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Effect of mesenchymal stem cell-derived from the amniotic membrane by Co-culture method on the expression of some genes associated with Alzheimer's disease in neural progenitor cells under the treatment of scopolamine

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Abstract

Background: Alzheimer's disease (AD) is a neurodegenerative disease with insidious onset and progressive destruction of behavioral and cognitive functions. Various therapies have been tested to improve or at least effectively change the course of AD. In recent years; stem cell therapy has emerged as a hopeful potential treatment for Alzheimer's. Stem cells can differentiate into various types of cells, including brain cells, potentially replacing damaged cells and improving cognitive function.
Objectives: In the present study we investigated the inhibitory effect of mesenchymal stem cells isolated from amniotic membrane (AM-MSCs) on neural progenitor cells (NPC) treated with Scopolamine.
Methods: NPC cell was provided by the Iranian Biological Resource Center. To expose these cells to Alzheimer's situations, scopolamine (0.05 mg/ml) treatment has been used. The inhibitory effect of mesenchymal stem cells isolated from the amniotic membrane was evaluated by using the co-cultivation method. The expression of amyloid beta (Aβ), TERM2, Tau, and ABCA7 genes, was assessed in NPC cells co-cultivated with AM-MSC by Real-time PCR. After the Co-culture of AM-MSC and NPC cells for 72 hours, we evaluated the expression of BDNF and CHAT protein in Co-cultured NPC cells by immunocytochemical test.
Results: Results of Real-time PCR and Immunocytochemistry showed that in Co-culture of AM-MSC with NPC decreased Aß, TREM2, and Tau gene expression and increased ABCA7 expression. As well, the expression of BDNF and CHAT protein enhanced.
Conclusions: AM-MSCs have attracted much consideration. MSCs have the capability of immune regulation, regeneration, and neuroprotection These cells are a potential candidate for cell therapy due to their easy accessibility and compliance with ethical issues.
Keywords: Mesenchymal stem cells, neural progenitor cell, TERM2, Tau, Aβ, ABCA7

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Background

Alzheimer's disease (AD) accounts for 60 to 80% of dementia cases in the elderly, according to the World Health Organization (10). More than ever, this number continues to increase (28). As a neurodegenerative disease with a high rate of disability, AD is characterized by the accumulation of extracellular amyloid plaques composed mostly of amyloid-beta (Aβ) peptides and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein (9). The pathophysiology of AD is still largely unknown, the amyloid cascade hypothesis, known as the main hypothesis, predicts the effect of the accumulation and aggregation of Aβ in a set of pathological events ultimately leading to AD (29). Current treatments that include some types of pharmacological therapies help improve the quality of life of patients; they do not change the course of the underlying disease pathology (32). So, it is critical to find innovative therapeutic strategies for AD (9). Cell therapy is considered to be a suitable process for treating AD. Mesenchymal stem cells (MSCs) have shown positive results in experimental models for the treatment of

neurological disorders (15). MSCs can potentially be functional in the treatment of AD by affecting the Aβ peptide, Tau protein, and cholinergic system (1). Interestingly, several achievements have been made in the treatment of AD using MSCs transplantation in recent years (32). However, the underlying mechanism by which MSCs provide treatment for AD has remained unclear.

Among the different types of MSCs, amniotic membrane-derived MSCs (AM-MSCs) and amniotic fluid (AF-MSCs) suggest better new prospects for regenerative medicine compared to other MSCs sources because of their ease of procurement, multipotency, reduced donor damage, low immune response, and acceptable ethical concerns (14). Despite the varying efforts in the treatment of AD with MSCs, the effect of AM-MSCs in this area is still open to question.

Neural progenitor cells (NPCs) are the source of central nervous system (CNS) cells making many types of glial and neuronal cells in the CNS. They are originated from both the CNS of developing embryos and in the neonatal, and mature adult brain, so they are not embryonic stem cells (22). Scopolamine, an antagonist of the muscarinic receptor, blocks the activity of the muscarinic acetylcholine receptor, causing transient cognitive amnesia and electrophysiological changes similar to those seen in AD. The use of NPCs with the optimal dose of scopolamine to produce a cell model appears to be a suitable method for AD study. This method provides a cost-effective and easier way to work which can help future research without ethical problems.

