Effect of polysaccharides from *Vitis vinifera* L. on NF-κB/IκB-α signal pathway and inflammatory factors in Alzheimer’s model rats

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**ABSTRACT**

The aim of this study was to investigate the mechanism of action underlying the effect of polysaccharide from *Vitis vinifera* L. (VTP) on the nuclear factor kappa B/inhibitor kappa B alpha (NF-κB/IκB-α) signaling pathway and related inflammatory factors in hippocampus of rats with Alzheimer’s disease (AD). Amyloid-β 25–35 was injected into the hippocampus to establish AD model rats, and the rats were administered with donepezil and VTP. The levels of interleukin-1β, interleukin-6 and tumour necrosis factor-α in the serum were determined by enzyme-linked immunosorbent assay. The phosphorylation level, protein and gene expression of NF-κBp65 and IκB-α in hippocampus were detected by immunohistochemistry, western blot and real-time quantitative polymerase chain reaction (PCR), respectively. VTP effectively improved the learning and memory ability of AD rats. Transmission electron microscopy indicated that VTP reduced the toxic effects of amyloid-β 25–35 on neurons in AD rats. Enzyme-linked immunosorbent assay showed that VTP inhibited the expression of interleukin-1β, interleukin-6 and tumour necrosis factor-α in a dose-dependent manner. Compared with the model group, the expression levels of NF-κBp65 in the nucleus of the VTP group decreased, which was consistent with the Western blotting results. The expression of p-IκB-α and IκB-α, and, the mRNA level of NF-κBp65 and IκB-α in VTP group were significantly decreased compared to that in the model group, indicating that VTP has a therapeutic effect on AD. Its mechanism may be related to the inhibition of inflammatory response.

**Introduction**

The main pathological features of Alzheimer’s disease (AD), a neurodegenerative disease, include neurofibrillary tangles and senile plaques, which further induce inflammation [1]. AD patients have a strong focal inflammatory reaction. A large number of activated glial cells and obvious inflammatory markers such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α are found around senile plaques in the brains [2,3]. High expression of inflammatory factors is found to be highly associated with AD, and inflammatory factors contribute to amyloid-β (Aβ) production in AD patients [4,5]. Chronic inflammation can activate inflammation-related signal transduction pathways, in which nuclear factor kappa B (NF-κB) plays an important regulatory role in the regulation of inflammatory response [6,7]. The activity of NF-κB is inhibited by inhibitor kappa B alpha (IκB-α) [6,7]. The phosphorylation and degradation of IκB-α as well as nuclear accumulation of NF-κB result in increased NF-κB activity, which further induces the production of a large number of inflammatory factors (IL-1β, IL-6 and TNF-α) [8,9]. These inflammatory factors induce a series of inflammatory reactions that cause neuronal damage [8,9].

In recent years, studies have shown that polysaccharides from *Lycium barbarum* L., *Astragalus membranaceus* and *Ganoderma lucidum* have protective effects on nerve cells [10–12]. These plant polysaccharides have no cytotoxicity and little side effects on organisms [10–12]. In our previous studies, polysaccharides from *Vitis vinifera* L. (VTP) showed many effects, such as antioxidant effect and antiapoptotic effect [13–15]. However, whether VTP has an effect on AD through regulating neuroinflammation is unclear. Therefore, this study investigates the molecular mechanism of VTP in the treatment of AD by inhibiting neuroinflammation.

**Materials and methods**

**Animals**

A total of 90 male Sprague Dawley (SD) rats (pathogen-free; 7–8 weeks; 250 ± 20 g body weight) were purchased from the Animal Center of Xinjiang Medical...
University (Quality certificate number: No.650007000). All animal experiments were conducted according to the ethical guidelines of Xinjiang Medical University. All efforts were made to minimize animal suffering.

