

Original Article

Huperzine A protects neural stem cells against A β -induced apoptosis in a neural stem cells and microglia co-culture system

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Abstract: Objectives: This study aims to explore whether Huperzine A (HupA) could protect neural stem cells against amyloid beta-peptide A β induced apoptosis in a neural stem cells (NSCs) and microglia co-culture system. Methods: Rat NSCs and microglial cells were isolated, cultured and identified with immunofluorescence Assays (IFA). Co-culture systems of NSCs and microglial cells were employed using Transwell Permeable Supports. The effects of A β ₁₋₄₂ on NSCs were studied in 4 groups using co-culture systems: NSCs, A β +NSCs, co-culture and A β +co-culture groups. Bromodeoxyuridine (BrdU) incorporation and flow cytometry were utilized to assess the differences of proliferation, differentiation and apoptosis of NSCs between the groups. LQ test was performed to assess the amounts of IL-6, TNF- α and MIP- α secreted, and flow cytometry and Western blotting were used to assess apoptosis of NSCs and the expressions of Bcl-2 and Bax in each group. Results: IFA results showed that isolated rat NSCs were nestin-positive and microglial cells were CD11b/c-positive. Among all the groups, the A β +co-culture group has the lowest BrdU expression level, the lowest MAP2-positive, ChAT-positive cell counts and the highest NSC apoptosis rate. Smaller amounts of IL-6, TNF- α and MIP- α were being secreted by microglial cells in the HupA+A β +co-culture group compared with those in the A β + co-culture group. Also the Bcl-2: Bax ratio was much higher in the HupA+A β +co-culture group than in the A β +co-culture group. Conclusions: HupA inhibits cell apoptosis through restraining microglia's inflammatory response induced by A β ₁₋₄₂.

Keywords: Huperzine A, Alzheimer's disease, amyloid beta-peptide, neural stem cells, microglial cells

Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disease, the most common form of progressive intellectual failure in humans [1]. To date, its mechanism is not clear yet, but it is considered to be associated with loss of neurons in the brain resulted from inflammatory response caused by Amyloid beta-peptide (A β) deposits activating microglial cells [2]. However, it has not been confirmed yet whether inflammation caused by A β -induced microglia activation makes the environment in the brain not suitable to live and differentiate in for neural cells, leading to insufficient supplementation of neural cells.

Huperzine A (HupA), extracted from Chinese club moss *Huperzia serrata* (Qian Ceng Ta), is a

potent, reversible and selective inhibitor of acetylcholinesterase (AChE) with a rapid absorption and penetration into the brain in animal tests [3, 4]. HupA has been used for AD treatment widely in China and in clinical trials for the treatment of age-related memory deficiencies in the United States (<http://www.clinicaltrials.gov/show/NCT00083590>). At the moment, the mechanism of treating AD with HupA is not fully understood yet. Studies have shown that preincubation with HupA could protect rat neurons against A β -mediated apoptosis [5, 6]. Most existing studies on treating AD with HupA target mature functional cells, only a few investigate the effects of HupA on neural stem cells (NSCs).

NSCs are neural precursor cells with capacity for self-renewal and multipotency. Neurons, astrocytes and oligodendrocytes are among

cells that NSCs can differentiate into [7]. Along with loss of neurons caused by A β -induced microglia activation, studies have shown that A β can suppress neural progenitor cell proliferation and differentiation, and induce apoptosis [8]. Based on this, we speculate that A β -induced microglia activation may indirectly harm NSCs' chances of survival, while HupA protects them against such an effect.

To test our hypothesis, we established co-culture systems of NSCs and microglia with Transwell Permeable Supports, added A β_{1-42} into microglial cell layers to simulate the inflammatory microenvironment in AD patients' brains. Effects of A β treated microglia on the survival of NSCs were studied in vitro to determine whether A β inhibits the production of neural cells through microglial cells. Also, effects of HupA on inflammatory response were evaluated in NSC-microglia co-culture systems. Meanwhile, the apoptosis rates of NSCs were assessed to determine the protective effects of HupA on NSCs.

Materials and methods

Immunofluorescence assays (IFA)

The cells were fixed with 4% paraformaldehyde for 15 min and then blocked with goat serum for 30 min. Nestin (Rat-401) mouse mAb (Cell Signaling Technology, USA) was added to identify NSCs and anti-CD11b/c antibodies (Abcam, USA) to identify microglial cells. The cells were rinsed with PBS after placing at 4°C overnight. FITC-labeled (green) and TRITC-labeled (red) goat anti-mouse IgG (Cell Signaling Technology, USA) secondary antibodies were then added for microglial cells and NSCs, respectively. Together, they were incubated for 1 h in the dark. DAPI was used to stain the nuclei for 5 min and images were taken with a fluorescent microscope (Olympus, Japan).

