

κ -carrageenan-derived pentasaccharide attenuates $A\beta_{25-35}$ -induced apoptosis in SH-SY5Y cells via suppression of the JNK signaling pathway

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Abstract. β -amyloid ($A\beta$)-mediated neuronal apoptosis is an important pathological feature of Alzheimer's disease (AD). Inhibiting apoptosis induced by $A\beta$ may lead to the development of a potential therapeutic target for AD treatment. κ -carrageenan-derived pentasaccharide (KCP) extracted from marine red algae is involved in a variety of biological activities and may be an effective in the treatment of AD. The present study aimed to investigate the neuroprotective effect of KCP against $A\beta_{25-35}$ -induced neurotoxicity in SH-SY5Y cells, and to examine the potential underlying mechanisms. The results of the present study revealed that pretreatment with KCP significantly attenuated $A\beta_{25-35}$ -induced loss of cell viability and apoptosis, as evaluated by MTT assays and annexin V/propidium iodide staining, respectively, in a dose-dependent manner. Furthermore, KCP downregulated the protein expression levels of $A\beta_{25-35}$ -induced cleavage caspase 3 by inhibiting the overactivation of the JNK signaling pathway. The results of the present study indicated that KCP attenuated $A\beta_{25-35}$ -induced neuroblastoma cell cytotoxicity, suggesting that KCP may be a potential therapeutic agent for the treatment of AD.

Introduction

Alzheimer's disease (AD) is the most frequent cause of dementia in the elderly. The global incidence rates of dementia reached ~24 million in 2012, and is predicted to double every 20 years until the year 2040 (1). Therefore, the prevention and treatment of AD will become a significant economic burden on

public health services in the future. As a progressive neurodegenerative disorder, AD is characterized by the accumulation of β -amyloid ($A\beta$) peptides, hyperphosphorylated tau proteins and neuronal loss. Resistance of amyloid deposition and tau protein aggregation has been the primary focus of research into the treatment of AD in recent decades. Although the exact etiology of AD remains unclear, neuronal apoptosis is a primary reason for the loss of neurons (2). Amyloid cascade theory is currently the leading theory for the etiology of AD (3). Immunotherapeutic methods have been demonstrated to reduce amyloid-induced cytotoxicity and neurodegeneration (4). The most notable of these immunotherapies are bapineuzumab and solanezumab. However, the results of phase III trials involving anti-amyloid treatment of AD with solanezumab and bapineuzumab have yielded disappointing results (5,6). Karran and Hardy (7) dismiss the overwhelming evidence obtained over the past few years demonstrating that anti- $A\beta$ therapy to remove brain $A\beta$ deposits in patients with AD was a clinical failure. The results of these phase III clinical trials involving solanezumab and bapineuzumab demonstrate that AD is a complex disease, involving a number of pathogenic factors. The use of anti-amyloid therapies for the treatment of AD remains controversial. Therefore, a combination of strategies to treat AD is required. Despite the known role of N-methyl-D-aspartate receptor antagonists and acetylcholinesterase inhibitors in improving cognitive function and delaying the progression of cognitive dysfunction, further exploration of the development of novel and more effective therapeutic strategies for AD treatment are urgently required (8). $A\beta_{25-35}$ is a synthetic peptide constituting 11 amino acids, which is not detected under normal physiological conditions. However, various investigators have used it as a model for full-length peptide studies, as it is a biologically active fragment of $A\beta$ and contains the structure responsible for neurotoxicity. Previous studies have demonstrated that oxidative stress is involved in the generation of $A\beta_{25-35}$ toxicity, which in turn involves the disruption of calcium homeostasis, oxygen radicals and nitric oxide, ultimately leading to mitochondrial dysfunction. Mitochondrial cytochrome c release and apoptosis-linked proteases subsequently initiate apoptosis (9). Therefore, antioxidants may inhibit the apoptosis induced by $A\beta_{25-35}$. κ -carrageenan-derived oligosaccharide, extracted and purified from marine red algae, is an antioxidant (10), and exerts antiviral (11), anti-aggregant and anticoagulant (12)

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Abbreviations: $A\beta$, β -amyloid; AD, Alzheimer's disease; KCP, κ -carrageenan pentasaccharide; JNK, c-Jun N-terminal kinase

