

## Original Article

# Flavones from *vitis vinifera* L inhibits A $\beta_{25-35}$ -induced apoptosis in PC12 cells

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**Abstract:** Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extracellular deposits of beta-amyloid (A $\beta$ ) and neuronal loss. In this study, we evaluated the protective effects and the possible mechanism of action of flavones from *vitis vinifera* L (VTF) against A $\beta_{25-35}$ -induced cell damage in PC12 cells. The control group, model group and groups treated with different concentrations of VTF (20, 40, 80  $\mu$ g/mL) were established, and cellular ultrastructural changes were observed using transmission electron microscopy. Apoptotic cells were analysed by flow cytometry. The permeabilization of the cell membrane was detected with lactate acid dehydrogenase assays. Caspase-3 mRNA, bax mRNA and bcl-2 mRNA expression levels were measured using RT-PCR analyses. Protein (Akt, p-Akt<sup>Ser473</sup> and caspase-3) levels were assessed by western blotting. We found that VTF significantly and dose-dependently inhibited A $\beta_{25-35}$ -induced release of lactate dehydrogenase and led to increased survival of PC12 cells. VTF enhanced Akt phosphorylation in PC12 cells, and the protective effects were inhibited by LY294002. This was the first report on this research area, the data demonstrated that VTF reduced A $\beta_{25-35}$ -induced apoptosis not only by activating the PI3K/Akt pathway and expression of bcl-2, but also by suppressing the activity of caspase-3 and bax.

**Keywords:** Alzheimer's disease, cell apoptosis, PI3K/Akt, caspase-3, bcl-2, bax

## Introduction

Alzheimer's disease (AD) is the most common form of senile dementia, affecting millions of people worldwide. AD is a chronic neurodegenerative disease that is pathologically characterized by accumulation of intracellular neurofibrillary tangles (NFTs) and senile plaques (SP) in the extracellular space and neuronal loss, particularly in the hippocampus [1-3]. The "A $\beta$  hypothesis" has been widely accepted as one of the primary mechanisms of AD. It indicates that the A $\beta$  protein initiates the disease process, activating downstream neurotoxic mechanisms. Aggregated A $\beta$  plays a pivotal role in the pathogenesis of AD [4, 5]. Previous studies found that apoptosis was increased with the development and progression of AD [6-9]. A $\beta$  has been shown to cause neurotoxicity and cell apoptosis in vivo and in vitro [10-12], and various signalling pathways have been associated with the mechanisms underlying A $\beta$ -induced apoptosis thus far [13-17]. A $\beta$  accumulation is considered to be the distinct morphological

hallmark of early onset of AD [18]. To date, several studies have established AD cell models by using A $\beta$  to injure neurons [19, 20]. In addition, several studies indicated that proteins involved in A $\beta$ -induced apoptosis are potential therapeutic targets for the treatment of AD [21, 22]. Therefore, in this study, we chosen A $\beta_{25-35}$  treated PC12 cells as an AD cellular model.

Due to the increased ageing population and life expectancy, the incidence of this type of neurodegenerative disease has increased and is a serious threat to the health and quality of life of the elderly. AD is a complex disease, as the pathogenesis is unclear, and there is no effective treatment. *Vitis vinifera* L is one of the medicinal grape varieties that are known in the medical literature as "Shen Nong's herbal classic" and "Uygur Medicine Zhi". It is predominantly produced in Turpan and Hotan in the province of Xinjiang. It is used to treat mental restlessness in Uygur medicine. Flavonoids can slow down neurons damaged, and can delay the brain lesions [23, 24]. In this study, A $\beta_{25-35}$

**Table 1.** Primer sequences used for RT-PCR analysis

| cDNA           | Primers 5'-3'   | Product lengths |
|----------------|---|-----------------|
| $\beta$ -actin | Forward: GAGACCTTCAACACCCAGCC<br>Reverse: CCGTCAGGCAGCTCATAGCTC | 362 bp          |
| Bax            | F: CGGCGAATTGGAGATGAACTG<br>R: GCAAAGTAGAAGAGGGCAACC            | 161 bp          |
| Bcl-2          | F: GTCGCTACCGTCGTGACTT<br>R: CAGCCTCCGTTATCCTGGA                | 268 bp          |
| Caspase-3      | F: GGACCTGTGGACCTGAAAAA<br>R: GCATGCCATATCATCGTCAG              | 159 bp          |

was used to induce PC12 cells damage and to establish an AD model to investigate the VTF (Flavones from *Vitis vinifera* L) inhibitory effects and mechanism of nerve cell apoptosis.