Establishment of AD model

The AD model was established in rats after 1 week of normal feeding in the SPF laboratory. Briefly, rats were anesthetized using 3.0% sodium pentobarbital. The bilateral hippocampal CA1 region was obtained using the stereotaxic atlas and 10 μL of 2 μg/μL Aβ25–35 (Sigma, St. Louis, USA) solution was slowly injected into the hippocampal CA1 region (5 μL per side) in 10 min. The needle was left in place for 5 min. The wound was then sutured. Saline was injected as normal control.

Animal grouping

After modeling, the rats were randomly divided into six groups, including control group, model group, donepezil group, low-dose VTP group, medium-dose VTP group and high-dose VTP group, with 15 rats in each group. The rats in the donepezil group were treated with 0.5 mg/kg donepezil (Eisai China Inc., Shanghai, China). The rats in the low-dose, medium-dose and high-dose groups received 50 mg/ kg VTP, 150 mg/ kg VTP and 300 mg/ kg VTP, respectively. VTP was extracted and separated using a patented method (Patent No. 200710201354) [16]. Intragastric administration was performed once daily (1.0 mL/100 g) for 14 consecutive days. The rats in the control group and model group received an equal volume of saline.

Morris water maze test

Rats were subjected to double-blind water maze training on the 13th day of administration. Briefly, the rats practiced the maze for six consecutive days to learn their position relative to the escape platform in the positioning navigation experiment. The escape platform was placed in the third quadrant, and the rats were pooled from the first quadrant. Rats were given up to 90 s to find the escape platform. The required indices were recorded by a rat water maze system. After six days, the rats were subjected to the space exploration experiment of the Morris water maze. The escape platform was removed and the rats were pooled from the original position and allowed to swim for 90 s. The percentage of swimming time spent in the third quadrant within 90 s and the number of times the rats crossed the effective area (1.5 times of the original platform area) were recorded.

Sample collection

After water maze training, the rats were anesthetized with 10% chloral hydrate (0.4 mL/100 g). Blood was taken from the abdominal aorta and centrifuged at 3,000 rpm for 15 min to isolate the serum. Four rats in each group were selected for cerebral perfusion. Bilateral hippocampus was harvested (on ice) from the brain tissue of the remaining 11 rats. For 3 of the 11 rats, one side of the hippocampus was perfused with 4% paraformaldehyde for 24 h for immunohistochemistry assay. The remaining hippocampus tissues were stored at −80 °C.

Structure observation by transmission electron microscope

Three rats were randomly selected from each group and sacrificed by decapitation. The hippocampus was isolated from each rat, fixed in the electron microscope fixation liquid, dehydrated, embedded, sliced, and observed with electron microscope. The ultrastructural changes of hippocampal neurons were observed.

Enzyme-linked immunosorbent assay

The levels of TNF-α, IL-6 and IL-1β in serum were determined by enzyme-linked immunosorbent assay (ELISA). The Rat TNF-α ELISA Kit, Rat IL-6 ELISA Kit and Rat IL-1β ELISA Kit (Elabscience, Bethesda, United States) were used. The procedures were conducted according to the protocols provided by the kits. The absorbance value at 450 nm wavelength was read in a Multiskan MK3 microplate reader (Thermo, MA, United States).

Immunohistochemistry staining

The tissue sections were dewaxed in xylene and rehydrated in graded alcohols. Antigen retrieval was achieved by microwave. Then sections were incubated with 0.3% hydrogen peroxide to inactivate the endogenous peroxidase activity. After blocking with goat serum, the sections were incubated with primary antibodies against NF-κBp65 (CST Inc., Danvers, USA) at 4 °C overnight. After washing with PBS, horseradish peroxidase-conjugated secondary antibodies of IgG were added. Then the sections were developed with DAB chromogenic reagent. For the negative control, PBS instead of the primary antibody was used. The immunohistochemistry staining results were observed under a light
microscope. The cells with brown staining in the nucleus or cytoplasm were positive cells.