Key words: Alzheimer's disease, apoptosis, β -amyloid, neurotoxicity, c-Jun N-terminal kinase

effects. Compared with other antioxidants, κ -carrageenan oligosaccharides have small molecular weights, good solubility, and a wide range of biological activities. In addition, studies have demonstrated that $A\beta$ can directly activate the release of inflammatory cytokines in microglia, and induce a respiratory burst to generate a large volume of peroxide free radicals, which damage neurons (13). κ -carrageenan oligosaccharides have been demonstrated to inhibit the excessive activation of microglia (14). Stress-activated protein kinases belong to the serine/threonine kinase family (15). Of these, the c-Jun N-terminal kinases (JNKs) have been implicated in the pathogenesis of AD. The introduction of $A\beta$ peptides into primary cortical neuron cultures has been demonstrated to induce JNK activation and cell death (16). Increasing evidence indicates that the JNK signaling pathway is activated in AD in susceptible regions of the brain. $A\beta_{25-35}$ -induced neuronal dysfunction is, in part, mediated by alterations in signal transduction pathways including JNK (17). κ -carrageenan-derived pentasaccharide (KCP, the structure of which is presented in Fig. 1) is a typical κ -carrageenan oligosaccharide and, to the best of our knowledge, the neuroprotective effect of KCP against $A\beta_{25-35}$ -induced damage remains to be investigated. The present study examined the effect of KCP on $A\beta$ -induced apoptosis via the JNK signaling pathway in SH-SY5Y cells.

Materials and methods

Cells and reagents. SH-SY5Y human neuroblastoma cells were donated by the Institute of Neuroscience, Chongqing Medical University (Chongqing, China). $A\beta_{25-35}$ was purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) and was incubated at 37°C for 7 days prior to use (18). KCP was obtained from Primus Qingdao Huili Biotechnology Co., Ltd (Qingdao, China). Low glucose Dulbecco's modified Eagle's medium (DMEM), L-glutamine and trypsin were purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA), and fetal bovine serum (FBS) was from Tianjin Hao Yang Biological Company (Tianjin, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and the Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) kit were purchased from Nanjing KenGen Biotech Co., Ltd. (Nanjing, China). Rabbit anti-cleaved caspase 3 (cat. no. 9664), rabbit anti-total JNK (cat. no. 9252) and rabbit anti-phosphorylated (p)-JNK (cat. no. 9251) antibodies for western blotting were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-GAPDH (cat. no. AG019), anti-mouse horseradish peroxidase (HRP)-conjugated IgG secondary antibody (cat. no. A0216), and anti-rabbit HRP-conjugated IgG secondary antibody (cat. no. A0208) were obtained from Beyotime Institute of Biotechnology (Haimen, China). Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody (cat. no. ZF-0511) was obtained from Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd., (Beijing, China).

Cell culture. SH-SY5Y cells were cultured in low-glucose DMEM with 10% FBS, 1% L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. The media was replaced every two days.

Cells that had reached 70-80% confluence were used for experiments.

Experimental protocol. To determine a suitable concentration of $A\beta_{25-35}$, cells were divided into four groups. The control cells were cultured in normal medium, whereas the remaining three groups were exposed to 0, 12.5, 25 or 50 μ M $A\beta_{25-35}$ for 24 h. Subsequently, SH-SY5Y cells were divided into five groups, including control, $A\beta_{25-35}$ only, and three groups treated with $A\beta_{25-35}$ plus 25, 50 or 100 μ M KCP pre-incubated with SH-SY5Y cells for 2 h. Experiments were performed following an incubation period of 24 h.

Cell viability assay. SH-SY5Y cells were seeded at a density of 1×10^4 cells/well in 96-well plates. Following overnight drug treatment, cell viability was evaluated by the MTT assay. Briefly, 50 μ l MTT solution was added into each well, and following a 4-h incubation at 37°C, the supernatant from each well was carefully removed. DMSO (150 μ l) was added into each well to solubilize the formazan product. The plate was agitated for 10 min to ensure complete dissolution of formazan. Absorbance at a wavelength of 490 nm was measured using a SpectraMax® M2 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA).