## Materials and methods

### Chemicals and antibodies

The antibodies against  $\beta$ -actin were purchased from Sigma-Aldrich (MA, USA). The antibodies against phospho-Akt and Akt were purchased from Cell Signaling Technology (Boston, CA, USA). Caspase substrates were purchased from ProteintechGroupInc (CA, USA). Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were obtained from HyClone. WST-1 was purchased from Roche (Switzerland). A PI3k inhibitor, LY294002, was obtained from Cell Signaling Technology (Beverly, MA). Bcl-2 and Bax from Santa Cruz-Biotechnology (CA, USA). All other reagents were purchased from Sigma-Aldrich (MO, USA), unless otherwise indicated.

### Sample preparation

**Preparation of aggregated  $A\beta_{25-35}$  peptide:**  $A\beta_{25-35}$  was dissolved in deionized water to a concentration of 1 mmol/L and incubated at 37°C for 7 days. After aggregation, the sample was stored at 4°C.

### The preparation of flavones from *vitis vinifera* L

*Vitis vinifera* L was purchased from a Turpan Uygur medicine market. The preparation process is as follows: *Vitis vinifera* L were dried and the dried grapes were smashed; 95% ethanol was used for extraction for 3 times (2 hours each time), the extracting solution was merged for rotatory evaporation till no alcohol taste, extract with water after suspension, the purifi-

cation process of total flavonoids by AB-8 macroporousresin, water-95% ethanol gradient elution. Among them, 50% ethanol elution fraction was collected, vacuum drying at 60°C, get brown yellow powder---flavones from *Vitis vinifera* L (VTF).

### Cell culture and grouping

PC12 cells were obtained from the Shanghai Institute of Cellular Biology of the Chinese Academy of Sciences and maintained in high-glucose DMEM containing heat-inactivated foetal bovine serum (FBS) (10% v/v) in a humidified atmosphere of 5% carbon dioxide at 37°C.

PC12 cells were treated with  $A\beta_{25-35}$  in vitro for 24 h to establish an AD model. Cells were divided into the control group, model group (20  $\mu$ mol/L  $A\beta_{25-35}$ ) and VTF groups (20, 40, 80  $\mu$ g/mL).

For experiments involving kinase inhibitors, we had a control group, model group, VTF experimental group, LY294002 (50  $\mu$ mol/L) group, VTF+LY (50  $\mu$ mol/L) group.

### Cell ultrastructure observation

PC12 cells were seeded in 6-well culture plates at a density of  $5 \times 10^4$  cells per well. Then, the PC12 cells were washed 2 times in PBS and fixed by glutaraldehyde. Electron microscopy was used to observe the ultrastructural changes with a transmission electron microscope.

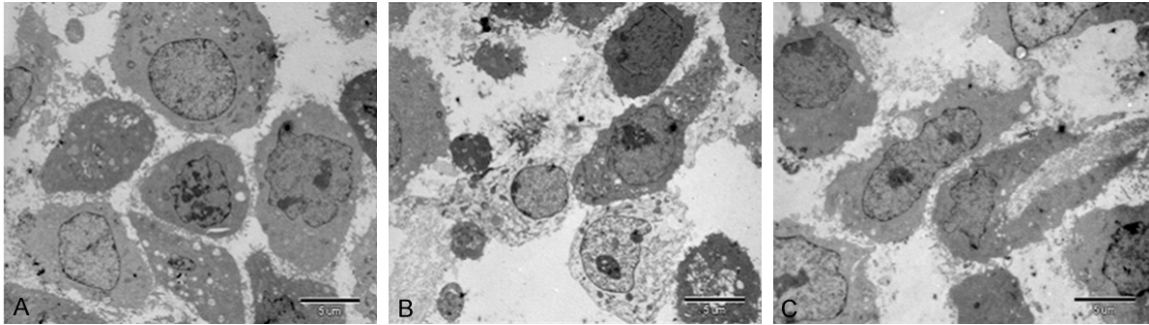
### Cell viability assay and LDH release assay

Cell viability was measured with a WST-1 assay and the cells were cultured for 24 h prior to the experimental VTF treatment. Optical density was measured by a microplate reader at 450 nm. Each group had 5 wells, and the experiment was repeated 3 times.

