGCGGTTCTGATAC-3'; iNOS forward 5'- TTGGAGCGAGTTGTGGATTG-3', reverse 5'-GGTCGTAATGTCCAGGAAGTAGG-3'; NF-κB forward 5'- CTGGAGCAAGCCATTAGCC-3', reverse 5'-GGTTATCAAAAATCGGATGTGAG-3' and β-actin forward 5'- GAGACCTTCAACACC CCAGC-3', reverse 5'-ATGTCACGCACGATTTCCC-3'. The data was analyzed with the Delta-Delta Ct method.

Drugs And Cell Culture

To prepare the aggregated Aβ1–42, powder Aβ peptides (Sigma, cat# A9810, USA) were dissolved in MiniQ H_2O to get a final stock concentration of 1 mg/ml (0.22 mM) followed by incubation at 37 °C for 7 days and further stored at 4 °C. Tetrandrine was the same to that used in the in vivo animal experiment. Microglial BV2 cells were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS, Gibco, cat# 10500064, USA), 100 U/ml of penicillin and 100 μg/ml streptomycins. In BV2 cells, tetrandrine pre-treatment was performed for 1 h and Aβ1–42 stimulation lasted for 12 h. Cell pellet or conditional medium was collected for indicated experiment. PC12 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum (Gibco, cat# 26050070, USA), 100 U/ml of penicillin and 100 μg/ml streptomycin. Preceding treatment, PC12 cells were firstly differentiated into neuron-like cells in medium containing 50 ng/ml nerve growth factor (NGF) for 7 days. Then, the cells were stimulated for 12 h with conditional medium from BV2 cells which was diluted at a ratio of 1:1 with cultural medium of PC12. All cells were maintained in incubator set at 37 °C with 5% CO_2 and 100% humidity. Cellular images were taken with an inverted microscope (Nikon, ECLIPSE TS100, Japan).

ELISA

After indicated treatment, the culture medium of BV2 cells was collected, clarified by centrifugation and stored at -80 °C. Concentration of secreted TNFα and IL-1β were determined with mouse TNFα ELISA kit (BeJing LiKe Tech. cat# DRE30030 ,China) and mouse IL-1β ELISA kit (BeJing LiKe Tech. cat# DRE30027 ,China) following the manufacturer's instruction.

Western Blot

0.1 g brain tissue was lysed in RIPA buffer. Protein concentration was determined by Pierce BCA protein assay kit (Thermo, cat# 23227, USA). 20 μg of total protein was used for Western blot as previously described [14]. Primary antibodies employed in this study were rabbit anti-NF-κB (Abcam, cat# ab32536, USA), rabbit anti-TLR4 (Abcam, cat# ab13867, USA), rabbit anti-COX-2 (Abcam, cat# ab15191, USA),

rabbit anti-iNOS (Abcam, cat# ab15323, USA) and mouse anti-GAPDH (Abcam, cat# ab8245, USA). Secondary antibodies were HRP-conjugated goat anti-mouse IgG (Sigma, cat#A3682, USA) and HRP-conjugated goat anti-rabbit IgG (Sigma, cat# A6154, USA).

CCK8 Assay

1×10^4 PC12 cells were seeded into the well of 96-well plate. After adhesion, the cells were differentiated for 7 days followed by tetrandrine and Aβ1–42 treatment as described above. Then, CCK8 reagent was added into the medium to a final concentration of 10% (v/v). After incubation for 3 h, optical density at 450 nm wave length was recorded with spectrophotometer. Each treatment was performed in triplicate.

Flow Cytometry

0.5×10^6 PC12 cells were seeded into 6-well plate. After differentiation and treatment with tetrandrine and Aβ1–42, cells were collected by standard typsin digestion. Then the cells were washed with PBS and subjected to Annexin V/PI staining according to the manufacturer's instruction (BD Bioscience, cat# 556547, USA) and analyzed with Aquios CL Flow Cytometry System (Beckman, USA). Three samples were included for each treatment.

Immunofluorescence

PC12 cells were seeded onto round sterile glass coverslip and subjected to differentiation and treatment as described above. For immunofluorescent staining, cells were fixed in cold 4% paraformaldehyde for 10 min. After washing, the cells were permeabilized with 2.5% triton X-100 for 10 min and blocked with 10% goat serum for 1 h at RT. Primary antibody was applied at 4 °C overnight. After washing with PBS, fluorescent secondary antibody incubation was performed at RT for 1 h. Next, the cells were washed with PBS and counter-stained with DPAI and mounted on glass slide. Antibodies used included rabbit anti-cleaved Caspase-3 (Abcam, cat# ab2302, USA), rabbit anti-Bcl-2 (Abcam, cat# ab182858, USA) and Alexs Fluor 488-conjugated goat anti-rabbit IgG (Abcam, cat# ab150077, USA). Photos were taken under a fluorescent microscope (Nikon, Eclipse Ci-S, Japan).

Statistics

The quantitative data were presented as mean ± SEM. Two-way analysis of variance (ANOVA) for repeated measures was used to analyze the escape latency data in Morris water maze test followed by Tukey's multiple comparison test. For additional multiple groups of quantitative data, one-way ANOVA followed by Tukey's post hoc test was used. $p < 0.05$ was regarded as statistical significance.

Results

Tetrandrine can reach brain tissue after intraperitoneal delivery in mice

To begin with the animal study, we initially investigated whether tetrandrine can cross blood-brain barrier and be distributed in the brain. We intraperitoneally injected the C57BL/6 mice with tetrandrine (40 mg/kg, the highest therapeutic dose used for animal study). Brain tissues were collected at different time points and subjected to homogenization, extraction and HPLC analysis as described in the methods. HPLC results showed that tetrandrine can be detected in the brain extract from 0.5 hour to even 12 hours post injection (Supplementary Fig. 1 and data not shown). This data indicated that tetrandrine can reach brain tissue after intraperitoneal delivery.

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Tetrandrine Treatment Improved The Cognitive Ability Of 5XFAD Mice

We already demonstrated that tetrandrine benefits the acute cognitive and memory injury induced by intra-hippocampus Aβ1–42 injection in our previous work [12]. To investigate whether tetrandrine could execute a similar therapeutic role in naturally occurring Alzheimer's disease, we chose the 5XFAD

transgenic mouse as the disease model which simultaneously over-express human APP and PS1 genes containing five familial Alzheimer's disease-associated mutations. The 5XFAD mice demonstrate chronic deposition of amyloid plaques in the brain accompanied with impairment of cognitive ability. To exclude the possible bias caused by genetic dose, hemizygous 5XFAD mice were used in the experiment. The 5XFAD mice were intraperitoneally injected every two days with different doses (10, 20 and 40 mg/kg) of tetrandrine for 2 months (from age of 5 months to 7 months). At the end of the treatment, Morris water maze test was used to assess the cognitive function of these mice. As shown in Fig. 1A, during the 5 days of training stage, the control wild type mice demonstrated steep decrease of average escape latency along training time. In contrast, the 5xFAD mice showed weak change of escape latency along the training time, indicating an impaired learning ability. Tetrandrine treatment reduced the escape latency in 5XFAD mice in a time and dosage-dependent manner. Notably, 5XFAD mice treated with 40 mg/kg tetrandrine presented a similar descending trend of escape latency with the wild type mice, suggesting significantly improved learning ability. In the stage of probe task, the 5XFAD mice tended to spend less time in the target quadrant with platform removed compared with the wild type mice, although without statistical significance. However, tetrandrine treatment improved the time spent by 5XFAD mice in the target quadrant, especially for the mice receiving 20 mg/kg tetrandrine (Fig. 1B). Besides, the 5XFAD mice showed significantly less number of times to cross the position of platform in the probe task compared with the normal mice, while tetrandrine treatment dose-dependently recovered it to a comparable level versus normal mice. Collectively, these findings suggest that tetrandrine improves the impaired learning and memory ability in Alzheimer's disease mice.

