

Original Article

Protective effect of bone marrow mesenchymal stem cells on PC12 cells apoptosis mediated by TAG1

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Abstract: Objective: This study aims to explore the protection effect of bone marrow mesenchymal stem cells (BMSCs) on PC12 cells apoptosis mediated by transient axonal glycoprotein 1 (TAG1). Methods: PC12 cells were divided into control group, A β_{25-35} group and BMSCs + A β_{25-35} group. The effects of BMSCs on PC12 cells treated by A β_{25-35} were detected using MTT, Hoechst 33258 and Annexin V-FITC/PI staining methods. The expression levels of TAG1, β -amyloid precursor protein (APP), AICD and p53 were determined by RT-PCR and Western blotting methods. The expression levels of Bax and Bcl-2 were determined by Western blotting method. The activity of Caspase 3 was detected by spectrophotometric method. Results: MTT results showed that cell activity decreased after the treatment of 20 μ M A β_{25-35} for 48 h ($P < 0.01$) while it increased in BMSCs + A β_{25-35} group ($P < 0.01$). Hoechst 33258 and Annexin V-FITC/PI staining results showed that A β_{25-35} could induce the apoptosis of PC12 cells while the apoptosis of PC12 cells was inhibited in BMSCs + A β_{25-35} group. RT-PCR and Western blotting methods showed that 20 μ M A β_{25-35} could increase the expression levels of TAG1, APP, AICD and p53 ($P < 0.01$) while they decreased in BMSCs + A β_{25-35} group ($P < 0.01$). 20 μ M A β_{25-35} could increase the expression levels of Bax and decrease the expression levels of Bcl-2 ($P < 0.01$), while the expression levels of Bax decreased and the expression levels of Bcl-2 increase in BMSCs + A β_{25-35} group ($P < 0.01$). 20 μ M A β_{25-35} could enhance Caspase 3 activity while it decreased in BMSCs + A β_{25-35} group ($P < 0.01$). Conclusions BMSCs with A β_{25-35} could inhibit the apoptosis of PC12 cells, which maybe related with TAG1/APP/AICD signal pathway.

Keywords: BMSCs, PC12 cells, TAG1, apoptosis

Introduction

Alzheimer's disease (AD) is a degenerative disease of the central nervous system; its main clinical features are the appearance of tangled nerve fibers and senile plaques in the brain. Apoptosis is the end result of nerve cells in many neurodegenerative diseases [1, 2]. β -amyloid protein (A β) is the main component of senile plaques, it deposits in the brain can cause loss or death of neurons, A β_{25-35} is its major segment [3, 4]. The formation of A β is the abnormal metabolism result of β -amyloid precursor protein (APP) in the nerve cell membrane. APP is a transmembrane protein and involved in the differentiation and regeneration of nerve cells, synaptic development, neural protection and other physiological processes. Transient axonal glycoprotein 1 (TAG1) is the ligand of APP. It can interact with APP and pro-

mote the release of AICD into the nucleus to regulate the expression of apoptosis related target gene and participate in the development of AD. TAG1/APP pathway inhibits neurogenesis in the development of the central nervous system and participates in the development of AD [5].

Bone marrow mesenchymal stem cells (BSCs) have the potential of stem cell differentiation and self-renewal. It is easy to draw materials, culture and proliferate in vitro and autologous BSCs transplantation can avoid immune rejection. In recent years, BSCs has gradually applied to the treatment of diseases of the nervous system, such as AD, Parkinson and so on [6-8]. BSCs or its supernatant can significantly improve the survival rate of nerve cells and inhibit their apoptosis [9-11]. The effects and its mechanism of BSCs on PC12 cells apopto-

Table 1. RT-PCR primers

Gene	Primer (5'-3')
TAG1	For: AGTCACAGCCTGTCCTCTAG Rev: ATCTGCCTATGCCTTGTTG
APP	For: AAAGAAGGCATGAGAGCATC Rev: GGAATTCAGGGTCTGGCGCTCAGGG
AICD	For: ATAGCGACAGTGATCGTCATC Rev: CTCCTCTGGGGTGACAGC
p53	For: CATCATCACACTGGAAGACTCC Rev: TTGCGGAGATTCTTCTCCTC
GADPH	For: AGCCACATCGCTCAGACA Rev: TGGACTCCACGACGTACT