Detection of apoptosis. SH-SY5Y cells were seeded onto 6-well plates at 2×10^5 cells/well prior to drug treatment. The cells were digested using trypsin without EDTA and collected at each time point, from two wells per sample. They were subsequently washed twice with PBS and centrifuged at $1,100 \times g$ for 5 min at the room temperature. The supernatant was discarded and 500 μ l binding buffer was added to the resuspended cells, followed by the addition of 5 μ l annexin V-FITC and 5 μ l PI. The cells were incubated for 15 min at room temperature in the dark. The apoptotic rate was assessed by flow cytometry using a BD Influx™ flow cytometer with the BD FACS™ software (BD Biosciences, San Jose, CA, USA).

Western blotting. SH-SY5Y cells were seeded into T25 cell culture flasks. Following drug treatment, cells were scraped and lysed in a lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin and 1 mM phenylmethylsulfonyl fluoride, and incubated on ice for 30 min. Following centrifugation at $13,350 \times g$ for 15 min at 4°C, the supernatant was collected into 1.5-ml Eppendorf tubes. Protein concentrations were determined by the bicinchoninic acid assay. Equal amounts of total protein (40 μ g) were loaded onto 10% and 12% SDS-PAGE gels, and the resolved proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology) for 60 min before incubating with primary antibodies (JNK, 1:800; p-JNK, 1:800; cleaved caspase 3, 1:1,000). GAPDH (1:800) served as a loading control. Proteins were detected using an Enhanced Chemiluminescence reagent (Advansta Inc., Menlo Park, CA, USA). The signals were quantified using Quantity One® software (version, 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunofluorescence. SH-SY5Y cells were seeded onto an 8x8 mm cell climbing film used to cover 12-well plates. Following drug treatment, cells were fixed with 4% paraformaldehyde at room temperature for 20 min and washed three times with PBS. Cells were lysed with 0.1% Triton X-100 for 10 min at 37°C, washed three times and blocked with 4% FBS for 30 min at 37°C. Cells were subsequently incubated with anti-cleaved caspase 3, 1:400, overnight at 4°C. Following washing with PBS, cells were incubated with an Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody (dilution, 1:400) for 1 h at 37°C, washed and stained with DAPI for 5 min. Finally, the cells were mounted with 50% glycerol and observed under a fluorescence microscope.

Statistical analysis. Data were obtained from three separate cultures and expressed as the mean \pm standard deviation. Statistical analyses were conducted in GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data from multiple groups were compared by one-way analysis of variance, whereas differences between two groups were compared by Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of varying concentrations of $A\beta_{25-35}$ on cell viability. The MTT assay was used to determine $A\beta_{25-35}$ -induced toxicity. SH-SY5Y cells that were exposed to $A\beta_{25-35}$ for 24 h demonstrated significant dose-dependent cytotoxicity (Fig. 2A). Compared with untreated cells, the survival of SH-SY5Y cells that were exposed to varying concentrations of $A\beta_{25-35}$ declined to $87.26 \pm 2.91\%$ at $12.5 \mu\text{M}$ ($P = 0.0489$), $67.29 \pm 2.37\%$ at $25 \mu\text{M}$ ($P = 0.0015$) and $47.58 \pm 1.19\%$ at $50 \mu\text{M}$ ($P = 0.0001$), with a maximal difference of $\sim 40\%$ at a concentration of $50 \mu\text{M}$ (Fig. 2A). Therefore, $50 \mu\text{M}$ $A\beta_{25-35}$ was selected for use in subsequent experiments.

Effect of increasing KCP concentrations on $A\beta_{25-35}$ -treated cells. The MTT assay was used to determine the protective effects of KCP against $A\beta_{25-35}$ -induced toxicity. Compared with the untreated group, the survival of cells exposed to $50 \mu\text{M}$ $A\beta_{25-35}$ significantly decreased to $43.08 \pm 2.08\%$ ($P = 0.001$), whereas pretreatment with various concentrations of KCP increased viability in a dose-dependent manner to $53.18 \pm 1.44\%$ ($P = 0.0162$) at $25 \mu\text{M}$, $75.38 \pm 2.23\%$ ($P = 0.0004$) at $50 \mu\text{M}$ and $85.70 \pm 2.12\%$ ($P = 0.0001$) at $100 \mu\text{M}$ (Fig. 2B). Therefore, KCP may significantly inhibit the cytotoxicity of $A\beta_{25-35}$ to improve the survival of SHSY5Y cells.