Tetrandrine treatment reduced amyloid plaques load and attenuated cell apoptosis in hippocampus

The ectopic production and deposition of Aβ-derived amyloid plaque is directly involved with the injury of brain neuron and positively correlates with the severity of cognitive impairment both in patients and animal models of Alzhemier's disease [13, 15]. We therefore investigated whether tetrandrine treatment could reduce the deposition of amyloid plaque in brain. As shown in Fig. 2, IHC staining against Aβ 1–42 showed that 5XFAD mice presented massively dispersed amyloid plaques across the sagittal section of the brain including hippocampus and cortex. In contrast, no amyloid plaque can be detected in control wild type mice. Interestingly, administration of tetrandrine significantly decreased the density of amyloid plaque, which happened in a dose-dependent way. The neuron loss in hippocampus contributes to the impairment of cognition, we then explored whether the reduction of amyloid plaques by tetrandrine treatment was accompanied with prevention of cell apoptosis by TUNEL assay. As illustrated in Fig. 3, 5XFAD mice demonstrated elevated cell apoptosis in hippocampus area compared with the wild type mice. however, tetrandrine treatment dose-dependently attenuated the ratio of apoptotic cell in hippocampus of 5XFAD mice.

Tetrandrine attenuated the inflammatory in the brain of 5XFAD mice

It has been well studied for the anti-inflammatory effect of tetrandrine [7–9, 11]. And persistent chronic inflammation is one of the key pathological features in Alzheimer's disease [3]. We therefore investigated whether the anti-inflammatory activity of tetrandrine contributes to improvement of Alzheimer's disease in 5XFAD mice. We firstly analyzed the inflammatory status in the brain tissues by quantitative RT-PCR. As revealed in Fig. 4, the 5XFAD mice showed significantly up-regulated expression of pro-inflammatory cytokines, including TNFα, IL-1β and IL-6. The expression of COX-2 and iNOS, two enzymes mediating the inflammatory reaction, were also significantly increased. Simultaneously, the core transcriptional factor of inflammation NF-κB also exhibited increased expression in 5XFAD mice. However, in 5XFAD mice treated with tetrandrine, the expression of all the inflammatory genes described above were down-regulated in a dosage-dependent fashion. These findings indicate that tetrandrine suppresses the inflammation in CNS of mouse of Alzheimer's disease.

Tetrandrine suppressed microglial activation induced by Aβ 1–42

Microglia are the primary cells dominating the chronic inflammation in the brain of Alzheimer's disease. We next investigated whether tetrandrine could suppress the inflammatory activation of microglia. The microglial cell line BV2 was used to study the effect of tetrandrine on microglial inflammatory activation. BV2 cells were firstly stimulated with different doses of aggregated Aβ 1–42 for 12 h and the secreted inflammatory cytokines, TNFα and IL-1β, in the supernatant were quantified by ELISA. As shown in Fig. 5A and B, all doses of Aβ 1–42 significantly promoted the secretion of both TNFα and IL-1β by BV2 cells, which also happened in a dose-dependent manner. We next queried whether tetrandrine could block the Aβ 1-42-stimulated activation of BV2 cell. We pre-treated the BV2 cells with different doses of tetrandrine before stimulation with 10 μM Aβ 1–42. The results in Fig. 5B and D showed that tetrandrine pre-treatment dose-dependently inhibited the production of TNFα and IL-1β in Aβ 1-42-stimulated BV2 cells, indicating the partial suppression of inflammatory activation.

To further confirm the inhibitory effect of tetrandrine on inflammatory activation of BV2 cells, the activity of NF-κB signal pathway was checked by Western blot. As illustrated in Fig. 6, Aβ 1–42 treatment induced remarkably increased expression of TLR4 and NF-κB. Moreover, expression of the downstream effective proteins iNOS and COX-2 were also up-regulated. However, tetrandine pre-treatment partially reversed the protein level of TLR4, NF-κb, iNOS and COX-2. Combined with the ELISA results, these findings demonstrated that tetrandrine attenuated the activation of BV2 cells.

Tetrandrine Ameliorated BV2-derived Neurotoxicity In PC12 Cells

To unravel the biological effects of inhibition of microglial activation by tetrandrine on the survival of neural cell, we used the conditional medium of BV2 cells to treat PC12 cells which were firstly differentiated into neuron-like cells in the presence of NGF and can be used to study the neural function and survival [16]. As showed in Fig. 7A, treatment with conditional medium from Aβ 1-42-stimulated BV2 cells caused notable neurotoxicity in PC12-derived neural cells, including shrinkage of the cell body and loss of neurites. However, tetrandrine pre-treatment of BV2 cells before Aβ 1–42 stimulation largely attenuated the toxicity of the conditional medium on PC12-derived neural cells which well preserved the outgrowth of neurites. In line with the morphological observation, CCK8 assay showed that conditional medium from Aβ 1-42-stimulated BV2 cells significantly impaired the viability of PC12-derived neural cells, which can be ameliorated by tetrandrine pre-treatment of the BV2 cells (Fig. 7B).

We next investigated the apoptosis status of PC12-derived neural cells. As revealed by double staining of Annexin V and PI followed by flow cytometry analysis, treatment of PC12-derived neural cells with conditional medium from Aβ 1-42-stimulated BV2 cells provoked significantly increased apoptosis in PC12-derived neurons. Notably, this increased apoptosis can be reversed by conditional medium from Aβ 1-42-stimulated BV2 cells pre-treated with tetrandrine (Fig. 8). The apoptosis of PC12-derived neurons was further validated by immunostaining of apoptosis-involved proteins as illustrated in Fig. 9. PC12-derived neurons treated with conditional medium from Aβ 1-42-stimulated BV2 cells demonstrated increased expression of apoptosis executor cleaved Caspase-3 and decreased expression of anti-apoptotic Bcl-2. However, the expression trends of cleaved Caspase-3 and Bcl-2 were reversed by conditional medium from Aβ 1-42-stimulated BV2 cells pre-treated with tetrandrine. In conclusion, these findings indicated that tetrandrine can attenuate the microglia-mediated neurotoxicity.