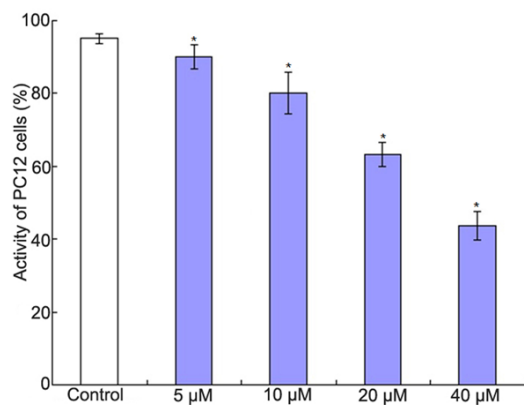


Figure 1. The effect of Aβ₂₅₋₃₅ on the activity of PC12 cells. *P<0.01 vs. control group.

sis induced by Aβ₂₅₋₃₅ remained unclear. PC12 cells were the adrenal cells of rats with the properties of neurosecretory cells and neurons; they were often used as an experimental model of neuron. In this study, we explored the effects and mechanism.

Materials and methods

Experimental animals

SD rats (weight 200±20 g) were purchased from Shanghai silaike experimental animal limited company. All animal protocols were approved by the national Animal Care and Use Committee. Rats were fed in a specific-pathogen-free facility with free access to food and water under a constant temperature (23±2°C).

Cell culture

PC12 cells were purchased from Chinese Academy of Sciences. BMSCs cells were isolat-

ed from SD rats. SD rats were killed by cervical vertebra dislocation and soaked in 75% alcohol for 5 min. Femur and tibia were taken out under sterile conditions and the muscle tissues attached to them were removed completely. The metaphysis was cut and bone marrow cavity exposed. The bone marrow cavity was washed with DMEM/F-12 medium to collect cells and they were centrifuged at 1000 r/min for 5 min. The cells were cultured with DMEM medium.

PC12 cells were cultured in DMEM medium with 10% fetal calf serum at 37°C with 5% CO₂ for 48 h. They were divided into control group, Aβ₂₅₋₃₅ group (5, 10, 20 and 40 μM Aβ₂₅₋₃₅ was added) and BMSCs + Aβ₂₅₋₃₅ group (BMSCs and PC12 cells co-cultured with Aβ₂₅₋₃₅).

MTT assay

The three group of PC12 cells were seeded at density 5000 cells/well in 96-well plates and cultured at 37°C with 5% CO₂ for 48 h. The cells were added 20 μl MTT (5 mg/ml) and incubated for an additional 4 h at 37°C. Then culture medium was removed and 150 μl of DMSO were added to each well with shaking at low speed for 10 min. The MTT solution was aspirated and optical densities (OD) of the supernatant were read at 570 nm using a Microplate Reader (Thermolux, Molecular Device Co). The experiments were repeated three times and the negative control was conducted using only cell-free culture medium (means ± SEM).

Flow cytometry analysis

Fluorescein Annexin V-FITC/PI double labeling was performed with the Annexin V-FITC apoptosis detection kit (Beckman) to detect the apoptosis of PC12 cells. The three group of PC12 cells were seeded in 6-well plates and cultured at 37°C with 5% CO₂ for 48 h. Then cells were stained with Annexin V-FITC and PI according to the manual of the kit. The apoptotic cells were determined with a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CELLQUEST software (BD Biosciences).

Hoechst staining

The three group of PC12 cells were seeded in 6-well plates and cultured at 37°C with 5% CO₂ for 48 h. Then they were stained with Hoechst staining kit according to the manual of the kit. They were observed under microscope.