Isolating and culturing of rat NSCs

Cells derived from hippocampus of newborn SD rats were resuspended with DMEM/F12 (Gibco) mixed with EGF (20 μ g/L), BFGF (20 μ g/L), B27 (2%), L-Gln (2%) and penicillin/streptomycin (1%). They were then seeded in 25 cm² vented culture flasks (1 \times 10⁹/L per flask) placed at 37°C in a 5% CO₂ incubator. Half of the solution was changed every 3-4 days. Subculturing

started on Day 7 and repeated once every 6 to 7 days. NSCs obtained from secondary cultures underwent IFA. Second-generation neurospheres were obtained to prepare single cell suspension. Bromodeoxyuridine (BrdU, 10 μ mol/L) was added and labeling was allowed for 24 h before the solution was replaced with a fresh batch. The neurospheres were transferred after 4 days to a 24 well culture plate coated with polylysine. IFA tests were performed to assess cell proliferation 2 days later. Second-generation NSCs were treated with DMEM/12F containing 10% FBS. After 10 days, immunofluorescence staining for GFAP and MAP2 was used to evaluate cell multipotency.

Purifying and culturing of rat microglial cells

Hippocampus of newborn SD rats was centrifuged after digested with 0.125% trypsin. The supernatant was discarded and the pellet was resuspended in DMEM/F12 containing 10% FBS. The single cell suspension was then transferred at a cell density of 5 \times 10⁸ to 75 cm² vented culture flasks coated with polylysine and incubated at 37°C in a 5% CO₂ incubator. After 2 days, the medium was replaced with a fresh batch. The flask was shaken by hand after 7 to 9 days to complete the separation of purified microglial cells. IFA tests were then performed on the microglial cells with CD11b/c, specific markers for microglia. Meanwhile, Hoechst 33258 was used for nuclear staining so that microglial cell numbers can be measured and purity can be assessed.

Co-culturing rat NSCs and microglial cells

Co-culture systems where cells shared medium without making any contact were employed with 6-well Transwell Permeable Supports. NSCs were seeded on Transwell inserts and purified microglial cells were cultured in 6-well culture plates at the lower compartment. To evaluate effects of microglial cells on the survival of NSCs before and after microglial cells were activated by A β_{1-42} , for NSCs group, NSCs were cultured on a Transwell insert and medium was added on a 6-well plate underneath; for A β +NSCs group, NSCs were seeded on a Transwell insert and medium containing 10 μ mol/L A β_{1-42} (US Biological) was added on the 6 well plate; for both co-culture and A β +co-culture groups, NSCs and microglial cells were seeded at a ratio of 1:1 on Transwell inserts

and in the wells of culture plates, respectively. For A β +co-culture group, 10 μ mol/L A β ₁₋₄₂ was also added to the lower compartment. All four groups were incubated at 37°C in a 5% CO₂ incubator for 96 h before undergoing tests.

To examine the protective effects of HupA on NSCs grown in co-culture systems, NSCs and microglial cells were added at a ratio of 1:1 onto Transwell inserts and culture plates underneath, respectively for co-culture, A β +co-culture and HupA+A β +co-culture groups. A β ₁₋₄₂ (10 μ mol/L) was added to the lower compartments of A β +co-culture and HupA+A β +co-culture groups. For HupA+A β +co-culture group, the microglial cells were treated with 1 μ mol/L HupA 4 hours ahead of seeding. Concentrations of IL-6, TNF- α and MIP-1 α were measured 24, 48 and 72 h after co-culturing started, and flow cytometry and Western blotting were performed to assess NSC apoptosis 72 h after co-culturing started.

Assessment of NSC proliferation with BrdU incorporation assays

Neurospheres were obtained to prepare single cell suspension. BrdU (10 μ mol/L) was added to label cells. Cells were collected after 24 h and resuspended. Cell suspension was then used in each group in co-culture systems. Neurospheres from all the groups were transferred after 96 hours' co-culturing to a 24-well culture plate coated with polylysine and cultured for 2 h. IFA was then performed as follows to examine expressions of BrdU in each group. The cells were fixed with 4% paraformaldehyde at room temperature and then treated with 0.3% Triton X-100. They were blocked with 10% goat serum before incubated with mouse anti-BrdU monoclonal antibodies (Santa Cruz Biotech, Dallas, TX, USA) overnight at 4°C in a humidified chamber and then for another 1.5 h in the dark with FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotech, Dallas, TX, USA). After nuclear staining with Hoechst 33258, the cells were observed and images were taken with a fluorescence microscope.