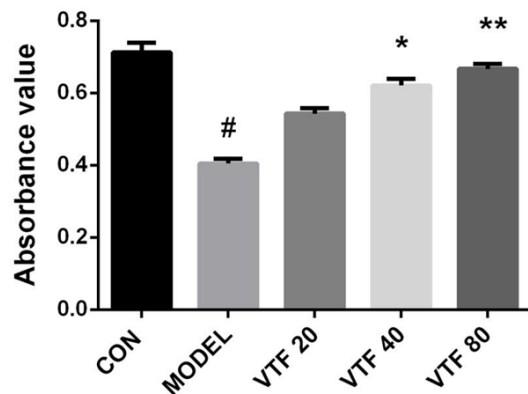
After treatment with  $A\beta_{25-35}$  for 24 h, the supernatant was collected separately, and LDH was detected according to the manufacturer's protocol. The absorbance of the samples was measured at 450 nm. Each group had 5 wells, and the experiment was repeated 3 times.

### Flow cytometric detection of apoptosis

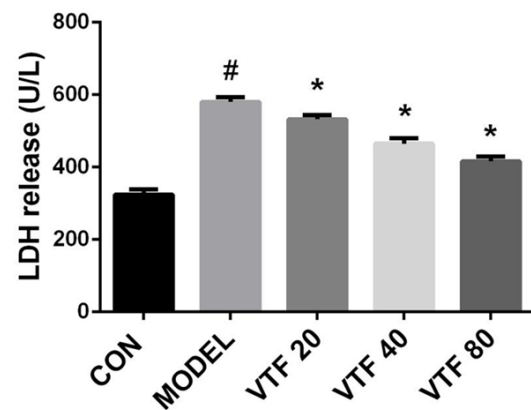
The cells were grouped as described above and seeded in 6-well plates. The cells were collected after digestion in each group. Briefly, PC12



**Figure 1.** Effect of VTF on cellular ultrastructure in PC12 cells induced by  $A\beta_{25-35}$  (transmission electron microscope,  $1\times 4000$ ). A. Control group; B. Model group; C. High-dose group.



**Figure 2.** Effect of VTF on absorbance value in PC12 cells ( $\bar{x} \pm s$ ). # $P < 0.01$  compared with the control group; \* $P < 0.05$ , \*\* $P < 0.01$  compared with the model group.



**Figure 3.** Effect of VTF on  $A\beta_{25-35}$ -induced damage in PC12 cells, as indicated by the LDH assay ( $\bar{x} \pm s$ ). # $P < 0.01$  compared with the control group; \* $P < 0.05$  compared with the model group.

cells were harvested by centrifugation, washed twice with ice-cold DMEM, resuspended in 100  $\mu$ L binding buffer, incubated for 15 min with 5  $\mu$ L Annexin V-FITC and 5  $\mu$ L PI at room temperature in the dark, and then analysed by flow cytometry.