Protective effect of BMSCs

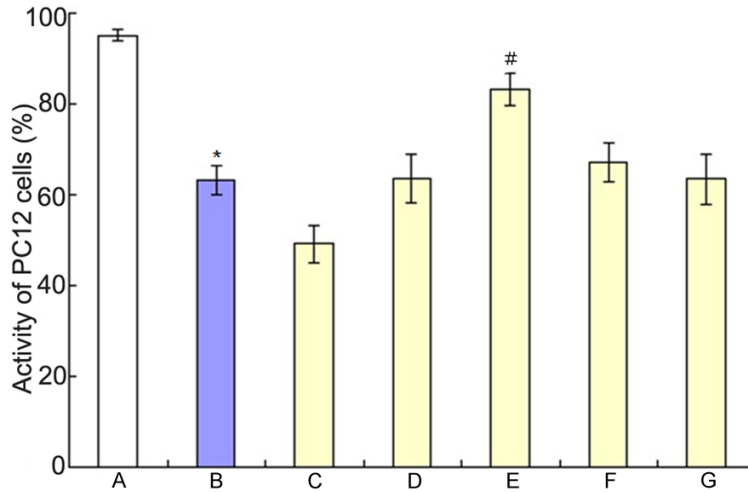


Figure 2. The effects of $A\beta_{25-35}$ on the activity of PC12 cells when co-culture of BMSCs and PC12 cells. A: Control group; B: $A\beta_{25-35}$ group; C: 1:30 of BMSCs and PC12 cells; D: 1:20 of BMSCs and PC12 cells; E: 1:15 of BMSCs and PC12 cells; F: 1:10 of BMSCs and PC12 cells; G: 1:5 of BMSCs and PC12 cells; * $P < 0.01$ vs. control group; # $P < 0.01$ vs $A\beta_{25-35}$ group.

Real-time PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Real-time PCR were performed using one-step RT-PCR kit according to the manual. Primers used in this study were shown in **Table 1**. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Quantifications of target genes mRNA was performed using the $2^{-\Delta\Delta Ct}$ method. The PCR products underwent electrophoresis on 2.5% agarose gel and were then visualized under UV illumination using ethidium bromide staining.

Western blotting

The cells were lysed with RIPA lysis solution. Total proteins were isolated and their concentration was determined by BCA kit according to the manual. Proteins were separated on 12% polyacrylamide gels and transferred to PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate primary antibody. Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The mem-

brane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to GAPDH.

Activity detection of Caspase 3

The three group of PC12 cells were seeded in 6-well plates and cultured at 37°C with 5% CO_2 for 48 h. The activity of Caspase-3 was detected using Caspase-3 spectrophotometric detection kit according to the manual. OD values at 405 nm were determined.

Statistical analysis

The data are expressed as mean \pm SD and analyzed with SPSS 17.0 software, t test was used to evaluate the differences among groups. A value of $P < 0.05$ was taken to denote statistical significance.

Results

Effect of $A\beta_{25-35}$ on the activity of PC12 cells

The effects of $A\beta_{25-35}$ on the activity of PC12 cells were shown in **Figure 1**. It showed that the activity of PC12 cells decreased with the increase of the dose of, we selected 20 μM $A\beta_{25-35}$ to do other experiments. The effects of $A\beta_{25-35}$ on the activity of PC12 cells when co-culture of BMSCs and PC12 cells were shown in **Figure 2**. The ratio of BMSCs and PC12 cells was 1:30, 1:20, 1:15, 1:10 and 1:5 respectively. It showed that the activity of PC12 cells increased to the max when the ratio of BMSCs and PC12 cells was 1:15.

Co-culture of BMSCs and PC12 cells on the PC12 cells apoptosis induced by $A\beta_{25-35}$

Flow cytometry analysis results were shown in **Figure 3** and Hoechst staining results were shown in **Figure 4**. They showed that $A\beta_{25-35}$ could induce the apoptosis of PC12 cells and the apoptosis was inhibited when co-culture of BMSCs and PC12 cells.