KCP reduces apoptosis in SH-SY5Y cells. To examine whether $A\beta_{25-35}$ -induced cell death was the result of apoptosis, flow cytometry was performed. Cells were stained with annexin V-FITC and PI and apoptotic cells were defined as annexin V⁺PI⁺. In the untreated group, the apoptotic rate was $3.57 \pm 0.95\%$, which increased to $40.63 \pm 2.0\%$ ($P < 0.0001$; Fig. 3) on exposure to $50 \mu\text{M}$ $A\beta_{25-35}$. Following pretreatment of SH-SY5Y cells with various concentrations of KCP, the apoptotic rate decreased to $31.40 \pm 1.18\%$ ($P = 0.0023$) at $25 \mu\text{M}$, $20.00 \pm 1.85\%$ ($P = 0.0002$) at $50 \mu\text{M}$ and $11.57 \pm 1.25\%$ ($P < 0.0001$) at $100 \mu\text{M}$ (Fig. 3). Therefore, KCP signifi-

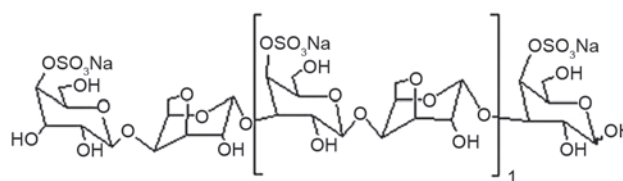


Figure 1. Chemical structure of κ -carrageenan-derived pentasaccharide.

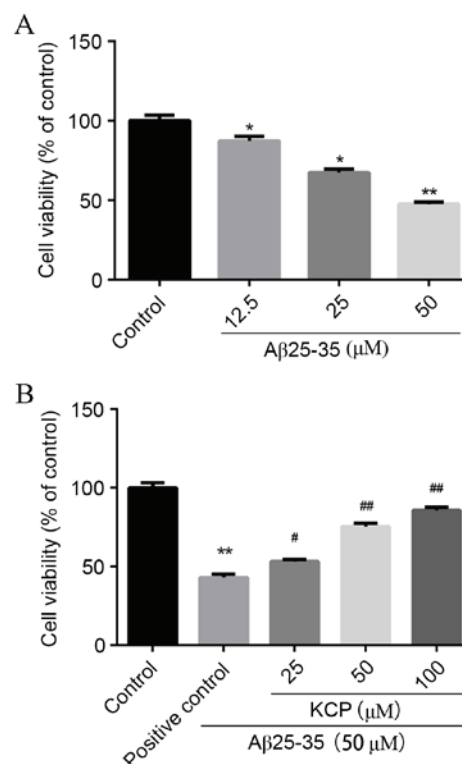


Figure 2. Effect of $A\beta_{25-35}$ and KCP on the viability of SH-SY5Y cells, as assessed by MTT assays. (A) Cells were treated with 12.5, 25 or $50 \mu\text{M}$ $A\beta_{25-35}$ for 24 h. $A\beta_{25-35}$ decreased cell viability in a dose-dependent manner. (B) Cells were pretreated with 0, 25, 50 or $100 \mu\text{M}$ KCP for 2 h followed by exposure to $50 \mu\text{M}$ $A\beta_{25-35}$ for 24 h. KCP treatment attenuated $A\beta_{25-35}$ -induced cytotoxicity in a dose-dependent manner. Data are expressed as the mean \pm standard deviation ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ vs. control; # $P < 0.05$ and ## $P < 0.01$ vs. $A\beta_{25-35}$ alone. $A\beta$, β -amyloid; KCP, κ -carrageenan pentasaccharide.

cantly reduced the rate of apoptosis induced by $A\beta_{25-35}$, in a dose-dependent manner.

Evaluation of cleaved caspase 3 protein expression levels in SH-SY5Y cells treated with $A\beta_{25-35}$ in the absence or presence of KCP. Cleaved caspase 3 is an important marker of apoptosis; therefore its protein expression levels in SH-SY5Y cells were analyzed by western blotting (Fig. 4A). SH-SY5Y cells treated with $A\beta_{25-35}$ ($50 \mu\text{M}$) demonstrated increased protein expression levels of cleaved caspase 3 ($P < 0.0001$; Fig. 4A), indicating activation of apoptosis. However, following pretreatment with KCP, the protein expression levels of cleaved caspase 3 decreased significantly in a dose-dependent manner ($P = 0.0193$, $P = 0.0002$ and $P < 0.0001$, respectively; Fig. 4A). These results were verified by immunofluorescence (Fig. 4B).