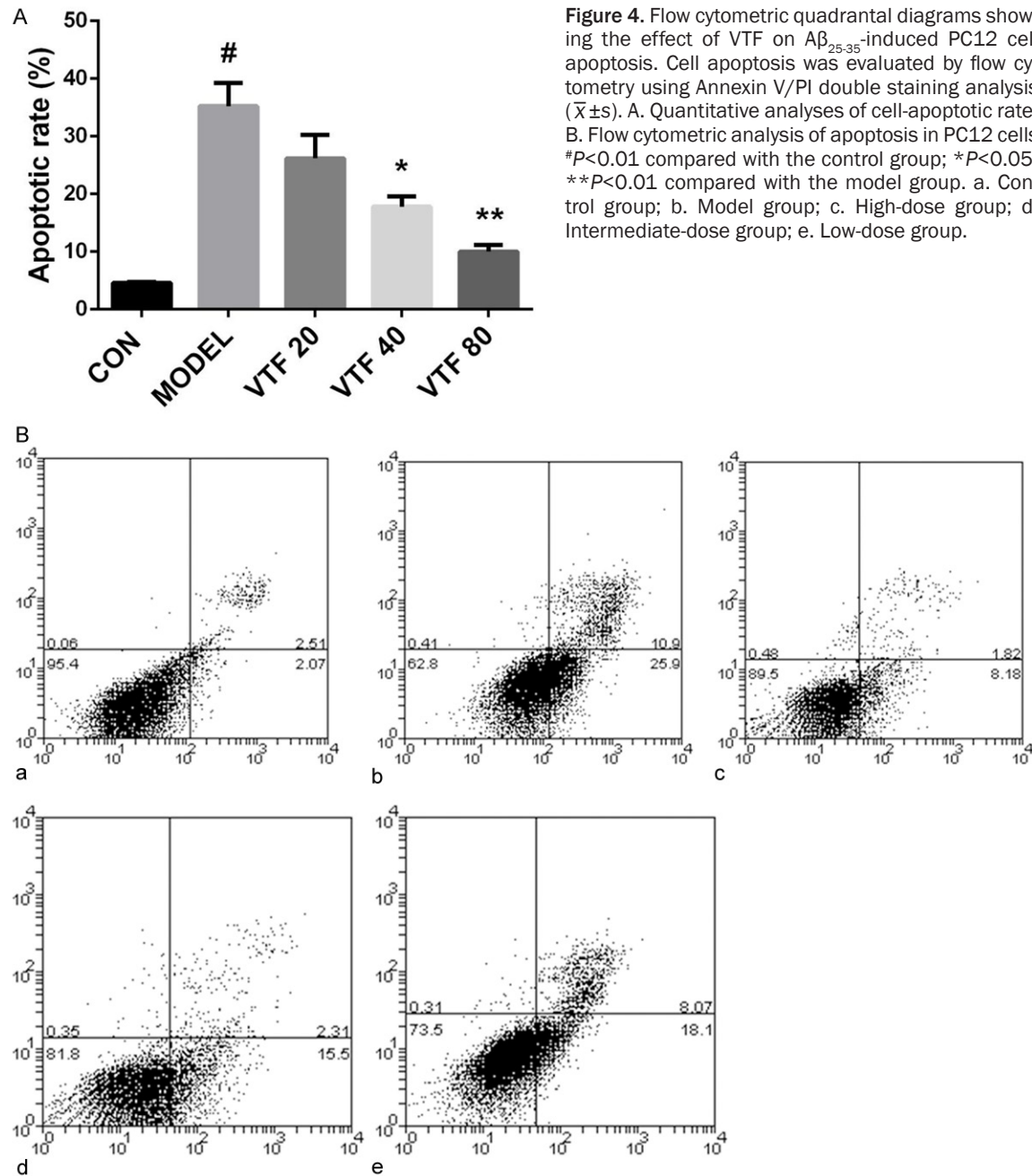
#### Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression levels were analysed via RT-PCR. cDNA was reverse transcribed from 1  $\mu$ g total mRNA using oligo dT. The PCR amplification protocol was 35 cycles at 94°C for 5 min and 30 s, at 57°C for 30 s and at 72°C for 30 s, with a 10 min final synthesis step at 72°C. The amplification products were electrophoresed on a 2% agarose gel containing ethidium bromide. Dissolution curve analyses were performed on the amplified PCR products. Sequence Detection Software 2.2 was used to analyze the data and calculate the Ct value. The relative expression quantities of

each sample (bcl-2/ $\beta$ -actin, bax/ $\beta$ -actin, caspase-3/ $\beta$ -actin) and reaction conditions are presented in **Table 1**.