Discussion

Alzheimer's disease is a chronic neurodegenerative disorder which is tightly associated with inflammation mediated by microglia. Anti-inflammation intervention is thought to be a plausible strategy to treat this disease [3]. Tetrandrine is a natural product isolated from Stephania tetrandra which shows a wide range of bioactivities [17]. In this study, we investigated the therapeutic effects of tetrandrine on Alzheimer's disease in a mouse model (5XFAD mouse) with similar pathological process to the clinical disease. Our results demonstrated that administration of tetrandrine dose-dependently reduced the load of amyloid plaque in the brain and improved cell apoptosis in the hippocampus with the consequence of ameliorated cognitive behavior of the 5XFAD mice as tested in the Morris water maze (Fig. 1–3). RT-PCR indicated that inflammation-involved genes showed suppressed expression upon tetrandrine treatment (Fig. 4). In in vitro experiment, we found that tetrandrine can effectively suppress the Aβ 1-42-stimulated inflammatory activation of microglia (BV2) including the attenuated expression of inflammatory regulatory genes and the decreased secretion of pro-inflammatory cytokines (Fig. 5–6). Furthermore, we

showed that the suppressed activation of microglia by tetrandrine treatment resulted in significantly attenuated neurotoxicity as demonstrated in PC12-derived neural cells treated with conditional medium from microglia (Fig. 7–9). We previously proved that tetrandrine can improve the acute cognitive impairment in a rat model induced by Aβ 1–42 injection. Thus, combining the findings from this study, we can conclude that tetrandrine exhibits therapeutic effects on Alzheimer's disease.

Inflammation plays dual roles in the pathogenesis of Alzheimer's disease. It is proposed that properly controlled inflammation promotes the clearance of Aβ and therefore benefits the recovery of brain function. However, ectopic chronic inflammation deteriorates the disease progress [3]. Study in transgenic mouse model of Alzheimer's disease indicated that microglia from diseased mouse have decreased expression of Aβ clearance-involved receptors and enzymes. In contrast, these microglia showed increased expression of pro-inflammatory cytokines. Furthermore, treatment of the microglia with TNFα directly decreased the uptake of Aβ [18]. This suggests that inflammatory microenvironment can suppress the Aβ clearance ability of microglia and contribute to its deposition. Otherwise, reversal of inflammation should help the clearance of amyloid plaque. To support this point, there is an evidence showing that the anti-inflammatory treatment, even in short term, can reduce the load of amyloid plaque in the animal model of Alzheimer's disease [19]. In our study, we also observed a significant decrease of amyloid plaque in the brains of tetrandrine-treated 5XFAD mice, which can be attributed to the anti-inflammatory activity of tetrandrine.

Conclusions

Collectively, our findings have proven that tetrandrine is a promising candidate drug in the treatment of Alzheimer's disease. However, besides the exciting results from animal study, more clinical evidences are needed for its clinical application in future treatment of Alzheimer's disease.

List Of Abbreviations

RT-PCR, real-time PCR

TNFα, tumor necrosis factor alpha

IL-1β, interleukin 1 beta

IL-6, interleukin 6

COX-2, cyclooxygenase-2

iNOS, inducible nitric oxide synthase

NF-κB, nuclear factor kappa B

TLR4, Toll like receptor 4

5XFAD mouse, mouse harboring five mutation associated with familial Alzheimer's disease

APP, amyloid beta precursor protein

PS1, presenilin-1

FBS, fetal bovine serum

NGF, nerve growth factor

Declarations

Ethics approval and consent to participate

All animal experiments were approved and complied with corresponding regulations issued by the Animal Experimental Ethic Committee of Southwest Medical University.

Consent for publication

Not applicable

Availability of data and materials

The datasets involved with this study is available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Z Li and HL Wang designed the experiment. DF Ren, Y Fu, X Zhong, JY Yuan and CL Jiang performed the experiment. L Wang and HL Wang conducted the data analysis. HL Wang and DF Ren wrote the manuscript which was further revised by all members of the authorship.

