

Puerarin inhibits β -amyloid peptide 1-42-induced tau hyperphosphorylation via the Wnt/ β -catenin signaling pathway

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Abstract. Excessive tau protein phosphorylation is important in the pathogenesis and early abnormal signal transduction of Alzheimer's disease. Excessive phosphorylation of microtubules is associated with tau accumulation, which induces the formation of neurofibrillary tangles in neurons, leading to synaptic damage and ultimately, neurodegeneration. The present study aimed to investigate the possible mechanism underlying the inhibitory effects of puerarin on β -amyloid peptide (A β)1-42-induced tau protein hyperphosphorylation in SH-SY5Y cells. Following various treatments, the viability of SH-SY5Y cells was determined using the MTT assay, and cell morphology was observed under an inverted fluorescence microscope. Western blotting was used to detect tau phosphorylation, and the protein expression levels of glycogen synthase kinase (GSK)-3 β , phosphorylated (p)-GSK-3 β (Ser9), β -catenin and cyclin D1, which are the key factors mediating the Wnt/ β -catenin signaling pathway in SH-SY5Y cells. The results demonstrated that puerarin reversed the A β 1-42-induced decrease in SH-SY5Y cell viability. In addition, puerarin inhibited the degree of A β 1-42-induced tau phosphorylation at Ser396, Ser199 and Thr231 in SH-SY5Y cells, and reduced the expression of GSK-3 β by increasing the expression of p-GSK-3 β (Ser9). Furthermore, puerarin increased the protein expression levels of β -catenin and cyclin D1, which are key factors involved in the Wnt/ β -catenin signaling pathway. The results of the present study demonstrated that puerarin may attenuate A β 1-42-induced tau hyperphosphorylation in SH-SY5Y cells, by inhibiting the expression of GSK-3 β and activating the Wnt/ β -catenin signaling pathway; therefore, puerarin may exert protective effects against Alzheimer's disease.

Introduction

Alzheimer's disease (AD), also known as senile dementia, is a degenerative disease of the central nervous system that is predominantly characterized by progressive degeneration of cognitive function and memory loss. Previous studies have suggested that disorders of the Wnt/ β -catenin signaling pathway in the brains of patients with AD may be associated with pathological alterations (1,2), including formation of neurofibrillary tangles (NFTs) involving neurites and phosphorylated tau proteins, and neuronal loss and senile plaques triggered by β -amyloid peptide (A β) accumulation. The Wnt/ β -catenin signaling pathway may therefore be considered the core pathway linking A β neurotoxicity with tau hyperphosphorylation.

Glycogen synthase kinase (GSK)-3 β is a multifunctional serine/threonine kinase and a negative regulator of Wnt/ β -catenin signaling. Its abnormal expression is closely associated with the pathogenesis, pathological manifestations and treatment of AD (2). Previous studies have suggested that GSK-3 β is a protein kinase that may induce abnormal tau phosphorylation in the brains of patients with AD and phosphorylation of tau at numerous sites (3,4).

Puerarin is the main active ingredient derived from the Chinese herb root of *Pueraria lobata*, which is a selective inhibitor of GSK-3 β (5,6). Previous research has indicated that puerarin downregulates A β expression in the brain, inhibits the abnormal expression of tau protein induced by A β , reduces cellular apoptosis, and improves learning and memory in animals (7-10). Puerarin exhibits anti-AD activity by inhibiting the activity of GSK-3 β and activating the Wnt/ β -catenin signaling pathway. At present, it is unknown whether puerarin ameliorates AD via the Wnt/ β -catenin signaling pathway. Therefore, the present study aimed to investigate the role of oligomeric peptide A β 1-42 in SH-SY5Y cell impairment, and the inhibitory effects of puerarin on tau hyperphosphorylation via the Wnt/ β -catenin pathway.

Materials and methods

Drugs and reagents. The SH-SY5Y human neuroblastoma cell line was obtained from the Chinese Academy of Sciences, Kunming Cell Biological Research Institute (Kunming,