#### Western blotting analysis

The PC12 cells were lysed with RIPA buffer. After the protein concentration was measured, 50  $\mu$ g of the cellular proteins was separated by 12% PAGE (Beyotime, China). The proteins were electrotransferred to PVDF membranes (Millipore). After the membranes were blocked for 2 h with 5% non fat milk in TBST, they were incubated overnight with primary antibodies (anti-phospho-Akt, 1:1000; Anti-Akt, 1:1000; Anti-caspase-3, 1:1000) overnight at 4°C. The membranes were again washed 6 times for 5 minutes in TBST, and the blots were incubated with the secondary horseradish peroxidase-conjugated antibody (1:50,000) at room temperature for 2 h. The blots were washed again, and the proteins of interest were detected



**Figure 4.** Flow cytometric quadrantal diagrams showing the effect of VTF on A $\beta_{25-35}$ -induced PC12 cell apoptosis. Cell apoptosis was evaluated by flow cytometry using Annexin V/PI double staining analysis ( $\bar{X} \pm s$ ). A. Quantitative analyses of cell-apoptotic rate. B. Flow cytometric analysis of apoptosis in PC12 cells # $P < 0.01$  compared with the control group; \* $P < 0.05$ , \*\* $P < 0.01$  compared with the model group. a. Control group; b. Model group; c. High-dose group; d. Intermediate-dose group; e. Low-dose group.

using an enhanced ECL system (Thermo). The chemiluminescence signals were visualized with X-ray film.

#### Statistical analyses

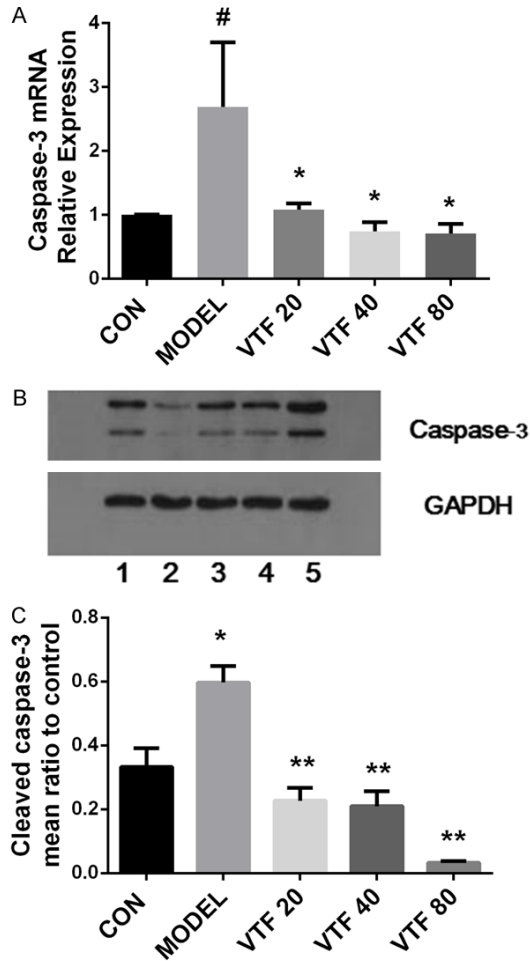
The data were analysed with SPSS 17.0 and presented as mean  $\pm$  standard error (SEM). One-way analysis of variance (ANOVA) was used to compare multiple groups.  $P < 0.05$  was considered statistically significant for all analyses.

## Results

### Effect of VTF on the cellular ultrastructure of PC12 cells

The cellular structure was normal in the control group (**Figure 1A**). The model group cells showed an irregular shape, shrinkage, loss of peripheral microvilli, expansion of the rough endoplasmic reticulum, cytoplasmic organelle swelling and vacuolar changes, increased





**Figure 5.** A. Caspase-3 mRNA in PC12 cells was quantified by RT-PCR. The levels of mRNA were calculated as nmol/nmol  $\beta$ -actin ( $\bar{x} \pm s$ ). <sup>#</sup> $P < 0.01$  compared with the control group; <sup>\*</sup> $P < 0.05$  compared with the model group. B, C. Caspase-3 was detected in PC12 cells treated with VTF. After PC12 cells were treated with VTF for 24 h, western blotting for caspase-3 and GAPDH was performed ( $\bar{x} \pm s$ ). <sup>\*</sup> $P < 0.05$  compared with the control group; <sup>\*\*</sup> $P < 0.01$  compared with the model group. 1. Control group; 2. High-dose group; 3. Intermediate-dose group; 4. Low-dose group; 5. Model group.

nuclear heterochromatin with chromatin agglutination and some pyknosis, and disappearance of the nucleolus. Model group cells displayed morphological changes in the early stage of apoptosis (**Figure 1B**). The high-dose group had a decreased number of injured cells. Cell morphology appeared normal, peripheral microvilli and the number of cell in divisions increased, and the nucleolus was large (**Figure 1C**).