Protective effect of BMSCs

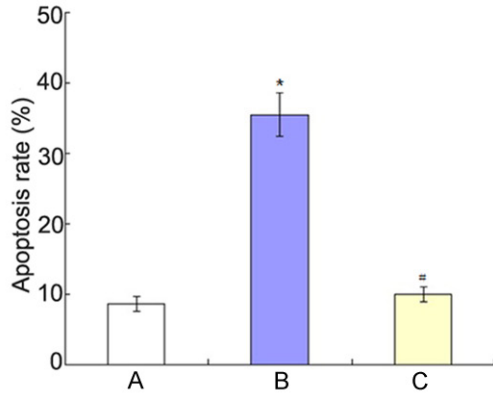
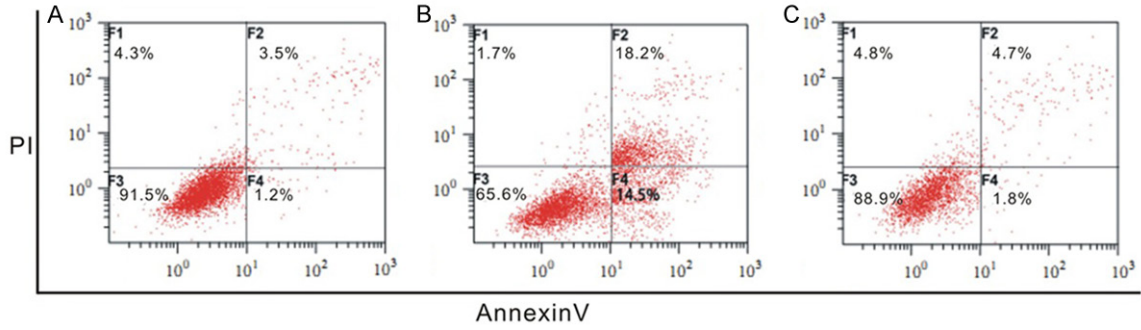


Figure 3. Co-culture of BMSCs and PC12 cells on the PC12 cells apoptosis induced by Aβ₂₅₋₃₅. A: Control group; B: Aβ₂₅₋₃₅ group; C: BMSCs + Aβ₂₅₋₃₅ group. *P < 0.01 vs. control group; #P < 0.01 vs. Aβ₂₅₋₃₅ group.

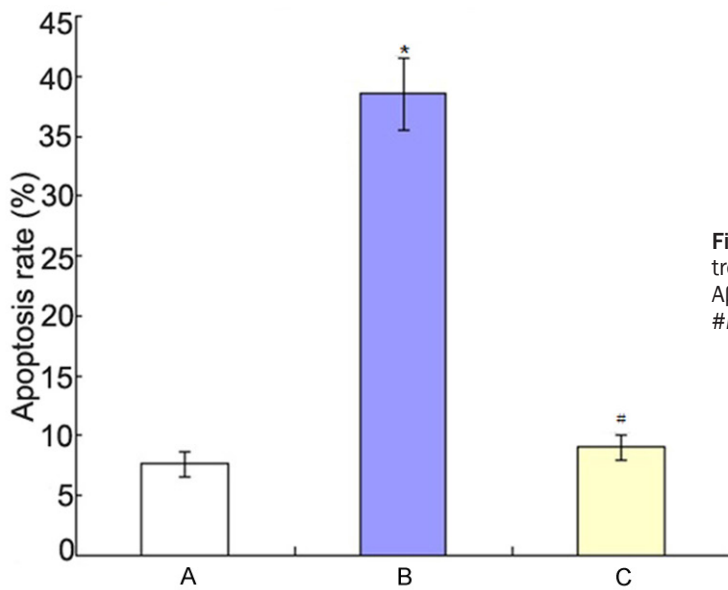
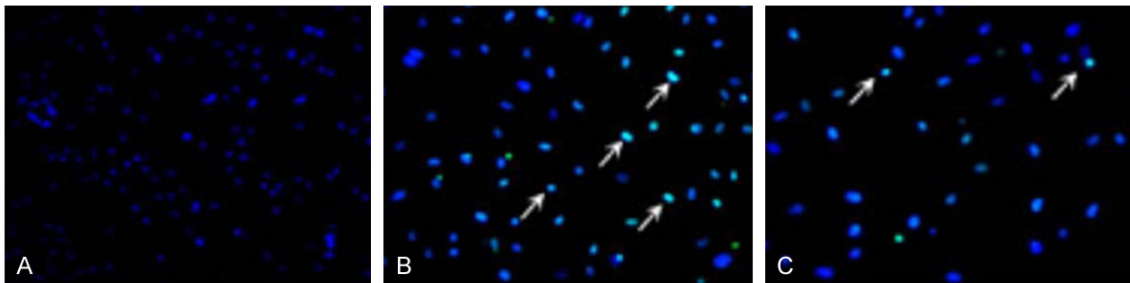


Figure 4. Hoechst staining results. A: Control group; B: Aβ₂₅₋₃₅ group; C: BMSCs + Aβ₂₅₋₃₅ group. *P < 0.01 vs. control group; #P < 0.01 vs. Aβ₂₅₋₃₅ group.