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China), and was used in the present study. Oligomeric peptide A β 1-42 (cat no. 107761-42-2) was purchased from GL Biochem (Shanghai), Ltd. (Shanghai, China) and puerarin (2 ml; 100 mg; 130,803) was obtained from Zhejiang CONBA Pharmaceutical Co., Ltd. (Zhejiang, China). The reagents used were as follows: anhydrous lithium chloride (LiCl; cat no. 213233; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), Dulbecco's modified Eagle's medium (DMEM; high glucose), and fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following antibodies were used: Rabbit anti-phosphorylated (p)-tau (Ser396; cat no. ab109390), rabbit anti-p-tau (Ser199; cat no. ab81268), rabbit anti-p-tau (Tau231; cat no. ab151559), rabbit anti- β -catenin (cat no. ab32572), rabbit anti-GSK-3 β (cat no. ab32391), rabbit anti-p-GSK-3 β (Ser9; cat no. ab75814), rabbit monoclonal anti-cyclin D1 (cat no. ab134175), mouse anti- β -actin (cat no. ab179467) (all from Abcam, Cambridge, MA, USA), goat anti-rabbit IgG (H+L) highly cross-adsorbed Alexa Fluor® Plus 555-conjugated secondary antibody (cat no. A32732) and goat anti-mouse IgG (H+L) highly cross-adsorbed Alexa Fluor® Plus 555-conjugated secondary antibody (cat no. A32727) (both from Invitrogen; Thermo Fisher Scientific, Inc.).

Cell culture and treatment. SH-SY5Y cells were cultured in DMEM (high glucose) supplemented with 10% heat-inactivated FBS, 100 μ g/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified incubator containing 5% CO₂. Cells were passaged once every 5-7 days, and cells in the logarithmic growth period were obtained.

MTT assay. The MTT assay was used to evaluate cell survival as a function of mitochondrial viability; the following procedure was performed at 37°C. SH-SY5Y cells were subcultured in 96-well plates at a density of 5x10⁵ cells/ml/well. After 24 h attachment, cells were treated with 10, 50, 100 and 150 μ mol/l puerarin. Following a 24 h pretreatment with puerarin, 30 μ mol/l oligomeric peptide A β 1-42 was added to the wells and the plate was cultured for a further 24 h. Subsequently, 20 μ l MTT (5 mg/ml PBS stock solution) was added to each well and the cells were incubated for 4 h at 37°C, after which, the medium was removed and the cells were treated with 150 μ l DMSO. The culture plate was gently agitated until the crystals were completely dissolved, and optical density values were measured at 540 nm using a microplate reader and the inhibitory ratio of cell proliferation was calculated.

Cell morphological analysis. SH-SY5Y cells in the logarithmic growth phase were seeded at a density of 5x10⁵ cells/ml/well in a 6-well plate. Cells were divided into the following groups: The control group, the AD model group (30 μ mol/l A β 1-42), the puerarin treatment groups (10, 50 or 100 μ mol/l), and the positive control group (10 mmol/l LiCl). After cell attachment on day 2, the medium in the 6-well plate was removed. Serum-free DMEM was added to the control and AD model groups; DMEM with puerarin, at a final concentration of 10, 50 and 100 μ mol/l, was added to the drug treatment groups; and DMEM combined with LiCl at a final concentration of 10 mmol/l was added to the positive control group; 50 μ l was added per well for all groups. After 24 h, with the exception of

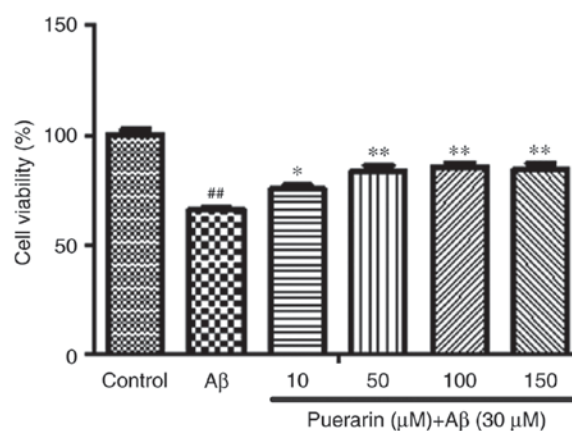


Figure 1. Effects of various doses of puerarin on the viability of A β 1-42-treated SH-SY5Y cells. Data are presented as the mean \pm standard deviation; n=5. ##P<0.01 compared with the control group; *P<0.05, **P<0.01 compared with the Alzheimer's disease model group. A β , β -amyloid peptide.

the control group, 30 μ mol/l A β 1-42 was added to the remaining groups. After culturing for a further 24 h, cell morphology was observed under an inverted fluorescence microscope. All procedures described were performed at 37°C.