In the present study, the effect of amniotic membrane-derived MSCs on scopolamine-treated NPCs was investigated using the co-culture method. For this, the gene expression and protein activity of several key molecular properties of AD, including Aβ, TREM2, Tau, ABCA7, BDNF, and CHAT were measured.

MATERIALS AND METHODS

MSCs isolation

The amniotic membrane was prepared from the Cesarean section of Milad Hospital (Iran, Tehran), with the acquiescence of the pregnant mother. For MSCs isolation, the membrane tissue was washed with phosphate-buffered saline (PBS) and then cut into several pieces. The membrane pieces were treated with trypsin to eliminate epithelial cells, after that the MSCs were released by collagenase and DNase as described previously.

Confirmation of the MSCs

For verification of MSCs, their surface markers were assayed using flow cytometry. For this, the MSCs were trypsinized and resuspended in PBS. 1×105 cells were placed in 1.5 mL tubes and incubated in FITC-conjugated primary antibodies (Becton Dickinson, Franklin Lakes, and NJ) and phycoerythrin (PE) as multicolor antibodies and isotype control antibodies for 1 h on the ice bath. After washing the MSCs with PBS solution, Flow MAX was used to analyze our cells.

Osteoblasts induction

Cells from passage 5 were used for differentiation into osteoblasts. 500 µL of the cells were added to a 6-well plate with the DMEM-Lg medium. Collagen was added to 3 wells of cell culture plate and another 3 wells were used for control cells. After 2 days, when the number of cells increased, the osteogenic differentiation medium, including 50 µg mL-1 of ascorbic acid, 5 mM β-glycerol phosphate, and 10 nM dexamethasone was added to the cells which were in 3 wells. Others were measured as control by adding DMEM-Lg medium. The cell culture medium was changed every 2 days. After 14 days, cell differentiation was detected with 2% alizarin red straining.

Adipocytes induction

After 5 passages, the cells were used for adipocyte differentiation. The centrifuged cells (500 µL) were added to a 6-well plate containing the DMEM-Lg medium. After 2 days and an increase in the cell number, the adipocyte differentiation medium (50 µg mL-1 ascorbic acid, 60 mM indomethacin, and 10 nM dexamethasone) was added to 3 wells. The DMEM-Lg medium was added to the remaining 3 wells as the control sample. These cell culture media were exchanged every 2 days for 14 days, then, oil red 2% was applied to stain the differentiated cells.

Cells treatment and co-cultivation

Neural progenitor cells (NPCs) were provided by the Iranian Biological Resource Center. To expose these cells to Alzheimer's situations, scopolamine treatment has been used. Different concentrations of scopolamine were tested to find an optimum concentration. Then, various treatments were performed by optimum concentration as indicated below.

a) NPCs alone.

b) NPCs treated with scopolamine.

c) Co-treatment of scopolamine-treated NPCs and amniotic membrane-derived MSCs.

For the co-culture process, an appropriate amount of MSCs were transferred to a 25 cm2 flask after exposure to a culture medium containing mitomycin C. After 24 h, treated NPCs were added to this flask.

RNA extraction

After 72 h of co-cultivation, RNA extraction was carried out using the RNA extraction kit (YTA Total RNA Purification Mini Kit) from different treatment conditions (NPCs alone, scopolamine-treated NPCs, and co-culture of scopolamine-treated NPCs and MSCs). In the next step, oligo-dT primer, and buffer (×10) including Tris-Hcl, Kcl, Mgcl2, and DTT were used to synthesize cDNA. Thenthereverse transcriptase enzyme was added to deoxyribonucleotide triphosphate (25). As well, the primers for target genes were designed for real-time polymerase chain reaction (PCR) (Table 1).

Real-Time PCR

The expression of AD genes marker in different treatments was performed using the real-time PCR technique (CFX96™ Real-Time PCR Detection System, Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene.

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SYBER Green was used for determining DNA concentration (26).

Table 1. The sequence of primers used in this study.