**Western blot**

The expression of IκBα, P-IκBα and p-NF-κBp65 was detected by western blot. Isolated total proteins and nucleoprotein extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking, the membranes were incubated with primary antibodies against GAPDH (Goodhere, Hangzhou, China), lamin B (Boster, Wuhan, China), P-IκBα and p-NF-κBp65 (CST Inc., Danvers, USA) at 4 °C overnight. Then the membranes were incubated with secondary antibodies of IgG at 37 °C for 2 h. Bands were visualized using an enhanced chemiluminescence kit (Pierce, Rockford, United States). The gray values were analyzed by ImageQuant software (GE Healthcare, Piscataway, NJ, USA). The relative expression levels were calculated based on the grey value of GAPDH or lamin B.

**Real-time quantitative polymerase chain reaction (PCR)**

The mRNA levels of NF-κB and IκB-α were detected by real-time quantitative PCR (RT-qPCR). Total RNA was extracted using Trizol Reagent (Invitrogen, CA, United States) according to the manufacturer’s instructions. Reverse transcription of total RNA was carried out with HiScript Reverse Transcriptase (RNase H) (Vazyme Bio-tech, Nanjing, China). RT-qPCR was performed with ABI7900 PCR (Illumina, CA, United states). Primes used in RT-qPCR were listed in Table 1 (Tsingke, Beijing, China). The specificity of all PCR products was confirmed by melting curve analysis. The expression was calculated using 2^{-ΔΔCt} as described previously [17].

**Statistical analyses**

The data were analyzed using SPSS 20.0 software. Data are expressed as mean values with standard error (± SEM). The Chi-square test was used to compare the differences between different groups. A P value of less than 0.05 was considered statistically significant.

**Results and discussion**

**Effect of VTP on memory learning in AD model rats**

The effect of VTP on the memory learning ability of AD model rats was detected by the water maze test. The results showed that the model group needed more time to find the platform than the remaining groups in the last 3 days of training (Figure 1(A)). On the 6th day, the escape latency of the model group was significantly increased than that of the control group (p < 0.01, Figure 1(B)). Compared with the model group, the escape latency of donepezil and low, medium and high-dose VTP groups were significantly reduced (p < 0.01, Figure 1(B)). Among these, the most significant change was observed in the high-dose VTP group (Figure 1(B)). In the space exploration experiment, the percentage of the retention time spent in the third quadrant and the number of times the rats crossed the effective area in the model group were significantly decreased compared to that of the control group (p < 0.01, Figure 1(C,D)); whereas the percentage of the retention time and the number of crossing times of the donepezil and low, medium and high-dose VTP groups were increased compared to those of the model group. These results suggest that VTP effectively improved the learning and memory ability of AD rats.

**Effect of VTP on ultrastructure of hippocampal neurons in AD model rats**

To investigate the effect of VTP on the ultrastructure of hippocampal neurons, transmission electron microscopy was used. In the control group, the nuclear membrane of nerve cells was smooth and the chromatin was fine and uniform; the structure of organelles in the cytoplasm was intact (Figure 2(A)). However, the features of apoptotic neurons, peripheral edema, nuclear chromatin condensation and margination as well as nuclear membrane rupture and folding were shown in the model group (Figure 2(B)). In the donepezil and low, medium and high-dose VTP groups, good neurons morphology, maintenance of nuclear membrane integrity with little folding, mild nuclear pyknosis with uniform chromatin and intact organelles in the cytoplasm were observed (Figure 2(C–F)). Altogether, these results indicate that VTP reduced the toxic effects of Aβ25–35 on neurons in AD rats.

**Effect of VTP on the release of inflammatory mediator in the serum of AD model rats**

Activation of microglial cells in the brain can produce and release a variety of inflammatory factors, such as
TNF-α, IL-6 and IL-1β, resulting in neuronal damage and neurological dysfunction [18–20]. To determine the effect of VTP on the release of inflammatory mediators in the serum of AD model rats, the serum levels of TNF-α, IL-6 and IL-1β were determined by ELISA. The results showed that the levels of TNF-α, IL-6 and IL-1β in the model group were significantly higher than those in the control group (p < 0.01, Figure 3). Administration of VTP significantly reduced the levels of IL-1β, TNF-α and IL-6 (Figure 3). These results demonstrated that the effect of VTP on learning and memory impairment in AD model rats may be related to the inhibition of neuroinflammation.