Protein expression detection by western blotting. Cells were treated as aforementioned. Subsequently, the medium was removed and cells were washed twice with pre-cooled PBS. Radioimmunoprecipitation assay lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β -glycerophosphate, 1 mM EDTA, 1 mM Na₃VO₄ and 0.5 mg/ml leupeptin; 200 μ l] was added to each well and the cells were lysed for 30 min over ice. The cell lysates were then centrifuged at 12,000 x g for 15 min at 4°C, the supernatants were collected, and protein content was quantified using the bicinchoninic acid method. Equal protein samples (20 μ g) from each group were separated by 10% SDS-PAGE and were transferred to polyvinylidene fluoride membranes by semi-dry transfer. The membranes were then blocked with 5% non-fat milk for 2 h at room temperature, after which the membranes were incubated with the following primary antibodies at 4°C overnight: Rabbit anti-p-tau (Ser396; 1:10,000), rabbit anti-p-tau (Ser199; 1:5,000), rabbit anti-p-tau (Thr231; 1:1,000), rabbit anti- β -catenin (1:5,000), rabbit anti-GSK-3 β (1:10,000), rabbit anti-p-GSK-3 β (Ser9; 1:10,000) and rabbit anti-cyclin D1 (1:10,000); β -actin (1:10,000) served as an internal control. After washing the membranes with TBS containing 20% Tween-20 (TBST), Alexa Fluor® Plus 555-conjugated secondary antibodies (1:1,000) were added to the membranes and incubated for 1.5 h in the dark at 37°C. Blots were visualized using chemiluminescence (Invitrogen; Thermo Fisher Scientific, Inc.) and were exposed to radiographic films. The same experiment was repeated three times. Density of the bands was measured and analyzed using a gel electrophoresis image analyzer (GS-900™ Calibrated Densitometer with integrated Image Lab™ v6.0 software; cat no. 1707991; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and are expressed as the absorbance ratio of the target protein to the internal control.