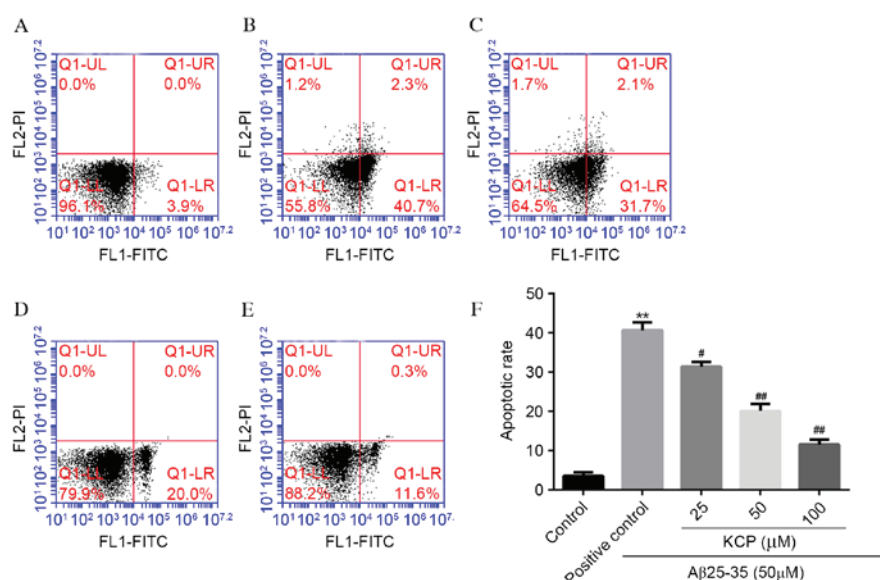


Figure 3. KCP protects SH-SY5Y cells against $A\beta_{25-35}$ -induced apoptosis. Cells were treated with $A\beta_{25-35}$ in the absence or presence of KCP and stained with annexin V-FITC and PI. Representative cytograms of (A) untreated, (B) $A\beta_{25-35}$ -treated, (C) $A\beta_{25-35}$ + 25 μ M KCP-treated, (D) $A\beta_{25-35}$ + 50 μ M KCP-treated and (E) $A\beta_{25-35}$ + 100 μ M KCP-treated cells are presented. Annexin V*PI⁺ cells (lower right quadrant) were defined as apoptotic. (F) Apoptotic rate of cells. Data are expressed as the mean \pm standard deviation (n=3). **P<0.01 vs. control; #P<0.05 and ##P<0.01 vs. $A\beta_{25-35}$ alone. KCP, κ -carrageenan pentasaccharide; $A\beta$, β -amyloid; FITC, fluorescein isothiocyanate; PI, propidium iodide.