Protective effect of BMSCs

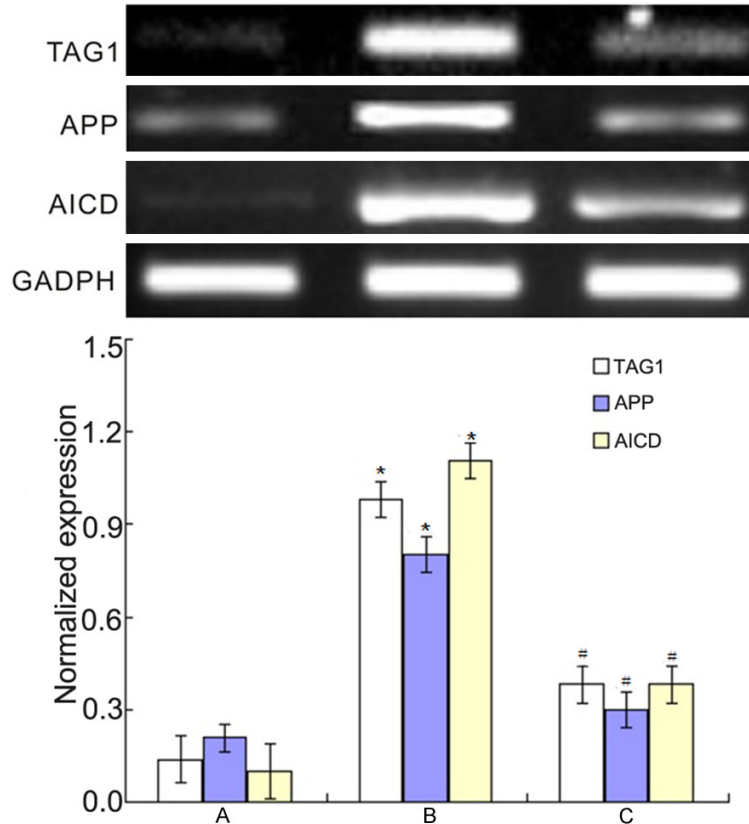


Figure 5. RT-PCR results of the changes of TAG1, APP and AICD expression levels. * $P < 0.01$ vs. control group; # $P < 0.01$ vs. $A\beta_{25-35}$ group.

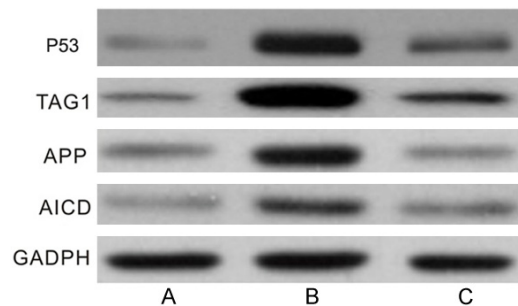


Figure 6. Western blotting results of the changes of TAG1, APP, AICD and p53 expression levels. A: Control group; B: $A\beta_{25-35}$ group; C: BMSCs + $A\beta_{25-35}$ group.

Changes of TAG1, APP, AICD and p53 expression levels

RT-PCR results were shown in **Figure 5**. It showed that $20 \mu\text{M}$ $A\beta_{25-35}$ could increase the expression levels of TAG1, APP, AICD and p53 ($P < 0.01$) while they decreased when co-culture of BMSCs and PC12 cells ($P < 0.01$). Western blotting results were shown in **Figure 6**. It showed the similar results as that of RT-PCR

results: $20 \mu\text{M}$ $A\beta_{25-35}$ could increase the expression levels of TAG1, APP, AICD and p53 ($P < 0.01$) while they decreased when co-culture of BMSCs and PC12 cells ($P < 0.01$).