Assessment of cell apoptosis with flow cytometry

NSCs were collected from each group. A flow cytometer was used and instructions of an

AnnexinV-FITC/PI detection kit (sigma) were followed to assess cell apoptosis.

NSC potency assessment

Immunofluorescent staining was performed 96 h after co-culturing started. The following antibodies were used: mouse anti-MAP2 (Abcam, USA) and rabbit anti-ChAT polyclonal antibody (Abcam, USA) as primary antibodies, FITC-labeled anti-mouse IgG and anti-rabbit IgG (Abcam, USA) as secondary antibodies. NSC potency was analyzed with a flow cytometer (FACS) and a CellQuest 3.0 analysis software.

LQ test

Supernatant was collected from each group. Instructions of LINCoplex Kit (Millipore, USA) were followed as follows to assess the concentrations of IL-6, TNF- α and MIP- α . Detection reagents were brought to room temperature. Buffer was pipette onto an assay plate to coat the filter plate. The plate was then shaken for 10 min at room temperature. 25 μ L supernatant, diluted standard sample, blank sample and quality control sample were added to wells on the plate, separately. Microplate mixtures were added too. Samples were incubated for 2 h at room temperature on a microplate shaker. The plate was rinsed twice with wash buffer twice before detection reagents were added to each well to detect antibodies. The cells were then incubated for 2 h at room temperature on a microplate shaker. Afterwards, streptavidin was added and the cells were incubated for 30 min. The plate was rinsed again before detection reagents were added. Luminex LiquiChip analytical system was utilized to detect the concentrations of inflammatory factors.

Western blot

The nuclear proteins of NSCs were extracted with a nuclear protein extraction kit (KeyGen bioTECH, China). The bicinchoninic acid (BCA) assay was used for protein quantization. A sample containing 15 μ g proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Semi-dry transfer was performed and proteins were transferred to PVDF membranes (Bio-Rad Laboratories, USA). Proteins were blocked with 5% milk made out of skim milk powder and 1 h later incubated with Bcl-2 (1:100), Bax (1:100)

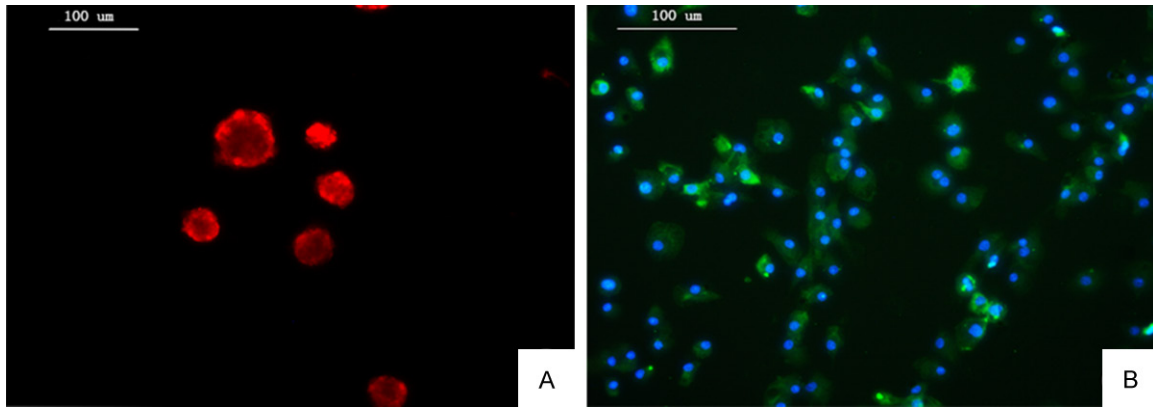


Figure 1. Identification of NSCs and microglial cells. A: NSCs were nestin-positive (red). B: Microglial cells were CD11b/c-positive (green). Nuclei were stained with DAPI (blue).

and β -actin (Cell Signaling, USA) primary antibodies and secondary antibodies.

Statistical analysis

SPSS 16.0 was utilized for statistical analysis. Data are presented as mean \pm standard deviation (SD). Statistical significance among groups was determined by one way ANOVA. LSD tests were performed to determine differences between groups. Differences were considered to be statistically significant where $P < 0.05$.

Results

Identification of NSCs and microglial cells

To identify isolated rat NSCs and microglial cells, IFA assays were performed. Nestin, a marker that expresses in neuroepithelial stem cells, is widely employed for identification of NSCs [9]. Neurospheres were tested positive for nestin (**Figure 1A**). Microglial cells were identified by immunofluorescence staining with CD11b/c. Microglial cells can be seen flat or oval-shaped with slender elongated processes projecting from cell bodies. IFA results showed more than 95% of microglial cells were CD11b/c- positive (**Figure 1B**).