**Effect of VTP on NF-κB/IκB-α signal pathway-related protein expression in hippocampus of AD model rats**

To study the effect of VTP on the NF-κB/IκB-α signal pathway-related protein expression in the hippocampus, immunohistochemistry staining and western blot were carried out. The immunohistochemistry results showed that the particles positive for NF-κBp65 were mainly located in the cytoplasm, occasionally in the nucleus (Figure 4). In the control group, only a small amount of cytoplasm was stained and coloured lighter (Figure 4(A)). Compared with the control group, a large volume of cytoplasm and nucleus was stained and coloured darker (Figure 4(B)). There were less NF-κBp65-positive particles...
in the VTP groups than that in the model group (Figure 4 (C–F)).

Furthermore, we detected the expression of NF-κB-p65, p-IκB-α and IκB-α by western blot. As shown in Figure 5, the expression of p-NF-κB-p65 protein in the hippocampus nucleus of the model group was 3.88-fold higher than that of the control group, and the difference was statistically significant (p < 0.01). Compared with the model group, the levels of p-NF-κB-p65 in the nucleus of donepezil and VTP groups were significantly reduced (P < 0.01). Among these, the most significant changes were observed in the high-dose VTP and donepezil group (Figure 5). Meanwhile, the expressions of p-IκB-α and IκB-α were also detected by western blotting. The results showed that the expression levels of p-IκB-α and IκB-α in the model group were significantly increased compared to that in the control group (P < 0.01, Figure 6). Compared with the model group, the expressions of p-IκB-α and IκB-α in VTP groups decreased, and the most obvious decrease was observed in the high-dose VTP group (P < 0.01, Figure 6). Finally, these results suggest that VTP inhibited the phosphorylation of IκB-α to further inhibit the NF-κBp65 nuclear translocation induced by Aβ25–35.

Figure 2. Electron microscopic observation of hippocampal CA1 neurons in each group. Control group (A), model group (B), donepezil group (C), low-dose VTP group (D), medium-dose VTP group (E), and high-dose VTP group (F).

Note: Scale bar = 5 μm (A, C, E and F), and 2 μm (B, D).

Figure 3. Expression of serum inflammatory factors in each group detected by ELISA. Expression of TNF-α (A), IL-6 (B), IL-1β (C).

Note: *P < 0.01 compared with the control group; **P < 0.05 compared with the model group; ***P < 0.01 compared with the model group.
Effect of VTP on the mRNA level of the NF-κB/IκB-α signaling pathway in the hippocampus of AD model rats

To further study the effect of the VTP on NF-κB/IκB-α signaling pathway at the gene expression level, total RNA of the hippocampus was extracted and RT-qPCR was performed. As shown in Figure 7, the mRNA levels of NF-κBp65 and IκB-α in the hippocampus of the model group were significantly higher than those in the control group ($P < 0.01$). Compared with the model group, the mRNA levels of NF-κBp65 and IκB-α in the donepezil and high-dose VTP groups were significantly decreased, and the difference was statistically significant ($P < 0.01$). These results show that VTP inhibits the activation of NF-κBp65.

The ‘Aβ hypothesis’ of the pathogenesis of AD has been widely accepted, which suggests that Aβ protein activates downstream neurotoxicity and thus causes AD.

Figure 4. Expression of NF-κBp65 in hippocampal slices of each group detected by immunohistochemical staining. Control group (A), model group (B), donepezil group (C), low-dose VTP group (D), medium-dose VTP group (E) and high-dose VTP group (F).

Note: Magnification 400×.

Figure 5. Expression of p-NF-κB-p65 in the hippocampus of each group. Western blot (A); relative expression (B).