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Figures

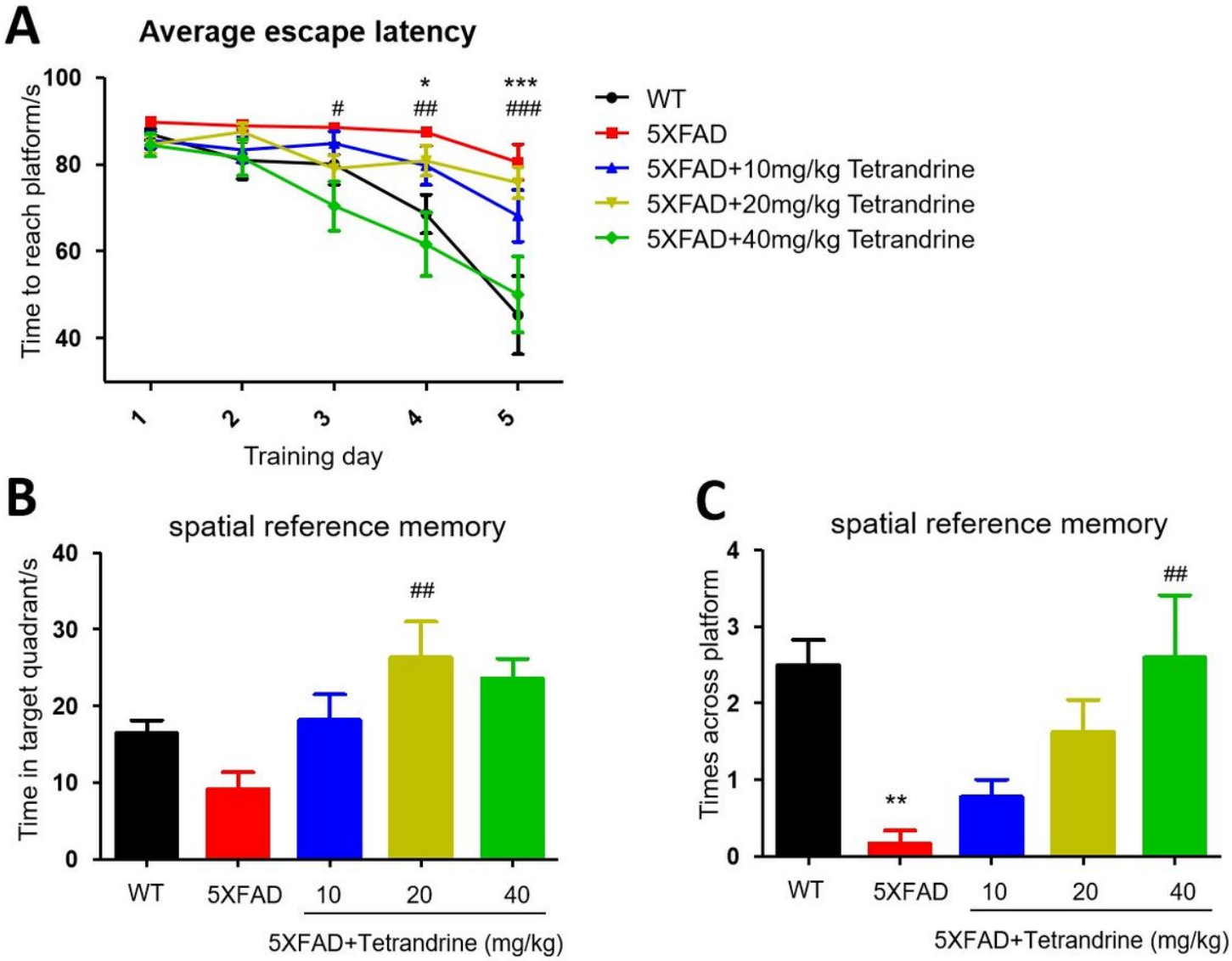

Figure 1

Tetrandeine improved cognitive ability of 5XFAD mice assessed by Morris water maze . A. The plotting of average escape latency in the 5 days' training. *, *** represented $p<0.05$ and $p<0.001$ respectively between 5XFAD and wild type group at corresponding time point. #, ## and ### represented $p<0.05$, $p<0.01$ and $p<0.001$ respectively between 5XFAD mice group receiving 40 mg/kg tetrandrine treatment and 5XFAD mice group at corresponding time point. B. The accumulative time spent in the target quadrant where the platform was removed in the probe test of the five groups of mice. ##, $p<0.01$ versus 5XFAD group. C. Number of times the mice crossed the location of the removed platform. **, $p<0.01$ versus wild type (WT) group. ##, $p<0.01$ versus 5XFAD group.

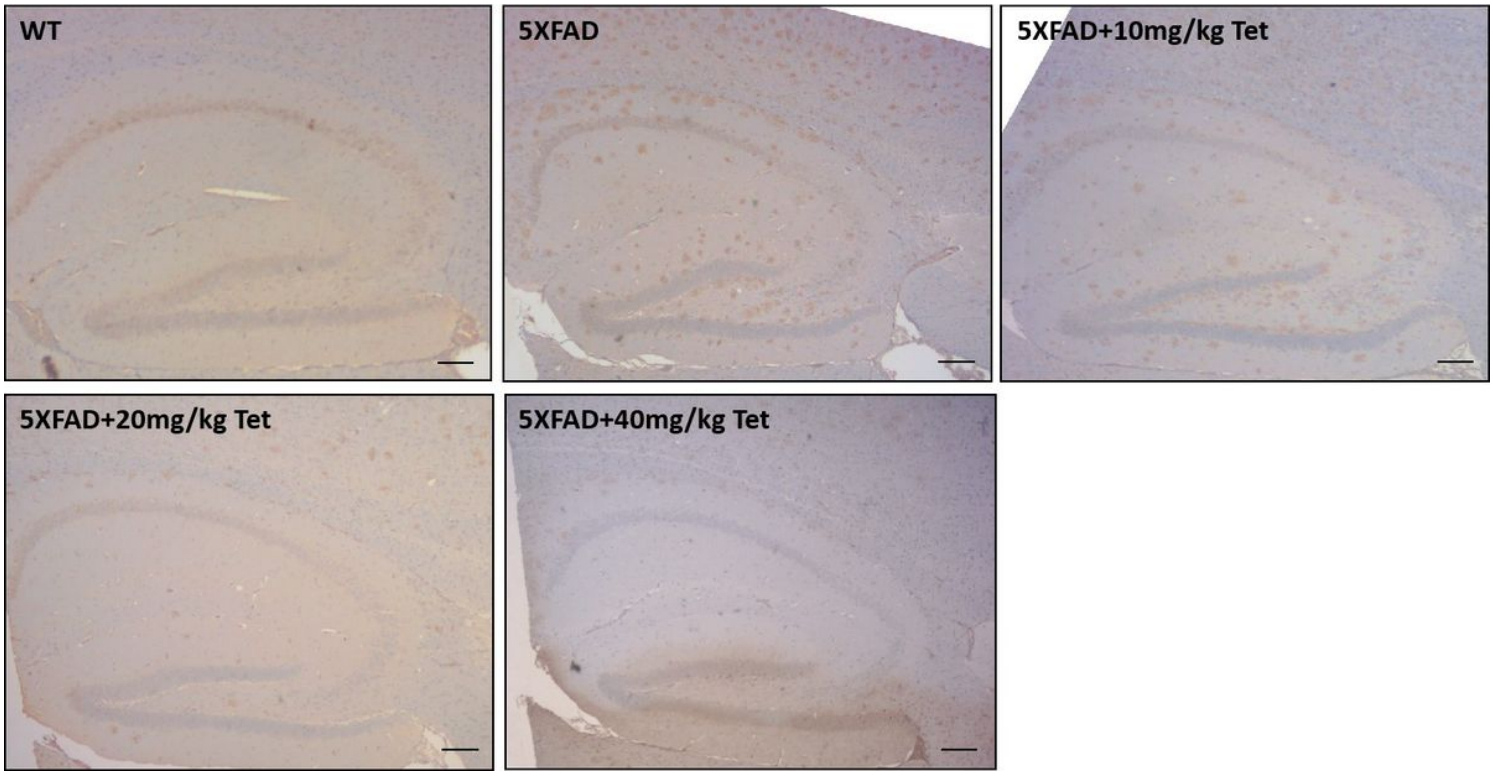

Figure 2

Administration of tetrandrine reduced the deposition of amyloid plaque in 5XFAD mice. Immunohistochemical staining against Aβ 1-42 to show the amyloid plaque in the brain. Representative images were shown for each mouse group. Scale bar, 200 μm.