#### Effects of VTF on cellular viability

When PC12 cells were pretreated with various concentrations of VTF (20, 40 and 80  $\mu\text{g/mL}$ ) for 4 h before treatment with  $\text{A}\beta_{25-35}$  for 24 h, the cell viability of the model group was decreased compared with that of the control group. Compared with the model group, the treatment group (20  $\mu\text{g/mL}$ ) slightly increase the cell viability, but this effect was not statistically significant ( $P > 0.05$ ), however, VTF significantly increased the cell viability at concentrations higher than 40  $\mu\text{g/mL}$  ( $P < 0.05$ ), at 80  $\mu\text{g/mL}$  ( $P < 0.01$ ), it markedly augmented the cell viability of  $\text{A}\beta_{25-35}$  peptide-treated cells (**Figure 2**).

#### Effect of VTF on LDH release

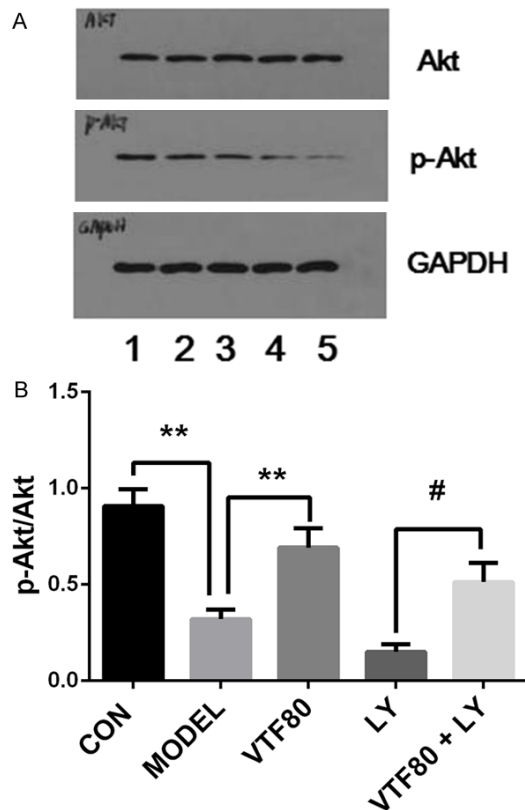
To evaluate the effect of VTF on  $\text{A}\beta_{25-35}$ -induced injury, which is characterized by cell membrane disruption and release of cellular contents, we measured LDH release as an index of PC12 cells damage. When the cells were incubated with 20  $\mu\text{mol/L}$   $\text{A}\beta_{25-35}$  for 24 h, LDH release significantly increased to  $580.00 \pm 13.6$  U/L (**Figure 3**); However, in cells pretreated with different doses of VTF, LDH release was significantly decreased compared with the model value ( $P < 0.05$ ). The effect of VTF did not significantly differ among the three concentrations tested.

#### Effect of VTF on apoptosis in PC12 cells

Apoptotic rates were detected by flow cytometry (**Figure 4**). The model group had an increased percentage of apoptotic cells ( $P < 0.01$ ) compared to that of the control group. The treatment group (40  $\mu\text{g/mL}$ ) had a reduced percentage of apoptotic cells compared to that of the model group ( $P < 0.05$ ). In addition, the apoptotic rate in high-dose group (80  $\mu\text{g/mL}$ ) was significantly lower than model group ( $P < 0.01$ ).