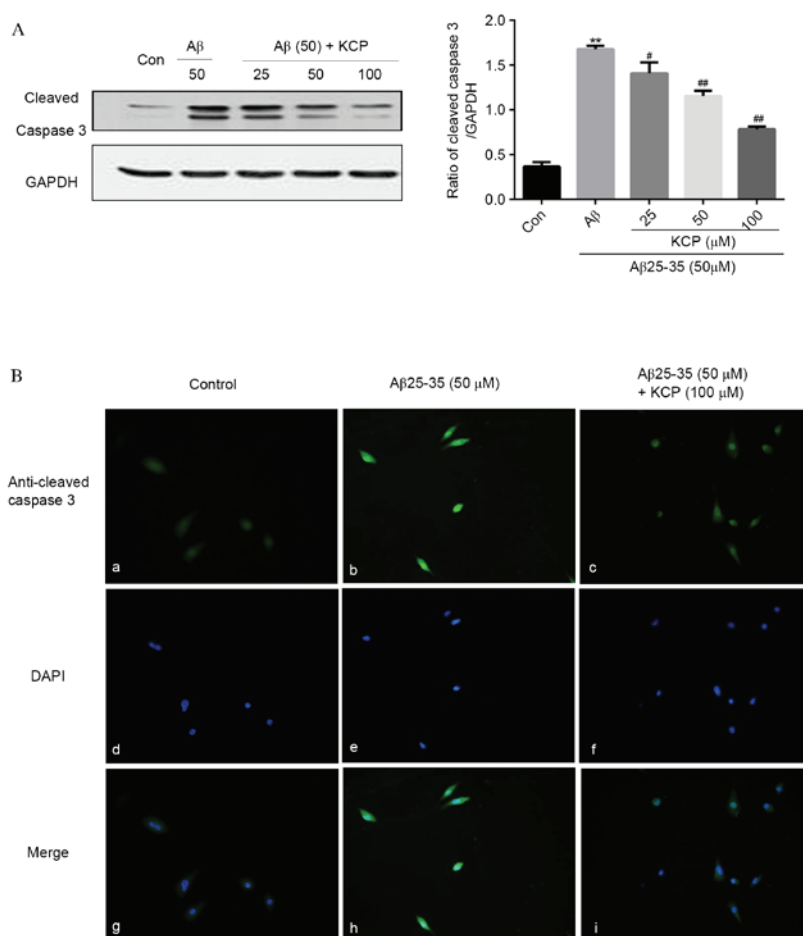


Figure 4. Effect of KCP on $A\beta_{25-35}$ -induced cleaved caspase 3 expression in SH-SY5Y cells. (A) Protein expression levels of cleaved caspase 3 in SH-SY5Y cells pre-incubated with KCP for 2 h and treated with $A\beta_{25-35}$ for 24 h, as detected by western blotting. KCP pretreatment reduced the protein expression levels of cleaved caspase 3 induced by $A\beta_{25-35}$ in a dose-dependent manner. The relative intensity of cleaved caspase 3/GAPDH was calculated. Data are expressed as the mean \pm standard deviation (n=3). *P<0.05 and **P<0.01 vs. control; #P<0.05 and ##P<0.01 vs. $A\beta_{25-35}$ alone. (B) Western blotting results were confirmed using immunofluorescence. Cells were stained with anti-cleaved caspase 3 (green) and DAPI (blue). Magnification, x40. KCP, κ -carrageenan-derived pentasaccharide; $A\beta$, β -amyloid.

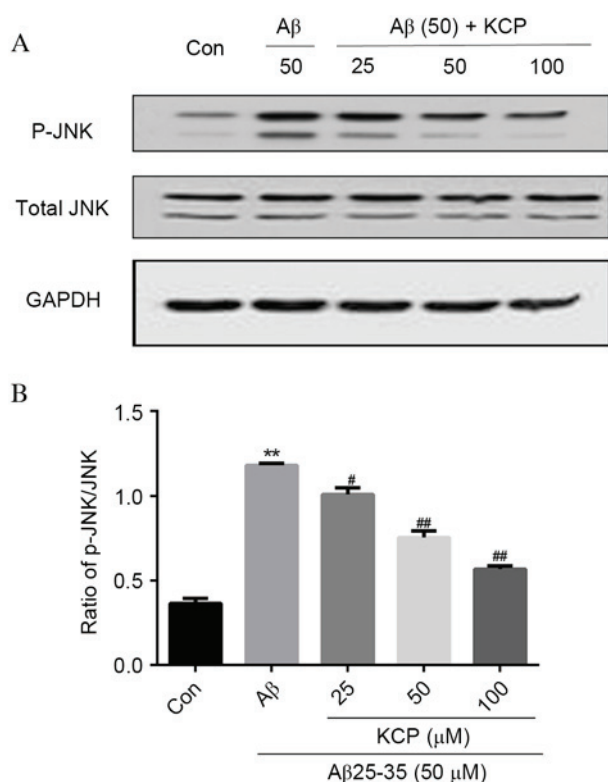


Figure 5. Effect of KCP on $A\beta_{25-35}$ -induced JNK signaling in SH-SY5Y cells. (A) Protein expression levels of p-JNK and total JNK in SH-SY5Y cells pre-incubated with KCP for 2 h and treated with $A\beta_{25-35}$ for 24 h, as detected by western blotting. (B) Quantification of western blotting. KCP pretreatment reduced the ratio of p-JNK/JNK induced by $A\beta_{25-35}$ in a dose-dependent manner. Data are expressed as the mean \pm standard deviation ($n=3$). * $P<0.05$ and ** $P<0.01$ vs. control; # $P<0.05$ and ## $P<0.01$ vs. $A\beta_{25-35}$ alone. KCP, κ -carrageenan-derived pentasaccharide; A β , β -amyloid; JNK, c-Jun N-terminal kinase; p, phosphorylated.