$A\beta_{1-42}$ inhibits proliferation and differentiation and promote apoptosis of NSCs in co-culture systems

To study effects of $A\beta_{1-42}$ on proliferation of NSCs in co-culture systems, BrdU incorporation assays were used to assess NSC proliferations in each group. Results showed that $A\beta_{1-42}$ treatment significantly inhibited NSC proliferation. No significant difference was observed between the co-culture group and the NSCs group

($P > 0.05$), while differences between the $A\beta$ +co-culture group, the $A\beta$ +NSCs group and the co-culture group were all statistically significant ($P < 0.01$). This suggests microglial cells that were not treated with $A\beta_{1-42}$ have no significant effect on NSC proliferation and that $A\beta_{1-42}$ could not only exert direct effects on NSC proliferation but also magnify the inhibitory effects of microglial cells on NSC proliferation (**Figure 2A-E**).

To investigate effects of $A\beta_{1-42}$ on differentiation of neurons in co-culture systems, IFA was performed to detect MAP2-positive and ChAT-positive cells. Results showed there were more MAP2-positive and ChAT-positive cells in the NSCs group than in the co-culture group ($P < 0.01$, **Figure 2F**). Also, there were less MAP2-positive and ChAT-positive cells in the $A\beta$ +NSCs group compared with the co-culture group, and the differences are statistically significant (ALL $P < 0.05$).

Results from flow cytometry showed that there was no significant differences between the NSC apoptosis rates in the co-culture group and the NSCs group ($P > 0.05$) while NSC apoptosis rate in the $A\beta$ +co-culture group was higher than those in the co-culture group and the $A\beta$ +NSCs group ($P < 0.05$). The results indicate that $A\beta_{1-42}$ promotes differentiation and apoptosis while inhibits proliferation of NSCs (**Figure 2G**).

HupA inhibits $A\beta_{1-42}$ -induced secretion of IL-6, TNF- α and MIP-1 α by microglia

Secretions of IL-6, TNF- α and MIP-1 α were compared between the co-culture, the $A\beta$ +co-culture and the HupA+ $A\beta$ +co-culture groups