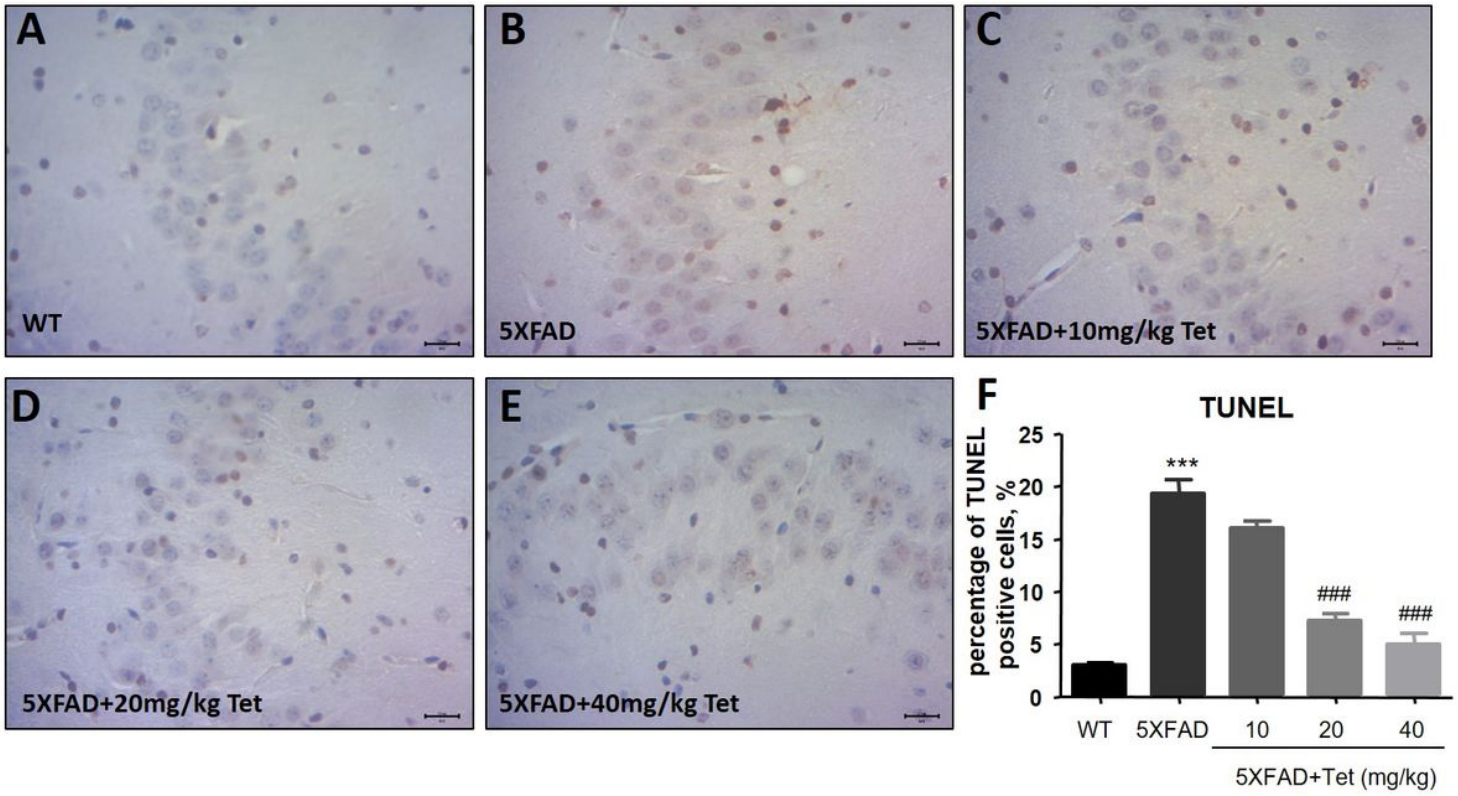

Figure 3

TUNEL assay to detect the cell apoptosis in the hippocampus. A-E, Representative image of TUNEL in the hippocampus (CA3 region) of mice with indicated treatment. Scale bar, 20 μm. F, Quantitative analysis of TUNEL-positive cells in the hippocampus region. ***, $p<0.001$ versus WT group. ###, $p<0.001$ versus 5XFAD group.

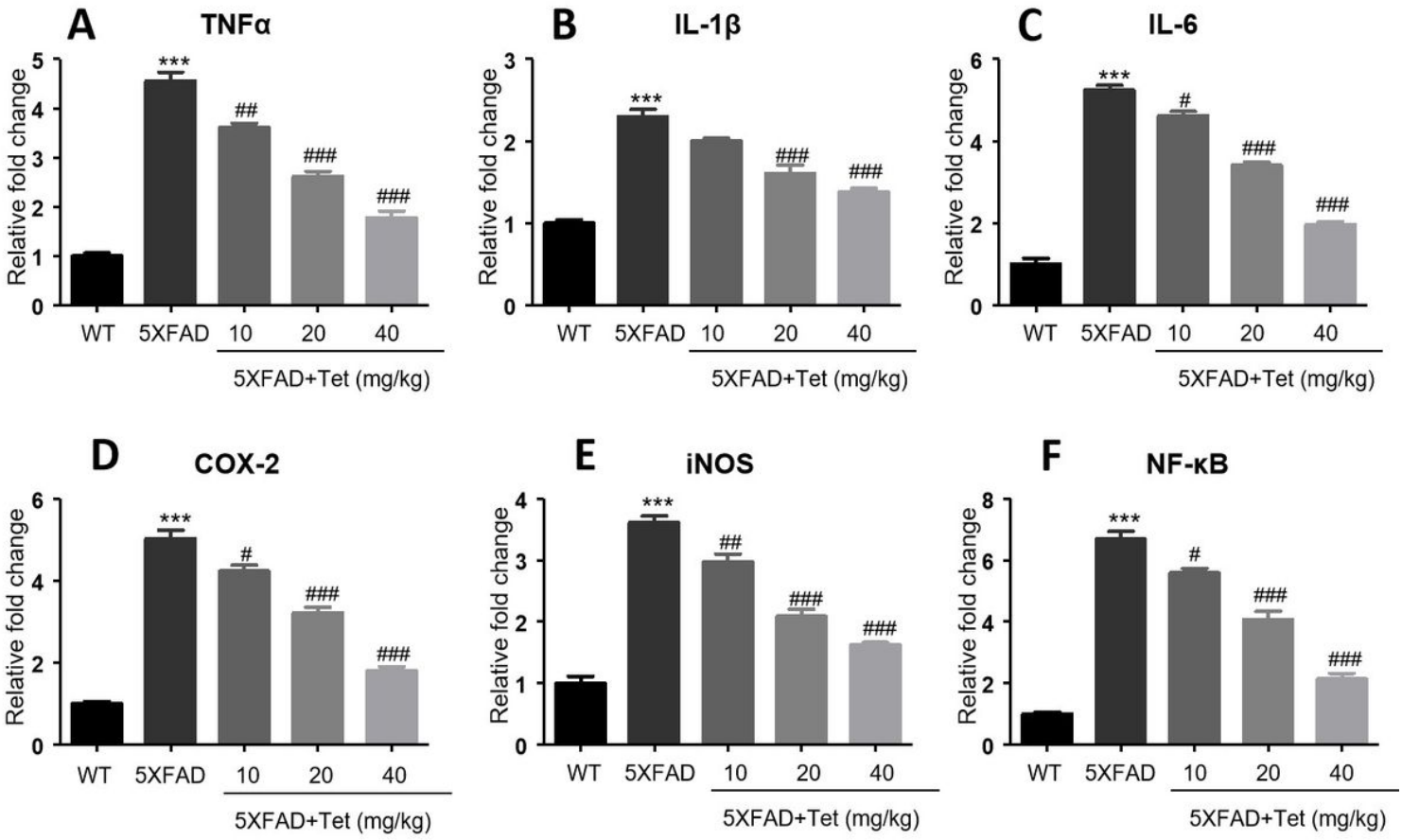

Figure 4

Tetrandrine down-regulated inflammation-involved gene expression. RT-PCR was performed to quantify the expression of TNFα (A), IL-1β (B), IL-6 (C), COX-2 (D), iNOS (E) and NF-κB (F) in the brain. ***, $p<0.001$ versus WT group. #, $p<0.05$ and ###, $p<0.001$ versus 5XFAD group.