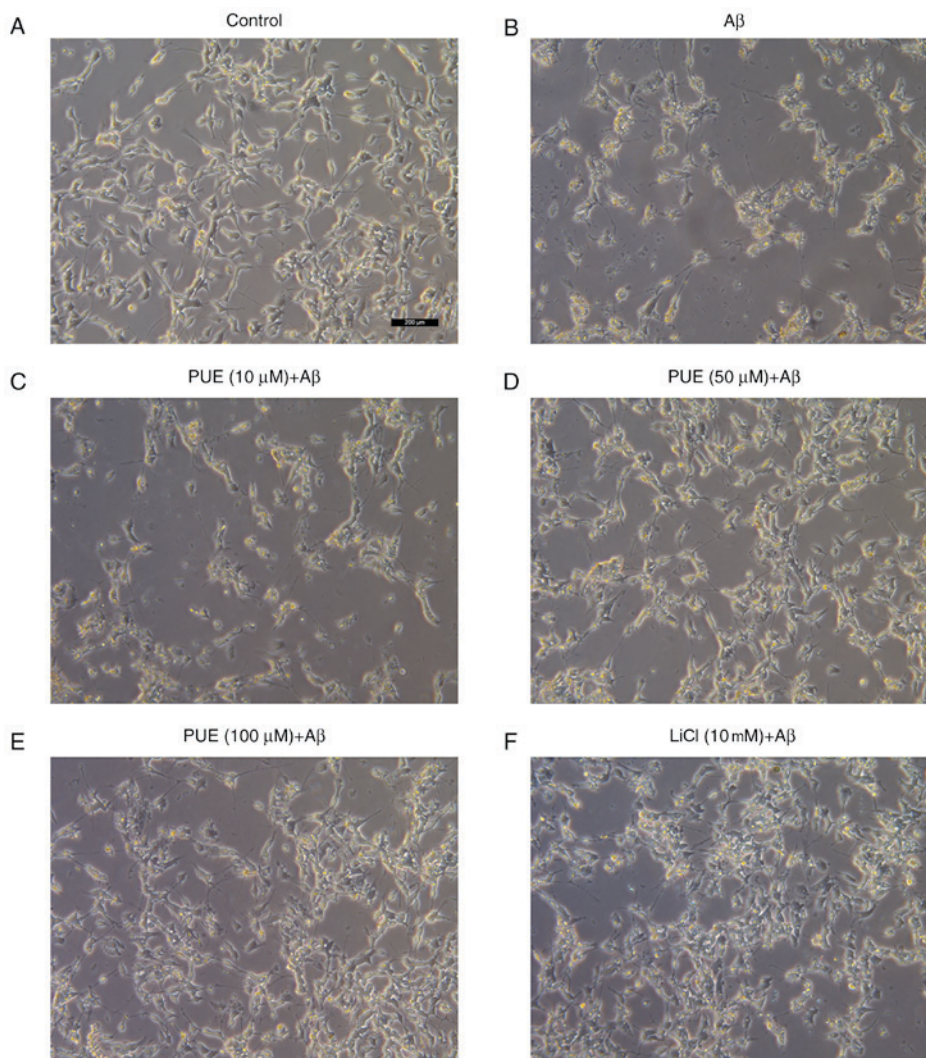


Figure 2. Effects of puerarin on the morphology of SH-SY5Y cells damaged by A β 1-42. (A) Control cells; (B) cells treated for 24 h with 30 μ M A β 1-42; (C) cells treated for 24 h with 10 μ M puerarin + 30 μ M A β 1-42; (D) cells treated for 24 h with 50 μ M puerarin + 30 μ M A β 1-42; (E) cells treated for 24 h with 100 μ M puerarin + 30 μ M A β 1-42; (F) cells treated for 24 h with 10 mM LiCl + 30 μ M A β 1-42. Scale bar: 200 μ m. A β , β -amyloid peptide; LiCl, lithium chloride.

Statistical analysis. Data are presented as the mean \pm standard deviation. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) were used to conduct statistical analyses. Data were statistically analyzed using one-way analysis of variance, with either the Kruskal-Wallis H, the least significant difference or the Games-Howell post hoc tests depending on the data. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of puerarin on the viability of A β 1-42-treated SH-SY5Y cells. As presented in Fig. 1, 30 μ mol/l A β 1-42 significantly reduced the viability of SH-SY5Y cells compared with the control group ($P < 0.01$). However, treatment with puerarin (10, 50, 100 and 150 μ mol/l) significantly enhanced the viability of SH-SY5Y cells in a dose-dependent manner, compared with the A β group ($P < 0.05$). These results suggested that puerarin may inhibit A β 1-42-induced decreases in SH-SY5Y cell viability and exert a cytoprotective effect.

Effects of puerarin on the growth of A β 1-42-treated SH-SY5Y cells. As presented in Fig. 2, cells in the control group were normal with high refraction, clearly visible axons and adherence, when observed under an inverted microscope. A day after the addition of A β 1-42 to the model group, cell diopeter was degraded, cell processes were significantly shortened or broken, and the cell number was decreased. Cell morphology in the puerarin (10, 50 and 100 μ mol/l) + A β group was markedly improved compared with that of the model group. Puerarin inhibited the loss of cellular synapse, and partially enhanced cell diopeter and number in a dose-dependent manner. The effects of puerarin (50 μ mol/l) were comparable to those of LiCl, thus indicating that various doses of puerarin protected SH-SY5Y cells from A β 1-42-induced damage.

Effects of puerarin on tau protein phosphorylation in A β 1-42-treated SH-SY5Y cells. As presented in Fig. 3, compared with the control group, the levels of tau phosphorylation at Ser396, Ser199 and Thr231 sites were significantly increased in the AD model group ($P < 0.01$). Conversely,

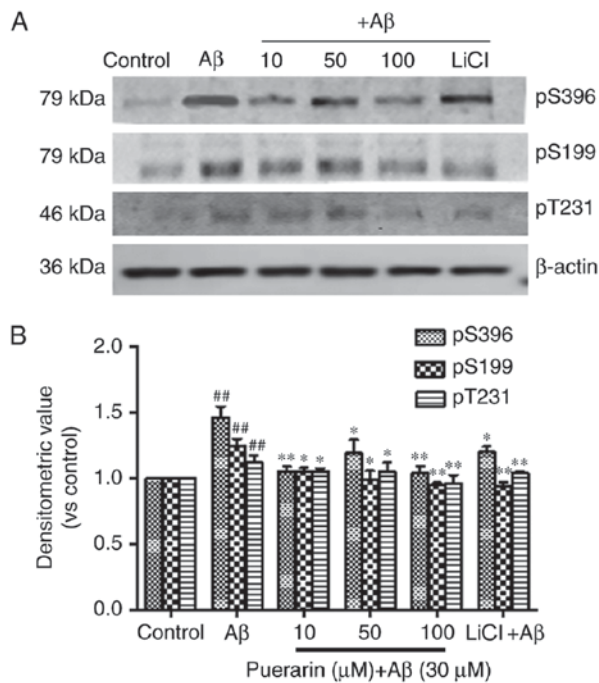


Figure 3. Effects of puerarin on tau phosphorylation at different sites in A β 1-42-treated SH-SY5Y cells. (A) Western blot bands. (B) Graph of protein content. Data are presented as the mean \pm standard deviation; $n=3$. $^{##}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the Alzheimer's disease model group. A β , β -amyloid peptide; LiCl, lithium chloride; p, phosphorylated; S, Ser; T, Thr.

the levels of tau phosphorylation in the puerarin and LiCl groups were much lower than in the model group ($P<0.05$). The levels of tau phosphorylation were most significantly decreased following pretreatment with 100 μ mol/l puerarin. Therefore, a specific dose of puerarin, similar to that of the GSK-3 β inhibitor LiCl, inhibited tau hyperphosphorylation in A β -treated SH-SY5Y cells.