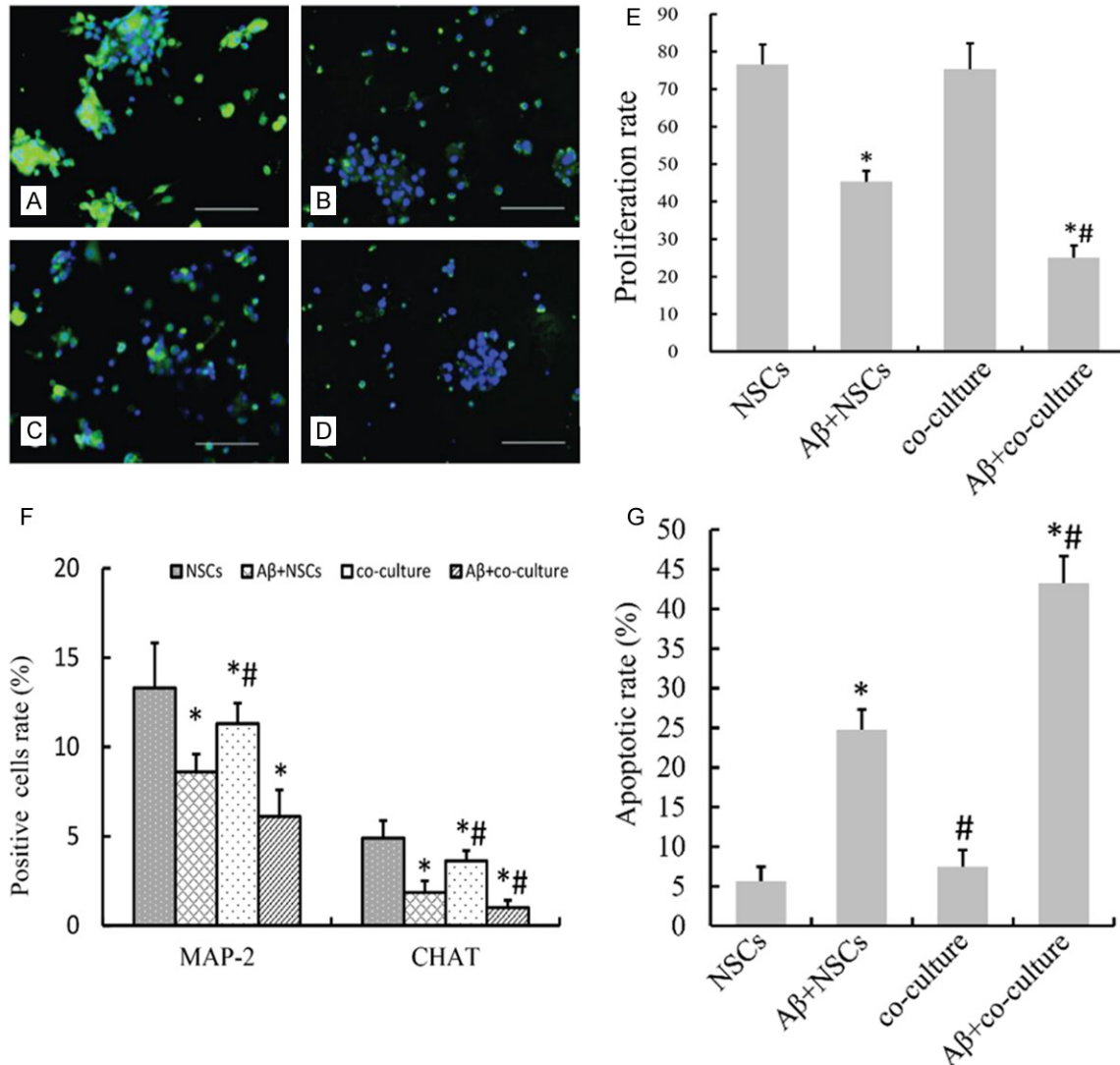


Figure 2. Aβ₁₋₄₂ inhibits proliferation and differentiation and promotes apoptosis of NSCs in co-culture systems. (A-E) BrdU incorporation assays were utilized to examine effects of Aβ₁₋₄₂ on NSC proliferation (scale bar =100 μm). (A) NSCs; (B) NSCs treated with Aβ₁₋₄₂; (C) NSCs and microglial cells; (D) NSCs and microglial cells treated with Aβ₁₋₄₂. (E) NSC proliferation rate of (A-D) group showed in BrdU incorporation assays. **P*<0.05 vs. NSCs, #*P*<0.05 vs. Aβ+NSCs. F: Flow cytometry was performed to detect MAP2 and ChAT positive cells. **P*<0.05 vs. NSCs, #*P*<0.05 vs. Aβ+NSCs. The experiment was repeated 5 times. (G) NSC apoptosis rate. **P*<0.05 vs. NSCs, #*P*<0.05 vs. Aβ+NSCs. The experiment was repeated 5 times and the mean of the results was adopted.

using LQ test. Results showed that the concentrations of IL-6, TNF-α and MIP-1α were much higher 48 h after co-culturing started than 24 h after treatments. The concentrations of IL-6, TNF-α and MIP-1α in the Aβ group were 2.6, 4.2 and 3.4 times as high as those in NSCs group, respectively, 48 h after co-culturing started (*P*<0.05), while the concentrations of the three cytokines in the HupA group were 2.1, 1.8 and 2.3 times as high as the concentrations of those in the NSCs group, respectively, and much lower than those in Aβ group (*P*<0.05). The differences were bigger 72 h after co-cul-

turing started. The concentrations of the cytokines in Aβ were 2.7, 5.8 and 4.8 times as high as the concentrations of those in NSCs group, respectively (*P*<0.05), while the concentrations of the cytokines in HupA were much lower than those in Aβ group (*P*<0.05, **Figure 3**). The results suggest that HupA restrains inflammatory factors from being secreted.

HupA reduces Aβ₁₋₄₂-induced NSC apoptosis

IFA and Western blot were performed to study the effects of HupA on NSC apoptosis in NSC-