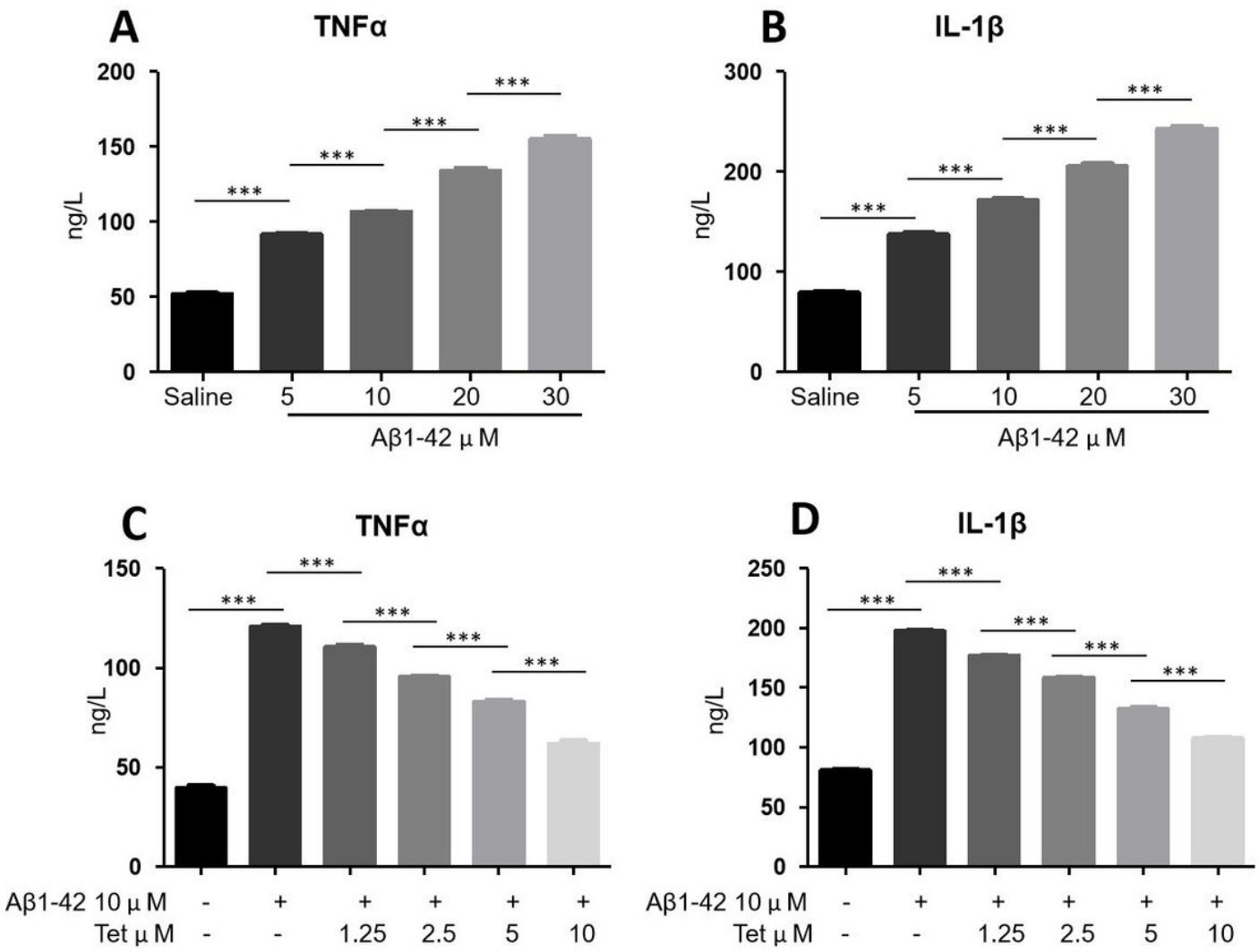

Figure 5

ELISA to show that tetrandrine inhibited Aβ 1-42-induced inflammatory activation in BV2 cells. BV2 cells were stimulated with different doses of Aβ 1-42 for 12 h. ELSIA was used to quantify the level of secreted TNFα (A) and IL-1β (B). In C and D, BV2 cells were firstly pre-treated with different dose of tetrandrine for 1 h followed by stimulation with 10 μM Aβ 1-42 for 12 h. TNFα and IL-1β level of the supernatant were determined by ELISA. ***, $p<0.001$.