Effects of puerarin on the expression of GSK-3 β and p-GSK-3 β (Ser9) in A β 1-42-treated SH-SY5Y cells. As shown in Fig. 4, treatment of SH-SY5Y cells with A β 1-42 for 24 h significantly reduced the levels of GSK-3 β phosphorylation at Ser9 (the inhibitory phosphorylated site) ($P<0.01$) and significantly increased the expression levels of GSK-3 β compared with the control group ($P<0.01$). The expression levels of p-GSK-3 β (Ser9) in the SH-SY5Y cells following treatment with puerarin and LiCl were significantly increased compared with in the model group ($P<0.05$); however, GSK-3 β expression was significantly decreased compared with the model group. A reduction in GSK-3 β expression and an increase in p-GSK-3 β (Ser9) expression were most prominent following pretreatment with 100 μ mol/l puerarin, thus suggesting that puerarin increased p-GSK-3 β (Ser9)-mediated inhibition of GSK-3 β , and GSK-3 β mediated A β 1-42-induced tau phosphorylation.

Effects of puerarin on the expression of β -catenin and cyclin D1 in A β 1-42-treated SH-SY5Y cells. As presented in Fig. 5, the expression levels of β -catenin and cyclin D1 were significantly reduced in SH-SY5Y cells following treatment with A β 1-42 compared with the control group ($P<0.01$). Compared with the model group, the expression levels of β -catenin and

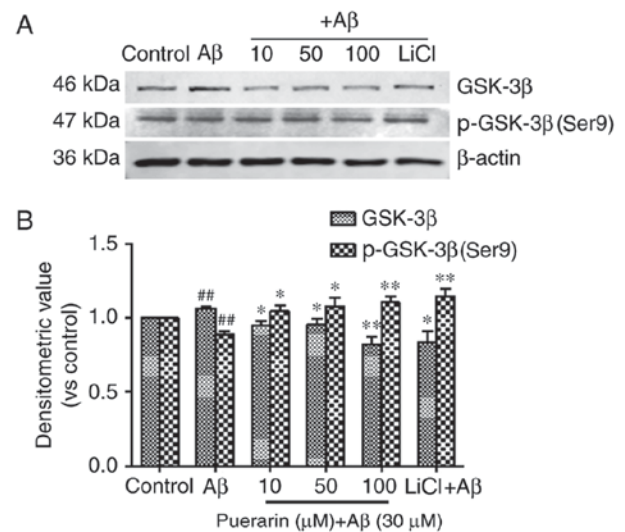


Figure 4. Effects of puerarin on the expression of GSK-3 β and p-GSK-3 β (Ser9) in A β 1-42-treated SH-SY5Y cells. (A) Western blot bands. (B) Graph of protein content. Data are presented as the mean \pm standard deviation; $n=3$. $^{##}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the Alzheimer's disease model group. A β , β -amyloid peptide; GSK-3 β , glycogen synthase kinase-3 β ; LiCl, lithium chloride; p, phosphorylated.