Changes of apoptosis related proteins Bax and Bcl-2

The effects of co-culture of BMSCs and PC12 cells on apoptosis related proteins Bax and Bcl-2 were shown in **Figure 7**. It showed that $20 \mu\text{M}$ $A\beta_{25-35}$ could increase the levels of Bax and decrease the levels of Bcl-2 ($P < 0.01$), while co-culture of BMSCs and PC12 cells could decrease the levels of Bax and increase the levels of Bcl-2 ($P < 0.01$).

Activity of Caspase 3

The effects of co-culture of BMSCs and PC12 cells on the activity of Caspase 3 were shown in **Figure 8**. It showed that $20 \mu\text{M}$ $A\beta_{25-35}$ could increase the activity of Caspase 3 ($P < 0.01$), while co-culture of BMSCs and PC12 cells could decrease the activity of Caspase 3 ($P < 0.01$).

Discussion

Previous studies showed that $A\beta$ could induce damage like AD in PC12 cells [12, 13]. The degradation of $A\beta$ was helpful for the treatment of AD [14]. In this study we found that the higher the concentration of $A\beta_{25-35}$, the lower the activity of PC12 cells. Other studies also found that PC12 and neuron cells showed typical morphological and biochemical characteristics of apoptosis after contacting with $A\beta$ [15, 16]. We selected $20 \mu\text{M}$ concentration of $A\beta_{25-35}$ to do the experiment, which is consistent with previous reports [17].

TAG1, also named Contactin1, CNTN2 or Axonin1, is a cell surface adhesion molecule and belongs to the immunoglobulin family. It expressed in the early stage of multiple neuronal axons then gradually reduced, while its spe-

Protective effect of BMSCs

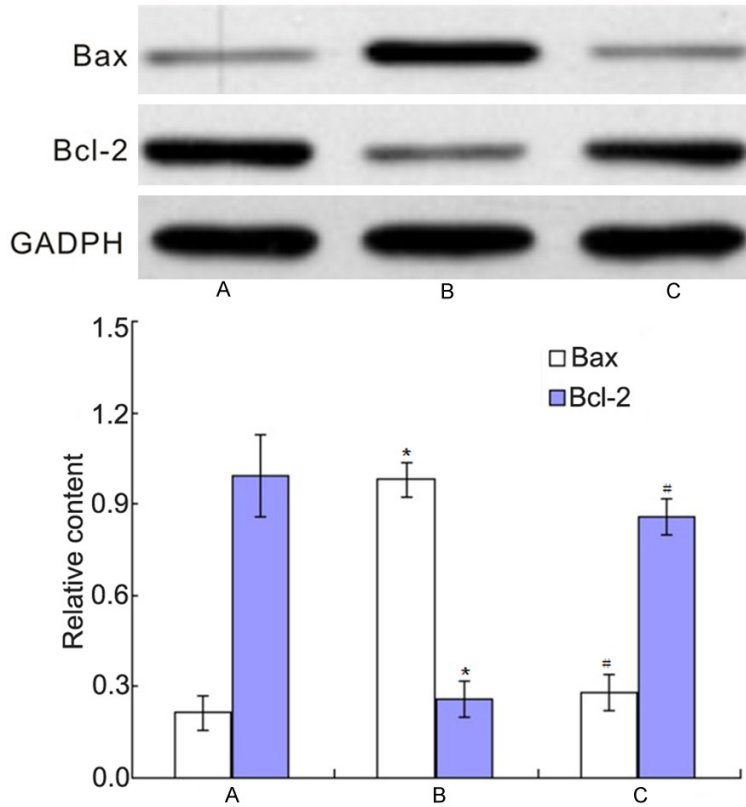


Figure 7. The changes of apoptosis related proteins Bax and Bcl-2. A: Control group; B: Aβ₂₅₋₃₅ group; C: BMSCs + Aβ₂₅₋₃₅ group. *P<0.01 vs. control group; #P<0.01 vs Aβ₂₅₋₃₅ group.