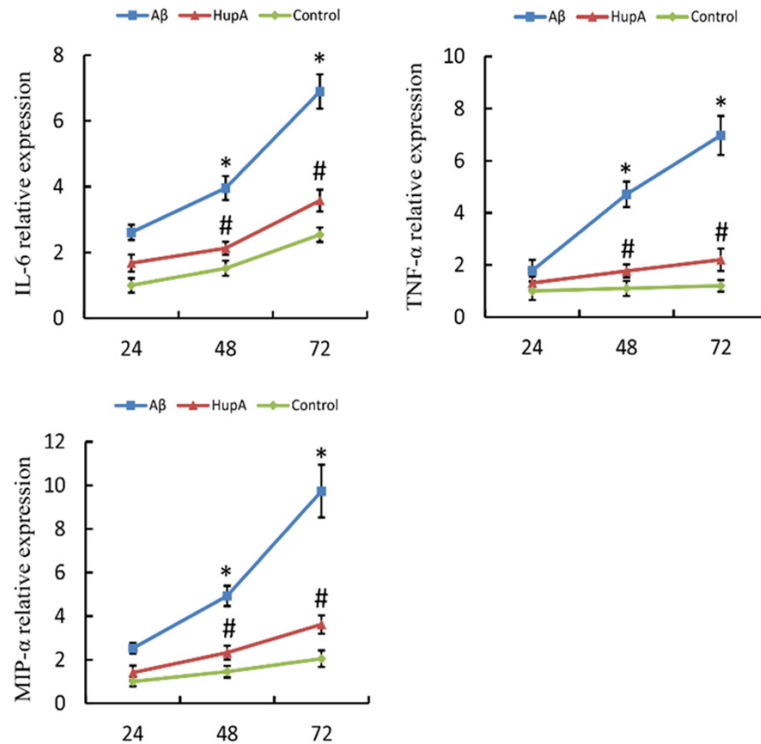


Figure 3. Concentrations of IL-6, TNF- α and MIP-1 α in each group. * $P < 0.05$ vs. NSCs, # $P < 0.05$ vs. A β +NSCs. The experiment was repeated 5 times and the mean of the results was adopted. A β : A β +co-culture group; HupA: HupA+A β +co-culture group; Control: co-culture group.

microglia co-culture systems with the presence of A β_{1-42} . Flow cytometry results showed that 72 h after co-culturing started, the apoptosis rate of NSCs in co-culture group was 4.3%, that in A β group was 24.6% and that in HupA group was 9.8%. The differences between the co-culture group and the A β +co-culture group, and that between the A β group and the HupA+A β +co-culture group are statistically significant ($P < 0.05$, **Figure 4**).

A study by Wong R, Zhang HY and Tang XC showed that HupA inhibits apoptosis induced by A β through raising the expression of anti-apoptotic protein Bcl-2 and decreasing the expression of pro-apoptotic protein Bax [6]. Expressions of Bcl-2 and Bax were analyzed by Western blot. Results showed that expressions of Bcl-2 and Bax proteins were the highest in the A β +co-culture group 72 h after treatment, with the lowest Bcl-2: Bax ratio ($P < 0.05$). The Bcl-2: Bax ratio is much higher in the HupA+A β +co-culture group compared with the A β +co-culture group. The difference is statistically significant ($P < 0.05$, **Figure 5**).

Discussion

In vivo and in vitro studies have shown that A β can activate microglial cells [10], and that activated microglial cells can release several types of cytokines, chemokines, complement and activation products of complement, leading to nonspecific inflammatory cell infiltration and causing chronic inflammation [11]. It still has not been confirmed whether this makes the environment in the brain not suitable for neural cells to live and differentiate in, resulting in endogenous and transplanted exogenous NSCs not being able to function properly, and eventually leading to insufficient supplementation of neural cells in AD patients' brain. This study established co-culture systems of NSCs and microglial cells. Proliferation and differentiation of NSCs were evaluated

before and after microglial cells were treated with A β_{1-42} . Results showed that microglial cells not treated with A β_{1-42} has no significant effects on proliferation of NSCs in co-culture systems, just as reported by past researches [12]. That the percentage of NSCs differentiating into neurons and cholinergic neurons was lower than normal level may have to do with some factors secreted by microglial cells under normal circumstances. Studies have shown that A β_{1-42} can inhibit proliferation of NSCs, promote their apoptosis and lower the percentage of NSCs differentiating into neurons and cholinergic neurons [13-15]. NSCs' capacity of self-renewal and multipotency was markedly restrained by A β_{1-42} in NSC-microglia co-culture systems, indicating inhibitive effects of A β_{1-42} on NSC survival may be magnified through microglial cells. These factors may be to blame for the hostile microenvironment inside the brains of AD patients that threatens the survival of endogenous and exogenous NSCs. This poses a challenge on tackling AD using NSCs but also sheds some light on it.