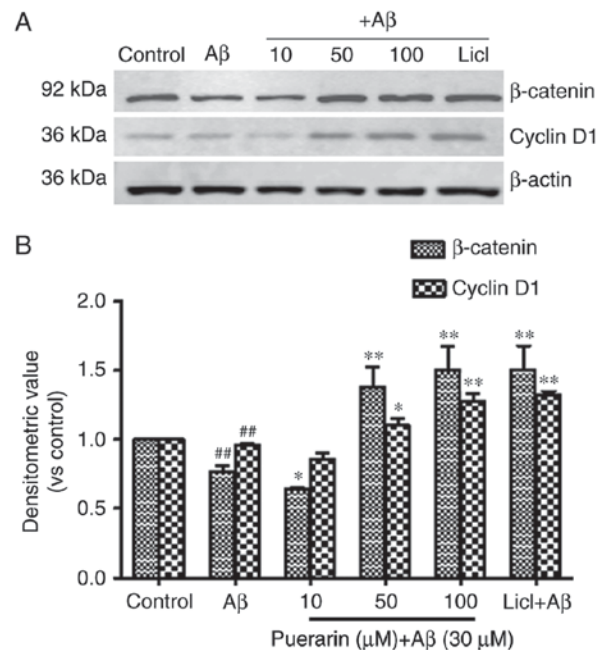


Figure 5. Effects of puerarin on the expression of β -catenin and cyclin D1 in A β 1-42-treated SH-SY5Y cells. (A) Western blot bands. (B) Graph of protein content. Data are presented as the mean \pm standard deviation; $n=3$. $^{##}P<0.01$ compared with the control group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the Alzheimer's disease model group. A β , β -amyloid peptide; LiCl, lithium chloride.

cyclin D1 were significantly enhanced in cells exposed to various doses of puerarin with increasing drug concentration, and in cells exposed to LiCl. The expression levels were significantly increased in cells treated with 50 and 100 μ mol/l puerarin groups compared with in the model group. These results suggested that puerarin induced neuroprotection via activation of β -catenin and cyclin D1 in the Wnt/ β -catenin

signaling pathway. It may be hypothesized that puerarin activates β -catenin and cyclin D1 expression by inhibiting the activity of GSK-3 β .

Discussion

Tau hyperphosphorylation is a key event in the pathogenesis of AD. Abnormal signal transduction in early AD triggers an imbalance in protein kinase and phosphatase levels, and the accumulation of hyperphosphorylated microtubule-associated protein tau in neurons leads to the formation of NFTs (11) resulting in synaptic damage and neural degeneration. Tau contains 21 tau-Ser and tau-Thr-phosphorylated sites; however, only a few sites serve a role in the microtubule-binding activity of tau. Nevertheless, abnormal phosphorylation at Ser396, Ser199 and Thr231 sites occurs in AD (12,13). In AD pathogenesis, the accumulation of A β is correlated with hyperphosphorylation of tau, and *in vitro* studies have demonstrated that A β induces tau hyperphosphorylation (14,15). In the present study, SH-SY5Y cells were treated with 30 μ mol/l oligomeric peptide A β 1-42. After 24 h, a cell model of tau hyperphosphorylation resembling AD was successfully established. Conversely, the levels of tau phosphorylation at the aforementioned sites were reduced, following pretreatment of the cells with various concentrations of puerarin. A reduction in tau protein phosphorylation was most prominent following treatment with 100 μ mol/l puerarin. Therefore, puerarin inhibited A β 1-42-induced hyperphosphorylation of tau.

GSK-3 β is the key protein kinase underlying tau phosphorylation. GSK-3 β is a kinase rich in Ser and Thr, and its activity is inhibited by phosphorylation at the Ser9 residue (16). The present study demonstrated that GSK-3 β expression was increased in A β 1-42-treated SH-SY5Y cells, whereas p-GSK-3 β (Ser9) expression was reduced. Treatment with puerarin and LiCl, which is a specific inhibitor of GSK-3 β , increased p-GSK-3 β (Ser9) expression and reduced GSK-3 β expression. It may be hypothesized that A β 1-42 triggers tau hyperphosphorylation predominantly via activation of GSK-3 β , whereas puerarin inhibits GSK-3 β activity by increasing p-GSK-3 β (Ser9) expression, which further inhibits tau hyperphosphorylation in A β -treated SH-SY5Y cells.

A previous study reported that Wnt/ β -catenin signaling is closely associated with the incidence and development of AD (2). GSK-3 β is a key negative regulator of the Wnt signaling pathway, which reduces the transcription and expression of β -catenin and the target gene cyclin D1 via phosphorylation and ubiquitination (4). Loss of regulation of normal cell growth and differentiation leads to neuron death (17). The present study suggested that puerarin may activate β -catenin and cyclin D1 in the Wnt/ β -catenin signaling pathway to inhibit GSK-3 β activity, and exert neuroprotective effects.

In conclusion, the present study demonstrated that puerarin is able to inhibit A β 1-42-induced tau hyperphosphorylation. The underlying mechanism is potentially mediated via inhibition of GSK-3 β , which may induce activation of β -catenin and the downstream factor cyclin D1, and reduce hyperphosphorylation of microtubule-associated tau in nerve cells,

consequently resulting in neuroprotection. These results provide novel ideas and strategies regarding the clinical treatment of AD with puerarin.

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