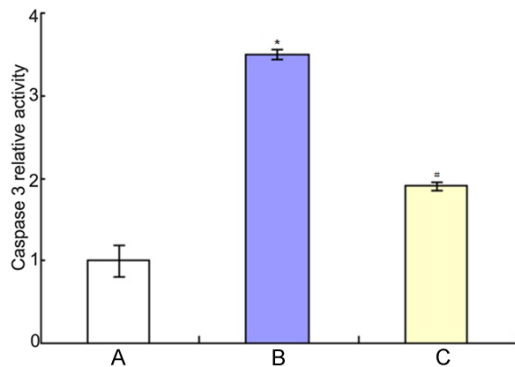


Figure 8. The changes of Caspase 3 activity. A: Control group; B: Aβ₂₅₋₃₅ group; C: BMSCs + Aβ₂₅₋₃₅ group. *P<0.01 vs. control group; #P<0.01 vs Aβ₂₅₋₃₅ group.

cific mechanism of action is unknown. In 2008, it was found that TAG1 could interact with APP to increase AICD expression in neural stem cells and neural precursor cells, which could regulate expression of target gene and inhibit neurogenesis [5, 18]. APP is a kind of type I

transmembrane glycoprotein and is a source of Aβ; it expressed in central nerve cells and participated in physiological processes such as signal recognition, cell adhesion, apoptosis and neural protection. APP formed AICD cut by secretory enzymes such as α, β, γ. AICD conducts the APP signal to the nucleus and is involved in the regulation of many target proteins. Grimm confirmed that degrading enzyme of Aβ depended on the regulation of AICD [19]. APP signaling pathway played an important role in the development of AD; AICD could be a new target for AD therapy [20]. AICD is the functional domain of APP molecules that play a biological function. In 2001, Cao reported that AICD and Fe65 and Tip60 formed protein dimer complex to regulate downstream gene expression [21]. TAG1 expression was up-regulated after injury [22]. In this study we found that TAG1 expression

was up-regulated in PC12 cells after treatment of 20 μM Aβ₂₅₋₃₅ and APP and AICD were also up-regulated, which suggested that TAG1/APP/AICD pathway played an important role in the development of AD.

Ozaki confirmed that AICD induced apoptosis through p53 dependent pathway [23]. P53 gene was tumor-suppressing gene and played an important role in cell cycle regulation, cell apoptosis, angiogenesis and so on. P53 expression increased in neurons in AD, which suggested that P53 played an important role in the apoptosis of neurons [24]. P53 could interact with Bax to induce the release of various pro apoptotic factors, which leading to the apoptosis of cartilage cells. Aβ was injected into the hippocampus or cortex of rats and mice, many nerve cells in the brain appeared apoptosis accompanied with decreased Bcl-2 and increased Bax [25]. Knock out of Bcl-xl or Bcl-2 in H9T cell lines could increase the expression of AICD and induce apoptosis of T cells [26].

Caspase is the executor of apoptosis. A β was injected into the hippocampus of mice; there was no apoptosis of neurons in Caspase 3 knock-out mice while there was many apoptosis of neurons in wild mice [27], which suggested that cell apoptosis could be significantly inhibited by resisting AICD/p53/Bax/Caspase 3 signaling pathway.

BMSCs have been widely used in the treatment of AD. It was confirmed that BMSCs supernatant could significantly resist apoptosis of PC12 cells induced by chronic alcohol [9]. In this study we found that co-culture of BMSCs and PC12 cells could significantly increase PC12 cell activity induced by A β_{25-35} which was consistent with previous report [10]. It was confirmed that BMSCs could delay the AD process in APP/PS1 transgenic mice [28]. BMSCs had significant therapeutic effect on AD induced by APP [7]. TAG1/APP/AICD/p53 pathway plays an important role in AD. We found that co-culture of BMSCs and PC12 cells could significantly decrease TAG1, APP, AICD and p53 expression in PC12 cells induced by A β_{25-35} . BMSCs could inhibit PC12 cells apoptosis induced by CoCl₂, which was related with up-regulation of Bcl-2 and Bcl-xl, down-regulation of Bax, Bak and Caspase 3 [10, 29]. We also found that co-culture of BMSCs and PC12 cells could significantly increase Bcl-2 and inhibit Bax and Caspase 3 activity in PC12 cells induced by A β_{25-35} .

In a word, 20 μ M A β_{25-35} could induce apoptosis of PC12 cells, co-culture of BMSCs and PC12 cells (1:15) could significantly inhibit apoptosis of PC12 cells induced by A β_{25-35} , which maybe associated with the TAG1/APP/AICD signaling pathway.

Disclosure of conflict of interest

None.

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