Role of the JNK signaling pathway in the protective effects of KCP against $A\beta_{25-35}$ -induced apoptosis in SH-SY5Y cells. The JNK signaling pathway mediates cellular apoptosis. In contrast to the untreated group, cells treated with 50 μ M $A\beta_{25-35}$ demonstrated considerably increased p-JNK protein expression levels ($P<0.0001$; Fig. 5B). However, treatment of cells with a combination of 50 μ M $A\beta_{25-35}$ and 25, 50 or 100 μ M KCP significantly decreased p-JNK protein expression levels in a dose-dependent manner ($P=0.0019$, $P<0.0001$ and $P<0.0001$, respectively; Fig. 5B) compared with cells treated with 50 μ M $A\beta_{25-35}$ alone.

Discussion

The present study demonstrated the protective effect of KCP against $A\beta_{25-35}$ -induced damage. Activation of caspase 3 and the JNK signaling pathway may be involved in this process. These findings indicated the neuroprotective potential of KCP by inhibiting the excessive activation of certain signaling pathways in neuronal cells.

Apoptotic neuronal cell death is a primary characteristic of numerous degenerative diseases, including AD. Caspases, a family of cysteine proteases, are critical for apoptosis in the central nervous system (19). Caspase 3, which is known as the final effector of apoptosis, splits into the two subunits

(17 and 19 kD) of cleaved caspase 3, and promotes apoptosis by stimulating certain apoptotic factors (20). The results of annexin V/PI staining and the protein expression levels of cleaved caspase 3 supported the finding that KCP may protect SH-SY5Y neuronal cells from $A\beta_{25-35}$ -induced apoptosis.

JNK is a serine/threonine kinase that is involved in cellular responses including proliferation, mitogenic stimuli, environmental stress and apoptosis (21). The expression and activation of JNK was first observed in the brains of patients with AD (22). In a subsequent study, JNK activation was observed in the primary cortical neuron cultures incubated with A β peptides and in transgenic mice overexpressing mutant PS1 (M146L), as well as in the human AD brain. In addition, incubation of A β peptides with primary cortical neuron cultures induced JNK activation and cell death (16). Furthermore, the activation of the JNK signaling pathway has been observed in the $A\beta_{25-35}$ -induced rat model of AD (23). Inhibiting the activation of the JNK signaling pathway attenuates the $A\beta_{25-35}$ -induced toxicity in primary neurons (24). To examine the importance of the JNK signaling pathway, a previous study used a JNK inhibitor, which improved learning and long-term memory in A β -injected rats (25). The JNK inhibitor, CEP-1347 (KT7515) protected PC12 cells and sympathetic neurons from A β -induced death, which indicates that the JNK signaling pathway acts relatively proximally and triggers the death mechanism (26). The present study confirmed that $A\beta_{25-35}$ activated the JNK signaling pathway; KCP inhibited the increased activation of this pathway, thereby inhibiting apoptosis. Although activation of the JNK signaling pathway appears necessary for $A\beta_{25-35}$ -induced cell death, it may be one of many possible mechanisms by which apoptosis is activated in these cells.

The present study has certain limitations, in that it focuses on the occurrence of apoptosis. It did not verify the specific process by which KCP attenuates $A\beta_{25-35}$ -induced apoptosis. Further studies are required to investigate this. However, the results of the present study suggested that KCP inhibits $A\beta_{25-35}$ -induced apoptosis of SH-SY5Y cells and that the JNK signaling pathway is involved in this process.

In conclusion, the results of the present study demonstrated a preliminary underlying mechanism to support the hypothesis that KCP possesses neuroprotective properties, and elucidates the specific role of JNK in this process. The attenuation of $A\beta_{25-35}$ -induced neuroblastoma cell cytotoxicity by KCP suggested that KCP may be a potential therapeutic agent for the treatment of AD.

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