Note: Lamin B was an internal control. Means ± SEM ($n = 3$). $^1P < 0.01$ compared with the control group; $^{**}P < 0.01$ compared with the model group.
Aggregation of $A\beta$ plays an important role in the pathogenesis of AD [22,23]. In this study, the animal model of AD was established using $A\beta_{25-35}$, the core fragment of $A\beta_{1-42}$ [24]. The Water maze test showed that the learning and memory ability of the model group decreased significantly, and AD-like manifestations were found. Compared with the model group, the spatial learning and memory capacity in the VTP and donepezil groups were improved significantly in the space navigation and space exploration experiments. In addition, VTP and Donepezil significantly reduced the nuclear pyknosis and apoptotic neurons, which indicated that VTP could decrease the toxic effect of $A\beta$ on AD model rats.

NF-$\kappa$B is an important nuclear transcription factor, and is closely related to brain injury and cerebrovascular disease [25,26]. In resting-state, NF-$\kappa$B and $\kappa$B-$\alpha$ in the
cytoplasm are in the form of trimer (inactive), and NF-κB cannot enter the nucleus [27,28]. When there is external stimulus, IκB-α is phosphorylated and degraded, therefore NF-κB is translocated into the nucleus and binds to target genes. NF-κB located in the nucleus could regulate the downstream expression of inflammatory genes, increase the inflammatory damage, and ultimately lead to neuronal damage in the brain and inflammatory response [27,28]. To further explore the mechanism of VTP against neuronal inflammation of the AD model rats, we detected the expression of NF-κB p65. The results showed that the expression of NF-κB p65 protein in the nucleus in the model group was significantly higher than that in the control group, and the mRNA level of NF-κB also showed the same trend. The expression of NF-κB p65 in the model group was significantly higher than that in the control group. These results suggest that NF-κB can be activated by Aβ25–35 and is translocated into the nucleus. Compared with the model group, the protein expression and mRNA level of NF-κBp65 were significantly decreased in the VTP groups, especially in the high-dose VTP group, which indicated that VTP could inhibit the activation of NF-κB induced by Aβ25–35.

After IκB-α is phosphorylated and degraded, NF-κB is activated and translocated from the cytoplasm to the nucleus. Therefore, IκB-α is as an important molecular switch in NF-κB activation [29]. To investigate whether VTP acts upstream of NF-κB activation, the expression, phosphorylation level and gene transcription of IκB-α protein were detected in the hippocampus. The mRNA levels of p-IκB and IκB-α in the model group were significantly higher than those in the control group, and the mRNA levels of p-IκB-α and IκB-α in VTP groups were lower than those in the model group, especially in high-dose VTP group. These results indicate that VTP may inhibit the phosphorylation of IκB-α induced by Aβ25–35 and further inhibit the activation of NF-κBp65.

TNF-α is a proto-proinflammatory cytokine that plays a key role in the pathogenesis of inflammation and AD. It can induce neuronal cell cycle events and promote neuronal toxicity [30]. IL-1 is a major proinflammatory cytokine and plays an important role in neuroinflammatory states and the activation of NF-κB [31]. IL-1β can also induce astrocyte activation to produce IL-6, and thus form a vicious circle [18]. Our study found that VTP inhibited the production and release of TNF-α, IL-6 and IL-1β.

Studies have shown that inhibition of NF-κB activity have great benefits in the treatment of inflammatory diseases (including AD) [32–34]. Combined with the ‘Aβ hypothesis’ and the ‘inflammatory response theory’, we propose that the mechanism of VTP on AD may be mediated through the NF-κB/IκB-α signalling pathway. VTP may inhibit the phosphorylation of IκB-α to further inhibit the NF-κBp65 nuclear translocation induced by Aβ25–35, and thereby control the immune and inflammatory cascade to protect the brain cells, improve memory capacity.

Conclusions
The results from this study demonstrate that VTP has a therapeutic effect on AD in a rat model. The mechanism underlying the therapeutic effect may be related to the inhibition of inflammatory response. The correlation between the expression of inflammatory cytokines TNF-α, IL-6 and IL-1β and the expression of phosphorylated NF-κBp65 signaling molecule and its role in the pathogenesis of AD are worthy of further study.

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Disclosure statement
All authors declare no financial competing interests. All authors declare no non-financial competing interests.

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