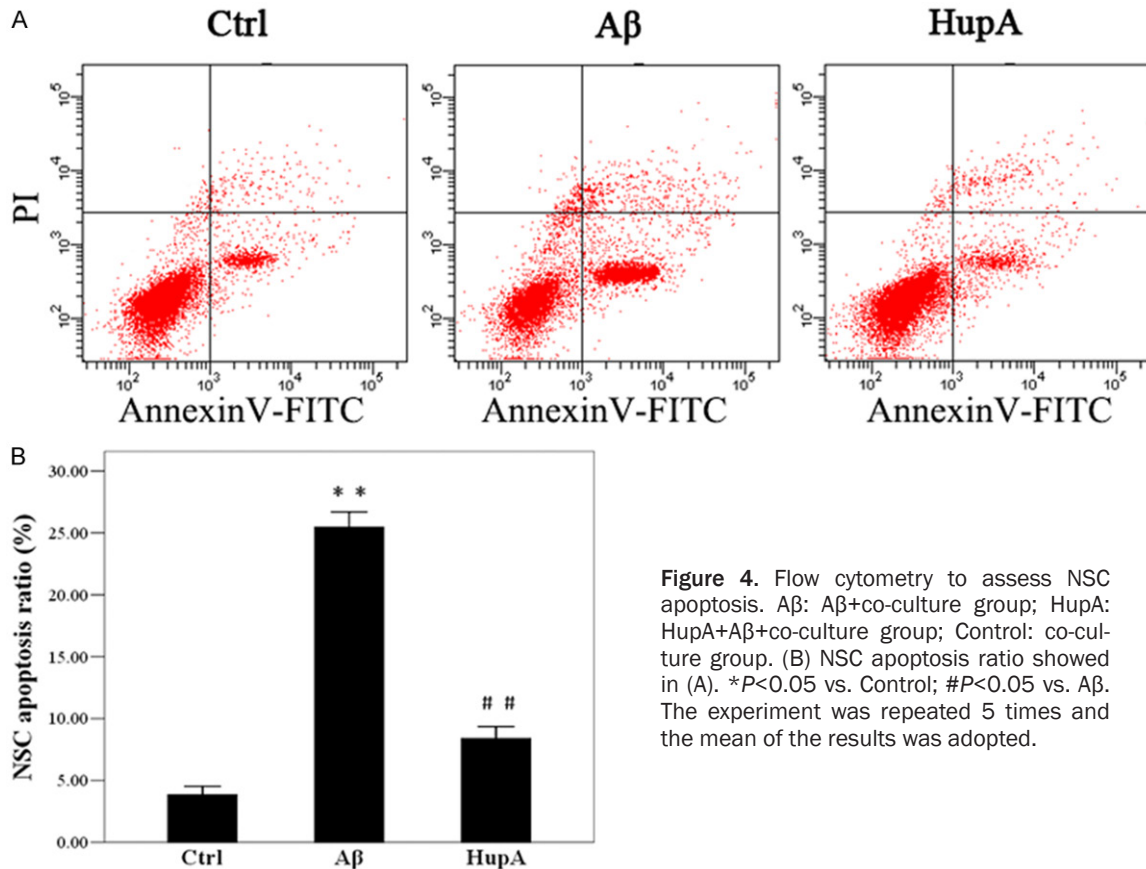


Figure 4. Flow cytometry to assess NSC apoptosis. Aβ: Aβ+co-culture group; HupA: HupA+Aβ+co-culture group; Control: co-culture group. (B) NSC apoptosis ratio showed in (A). * $P < 0.05$ vs. Control; # $P < 0.05$ vs. Aβ. The experiment was repeated 5 times and the mean of the results was adopted.

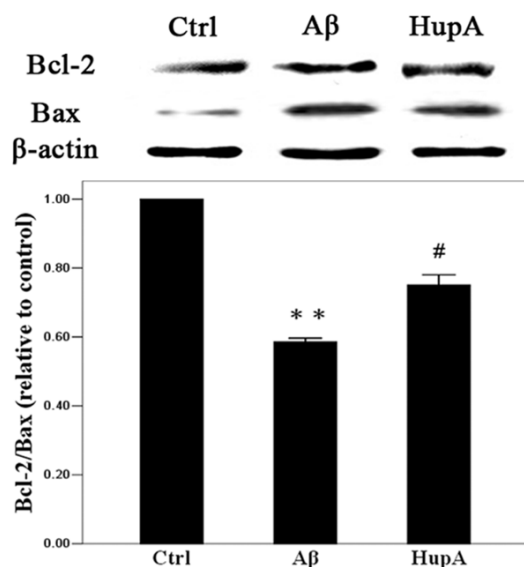


Figure 5. Western blot assay of Bcl-2 and Bax expressions. Western blotting was repeated 3 times and the mean of the results was adopted. * $P < 0.05$ vs. Control; # $P < 0.05$ vs. Aβ.