#### Effect of VTF on caspase-3 protein expression in PC12 cells

Western blot analysis of PC12 cells was performed to assess the effect of VTF on caspase-3 protein expression. An antibody against activated caspase-3 detected two bands at 32 kD and 17 kD. Western blot results showed that the level of cleaved caspase-3 in the model group was higher than in the control group

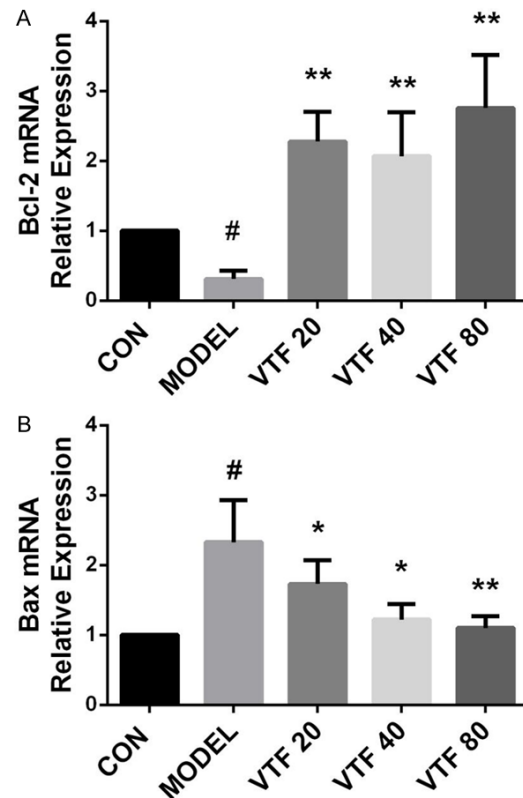


**Figure 6.** Proteins (Akt and p-Akt<sup>Ser473</sup>) were extracted and analysed by western blotting ( $\bar{X} \pm s$ ), \*\* $P < 0.01$  compared with the model group; # $P < 0.01$  compared with the LY group. 1. Control group; 2. VTF80; 3. VTF80+LY; 4. Model group; 5. LY.

( $P < 0.05$ ). The VTF group (20, 40 and 80  $\mu\text{g/mL}$ ) significantly decreased the level of cleaved caspase-3 ( $P < 0.01$ ), compared with the model group (Figure 5B, 5C). To investigate the mechanism of VTF on  $\text{A}\beta_{25-35}$ -induced apoptosis, we measured caspase-3 mRNA expression in PC12 cells by RT-PCR. As shown in Figure 5A, caspase-3 mRNA in the model group increased compared to the control group ( $P < 0.01$ ). Compared with the model group, the treatment groups had decreased expression of caspase-3 ( $P < 0.05$ ). VTF inhibits apoptosis by regulating expression of apoptosis related genes and proteins.

#### Effect of VTF on the PI3K/Akt pathway in PC12 cells

To further confirm the activation of Akt by VTF, we performed western blots to detect Akt and p-Akt<sup>Ser473</sup> in  $\text{A}\beta_{25-35}$ -treated PC12 cells with or without VTF. The expression of p-Akt/Akt in the VTF experimental group was higher than that in



**Figure 7.** Effect of VTF on  $\text{A}\beta_{25-35}$ -induced damage in Bcl-2 (A) and Bax (B) mRNA expression of PC12 cells ( $\bar{X} \pm s$ ), respectively, # $P < 0.01$  compared with the control group; \* $P < 0.05$ , \*\* $P < 0.01$  compared with the model group.

the model group, and the expression of p-Akt/Akt in the VTF+LY group was lower than that in the VTF group, which indicated that VTF enhanced Akt phosphorylation and LY294002 inhibited VTF-induced phosphorylation of Akt<sup>Ser473</sup>. Thus, the results showed that the protective effect of VTF was due, in part, to activation of the PI3K/Akt pathway (Figure 6).

#### Effect of VTF on bax and bcl-2 mRNA expression in PC12 cells

There was a significant ( $P < 0.01$ ) up-regulation of bax mRNA expression and down-regulation of bcl-2 mRNA expression in the model group as compared to the control group, indicating that  $\text{A}\beta_{25-35}$  can promote the apoptosis of PC12 cells. Compared with the model group, the high-dose VTF group (80  $\mu\text{g/mL}$ ) significantly reduced the mRNA levels of bax ( $P < 0.01$ ). However, the mRNA level of bcl-2 in each treatment group was largely upregulated than model group ( $P < 0.01$ ) (Figure 7).