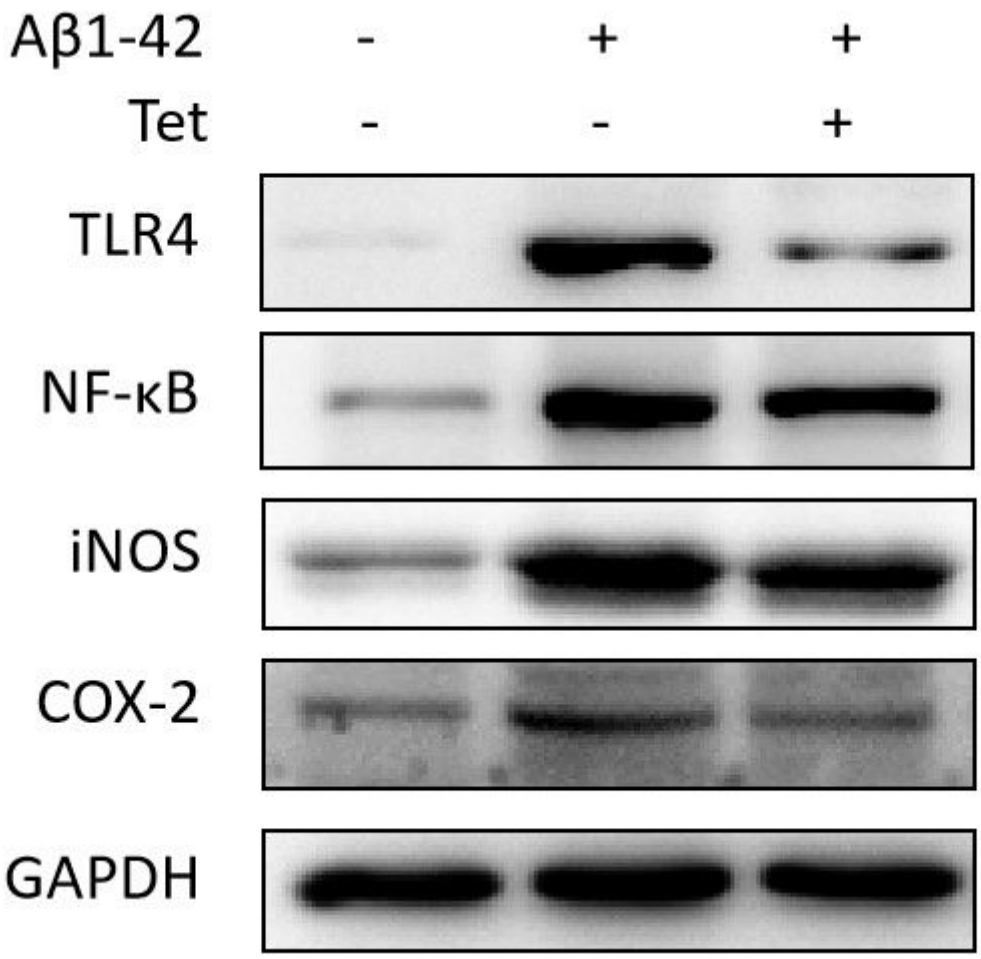

Figure 6

Western blotting to demonstrate that tetrandrine attenuated the inflammatory activation of BV2 cells induced by Aβ 1-42. BV2 cells were pre-treated saline or 10 μM tetrandrine for 1 h before stimulation of Aβ 1-42 for 12 h. Total protein was harvested to probe the expression of indicated proteins. GAPDH was used as loading control.

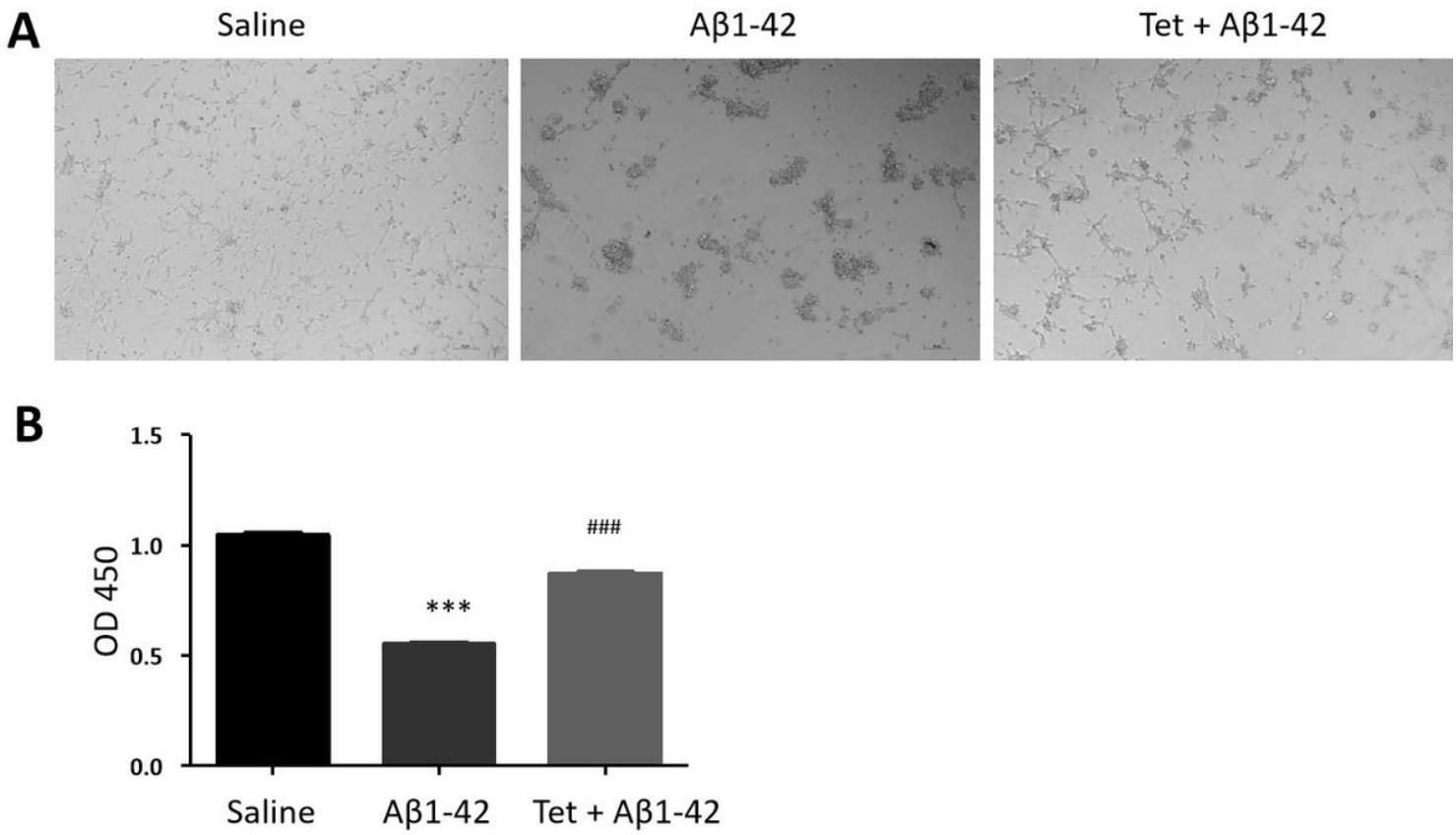

Figure 7

A. Morphological observation of PC12-derived neural cells treated with conditional medium (12 h) from BV2 cells pre-treated with or without 10μM tetrandrine (1 h) and stimulated with or without 10μM Aβ 1-42 (12 h). View magnification, X100. B. CCK8 assay to determine the cellular viability of PC12-derived neural cells treated as in A. ***, $p<0.01$ versus saline group. ###, $p<0.01$ versus Aβ 1-42 group.