In this study, as significantly smaller amounts of inflammatory factors such as IL-6, TNF-α and

MIP-1α were secreted with the presence of HupA, we presume this may be the way in which HupA exerts its protective effects on NSCs. Aβ that accumulates in the brain tissue of AD patients acts on microglia constantly, resulting in chronic inflammation. Chronic inflammation then leads to neuron apoptosis and inhibits neural cells from being produced, causing massive loss of neural cells in the central nervous system. This could be the cause of AD and the main reason that it deteriorates. HupA, as a cholinesterase inhibitor, has been applied in AD treatment and is effective to certain extent. A study by Wang J et al. suggested HupA makes the inflammatory response less severe through acting on cholinergic receptors [16]. This study utilized Transwell Permeable Supports and established co-culture systems of NSCs and microglial cells. The inflammatory microenvironment in AD patients' brains was simulated by exposing microglial cells to Aβ to examine the protective effects of HupA and NSCs. Results showed that, with the stimulus of Aβ₁₋₄₂, microglial cells secreted IL-6, TNF-α and MIP-α. These inflammatory factors passed through Transwell

semipermeable membranes, triggering apoptosis of NSCs. IL-6, a cytokine that expresses largely in AD patients, can promote inflammatory response through inducing the expression of phospholipase A₂ gene. TNF- α is an important pro-inflammatory cytokine that triggers and regulates cytokine storm in the inflammatory response. It can stimulate the expressions of cytokines such as IL-1, and these cytokines, in turn, continuously magnify the inflammatory response in various ways [17]. Therefore, inhibiting TNF- α and IL-6 from being produced is crucial for the control of chronic inflammation and the improvement of microenvironment for AD patients. Unlike TNF- α and IL-6, MIP-1 α is a member of the C-C subfamily of chemokines. MIP-1 α chemotaxis can cause microglial cells to accumulate around A β plaques in AD patients. Microglial cells, stimulated by A β , release inflammatory factors including MIP-1 α , forming a vicious circle (microglia activation in retinal degeneration). With the presence of HupA, significantly smaller amounts of IL-6, TNF- α and MIP-1 α were secreted by microglial cells, exerting strong anti-inflammatory effects. Studies conducted in the past by researchers showed this is realized as HupA restrains cholinesterase activities, increases acetylcholine, activates cholinergic receptors and eventually reduces the expressions of cytokines and chemokines [16, 18, 19]. Our study showed HupA can act on microglial cells directly, restraining inflammatory factors from being secreted by microglial cells, thus inhibit inflammatory response.

HupA is effective in AD treatment. A study by Zhang HY and Tang XC showed that HupA protect neurons through reducing A β production and acting on the signaling pathways of nerve growth factor [20]. This study focuses on the protective effects of HupA on NSCs. Results from flow cytometry and Western blotting showed that the Bcl-2: Bax ratio in NSCs in the HupA+A β +co-culture group was higher than that in the A β +co-culture group. Bcl-2 is an important anti-apoptotic protein while Bax is a pro-apoptotic one in Bcl-2 protein family and can antagonize the protective effects of Bcl-2. Whether cells live or die after stimulated by apoptosis signals depends on the ratio of Bcl-2 protein family's pro-apoptotic and anti-apoptotic proteins. When Bcl-2: Bax ratio is low, Bax forms homodimers to induce cell apoptosis; when Bcl-2: Bax ratio is elevated, Bax and Bcl-2

form heterodimers, inhibiting cell apoptosis [21].

We found that stimulated by A β ₁₋₄₂-mediated inflammatory response, the expressions of Bcl-2 and Bax were the highest in the A β +co-culture group, with the lowest Bcl-2: Bax ratio. LQ test revealed that treated with HupA, the amount of inflammatory factors being secreted was smaller in the HupA+A β +co-culture group than in the A β +co-culture group, with a much higher Bcl-2: Bax ratio, exerting inhibitive effects on NSC apoptosis. Results from Western blotting and flow cytometry both indicate HupA can inhibit NSC apoptosis. HupA reduces the amount of inflammatory factors being secreted, decrease NSC apoptosis rates, favoring neural cell survival.

To conclude, this study proved A β ₁₋₄₂ inhibits NSC proliferation, apoptosis and differentiation, suggesting A β ₁₋₄₂ magnifies its inhibitive effects on neural cell survival through microglial cells. Also, smaller amount of IL-6, TNF- α and MIP-1 α was secreted by microglial cells and the apoptosis level of NSCs was lower with the presence of HupA, indicating one mechanism for HupA to treat AD may be through restraining microglial cells from secreting cytokines and chemokines, and thus making inflammatory response less severe and the microenvironment more friendly for neural cells to live in and eventually raise their survival rate. However, the specific signaling pathways through which HupA acts on microglial cells, the mechanism and effects of HupA on proliferation and differentiation of NSCs await further studies.

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Disclosure of conflict of interest

None.

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