## Discussion

AD is increasing rapidly as life expectancy is prolonged; The disease is predicted to become a major health care issue and an important social problem worldwide [25]. AD is a progressive, neurodegenerative disorder clinically characterized by memory loss and cognitive dysfunctions [26-28]. This disease is closely linked to cell apoptosis. In the initiation and development of AD, apoptosis results in neuronal loss, aggravates the pathological changes of the nerve cells, and affects learning and memory [7]. The abnormal accumulation of A $\beta$  is believed to have toxic effects on neurons and can induce neuronal apoptosis in vitro and in vivo. Therefore, neuronal apoptosis has become an important target in the development of preventive and therapeutic treatments of AD.

A $\beta$  is the major component of senile plaques, onset of AD is closely related to the deposition and aggregation of A $\beta$  in the brain, and AD pathogenesis is linked to the neurotoxicity of A $\beta$  [29]. In AD animal and cell models, A $\beta$  is commonly used to induce damage [30]. In this study, we used 20  $\mu$ mol/L A $\beta_{25-35}$  for 24 h as the optimal standard concentration and time point for the AD model. The experimental results showed that the cell survival rate of the model group decreased, and LDH activity was significantly increased in the model group. However, compared with the model group, the VTF-treated cells had a significantly increased survival rate, and the content of LDH was significantly decreased. Furthermore, with the increase in concentration, the protective effect was enhanced.

The PI3K/Akt pathway is a central signal transduction pathway involved in cell growth, survival and metabolism [31]. PI3K is the major survival-promoting protein for neurons. The phosphorylation of Akt-1 has been reported to be critically dependent on PI3K activity [32]. In this study, we hypothesized that VTF protected PC12 cells against A $\beta_{25-35}$ -induced apoptosis via the PI3K/Akt pathway. VTF activated the PI3K/Akt pathway, significantly promoted Akt phosphorylation and reduced apoptosis of PC12 cells. Western blot analyses showed that the expression of p-Akt/Akt in the VTF experimental group was higher than that in the model group. Moreover, the ability of VTF to protect against A $\beta$ -induced apoptosis was significantly

reduced when the Akt inhibitor LY294002 was added. Taken together, these results suggest that the cytoprotective effect of VTF was associated with PI3K/Akt activation, which indicated that VTF may have therapeutic potential for preventing the development or delaying the progression of AD.

Caspase-3 is the final executor of cellular apoptosis. A key member of the caspase family, caspase-3 plays an important role in cell apoptosis. Previous evidence for a role of caspase-3 in AD included its activation in A $\beta$ -treated neurons and immunodetection in AD brains, and in both cases, its function was always considered the terminal step in the biochemical cascade leading to apoptotic cell death [33, 34]. Our results showed that the relative expression of caspase-3 decreased after VTF treatment. Consistently, VTF suppressed the expression of cleaved caspase-3 and protected neurons from apoptosis. In addition, the results provided support for the hypothesis that antagonism of apoptosis provides therapeutic benefits.

The bcl-2 family is intricately involved in neuronal apoptosis; Bcl-2 is an important endogenous anti-apoptotic gene that prevents death from apoptotic in neurons, while bax is the most important pro-apoptotic gene in this family. We also found that VTF significantly enhanced the expression of bcl-2 mRNA, and decreased the expressions of bax mRNA. Accordingly, we inferred that the inhibition of apoptosis about VTF partly related to down-regulation of bax mRNA expression and up-regulation of bcl-2 mRNA expression.

In conclusion, our results demonstrated that VTF reduced A $\beta_{25-35}$ -induced apoptosis not only by activating the PI3K/Akt pathway and expression of bcl-2 but also by suppressing the activity of caspase-3 and bax, indicating that VTF can inhibit cell apoptosis by regulating the expression of apoptosis-related genes and proteins. In summary, the data presented in this study further support the protective potential of VTF in ameliorating cell membrane damage and reducing apoptosis. Our data indicated that VTF may have a neuroprotective effect on A $\beta_{25-35}$ -induced impairments in learning and memory. VTF should be tested in animal models of AD before being considered as a candidate for a clinical trial to prevent AD progression in humans.

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## Disclosure of conflict of interest

None.

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