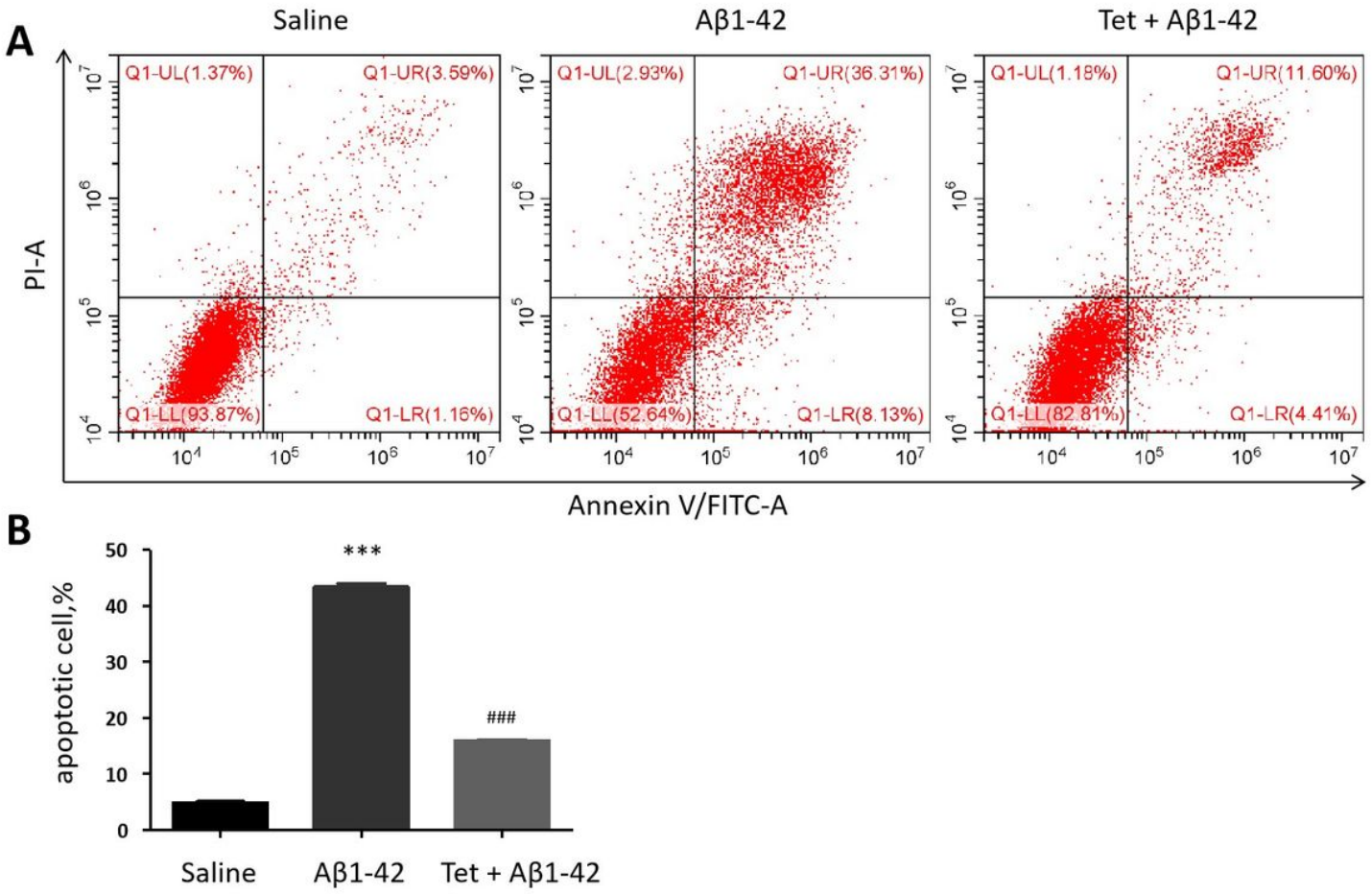

Figure 8

Annexin V/PI staining to determine the apoptosis of PC2-derived neural cells. The treatment of PC12-derived neural cells with conditional medium from BV2 cells is the same as Figure 7. A is the representative images of the flow cytometry plot of Annexvin V/PI staining. B is the quantitative analysis of the apoptotic rate. Early (Q1-LR) and late (Q1-UR) apoptosis are combined for the quantitative analysis. ***, $p<0.01$ versus saline group. ###, $p<0.01$ versus Aβ 1-42 group.

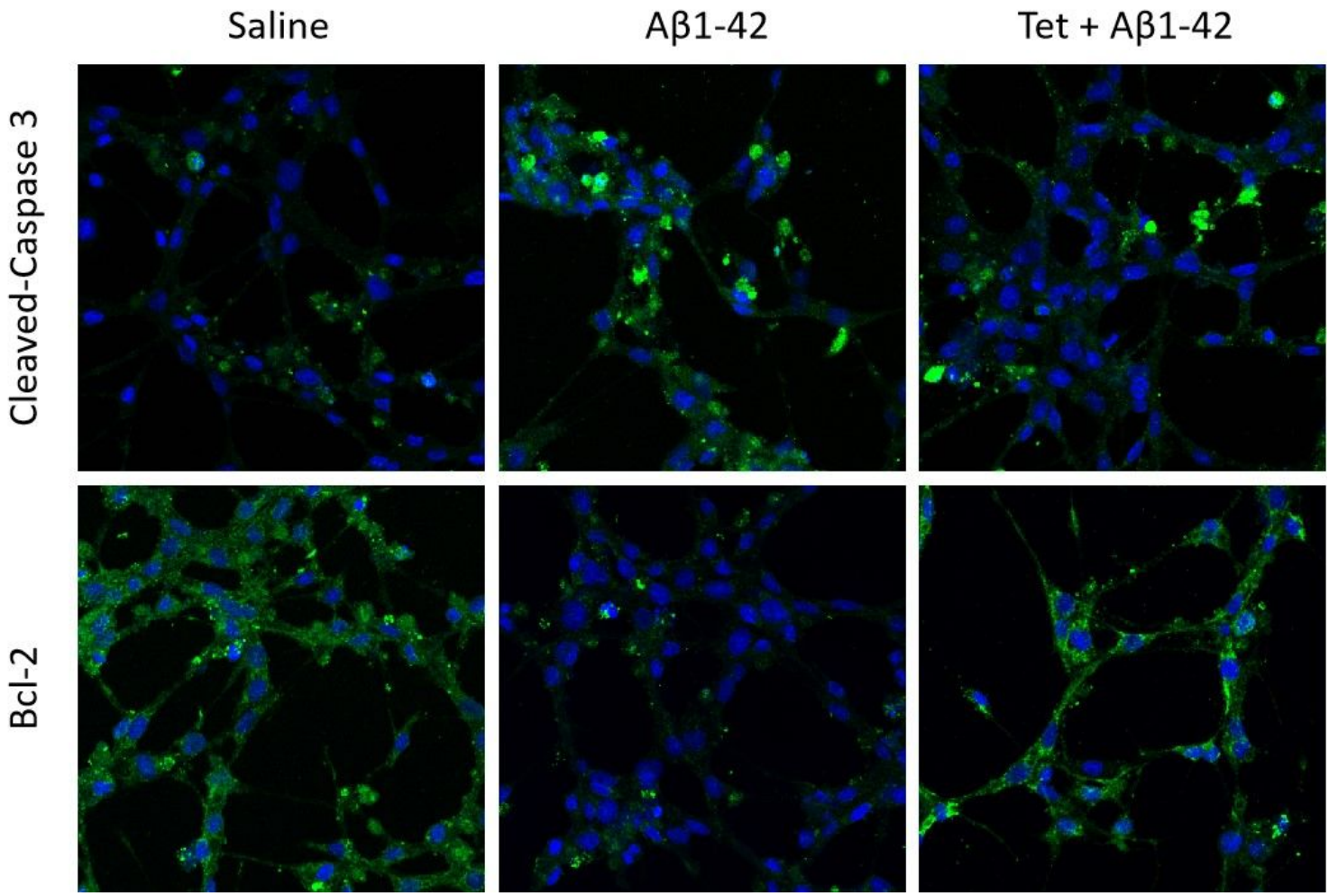

Figure 9

Immunofluorescent staining against cleaved-Caspase 3 and Bcl-2 in PC12-derived neurons. The treatment of PC12-derived neural cells with conditional medium from BV2 cells is the same as Figure 7. Cells were counterstained with DAPI (blue).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarymaterials.docx