Genes	Primers' sequence	Amplicon size (bp)
TREM2	Forward 5'- ATGATGCGGGTCTCTACCAGTG Reverse 5'- GCATCCTCGAAGCTCTCAGACT	98
Tau	Forward 5'- CCAGTCCAAGTGTGGCTCAAAG Reverse 5'- GCCTAATGAGCCACACTTGGAG	120
Aβ	Forward 5'- CCTTCTCGTTCCTGACAAGTGC Reverse 5'- GGCAGCAACATGCCGTAGTCAT	112
ABCA7	Forward 5'- CACTCTTCCGAGAGCTAGACAC Reverse 5'- CTCCATATCTGTGTCCGCAGCA	132
GAPDH	Forward 5'- CCACTCCTCCTTTGACGCT Reverse 5'- TTACTCCTTGGAGGCCATGTGGG	139

Immunocytochemistry (ICC) of BDNF and CHAT

The treated cells were fixed with 4% formaldehyde for 20 min at 4°C. After washing with PBS, 5% Tritone X-100 was used to make the cells permeable. In the next step, hemotoxin well as the primary antibodies were used and the cells were incubated at 2-8 C◦ for 24 h. Diluted secondary antibodies were applied at 37 C◦ for 1 h. After washing the cells with PBS, DAPI was added and the samples were analyzed to detect protein expression through fluorescent microscopy. The image analysis was performed with Image software (Bethesda, MD, USA).

Atistical analysis

The experiment was conducted according to a randomized plan. All the experiments were performed with three independent replicates. Statistical analysis was performed using analysis of variance (ANOVA) by SPSS Ver. 16 software (version 16, Chicago, IL, USA). Data were presented as mean values ± SD. The significance of the differences between the treatments was assessed using t-test at the level of $p \leq 0.05$.

Results

Growth characterization and Surface markers of AM-MSCs

The growth characteristics of AM-MSCs were displayed in Fig. 1. These cells showed typical properties like adherent growth, having a large rounded nucleus, and long and short cellular tails. In addition, they could maintain a strong mitotic capability.

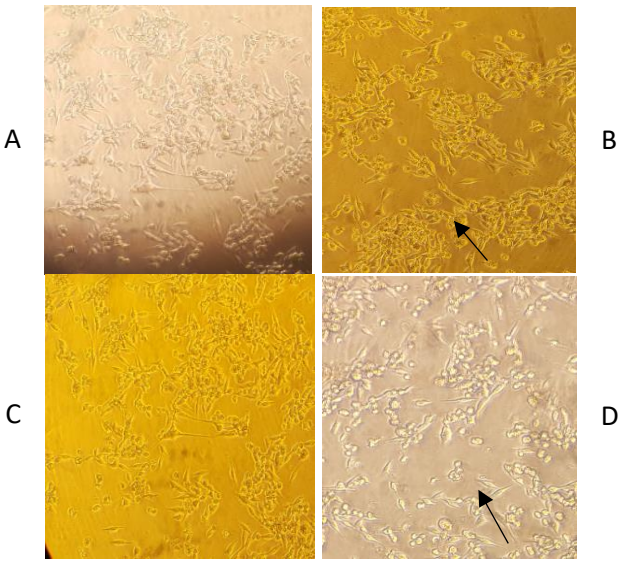

Figure 1. Morphology of amniotic membrane stem cell (AM-MSC). A: AM-MSC cells in passage 2, B: AM-MSC cells in passage 3, C: AM-MSC cells in passage 5, D: AM-MSC cells in passage 6. These images show AM-MSC spindle cells, they are part of the adhesive cells. These images were taken by a reverse microscope with 100x magnification. The dots in the figure indicate spindle-shaped mesenchymal cells.

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Then, the AM-MSCs were isolated and characterized with their surface antigens using flow cytometry. Flow cytometry analysis indicated that AM-MSCs were positive for mesenchymal-associated markers and negative for the hematopoietic cell antigens (Fig. 2). The results showed the expression of the CD29 (99.8 %), CD34 (0.983 %), CD105 (95.3 %), CD45 (0.519 %), respectively.

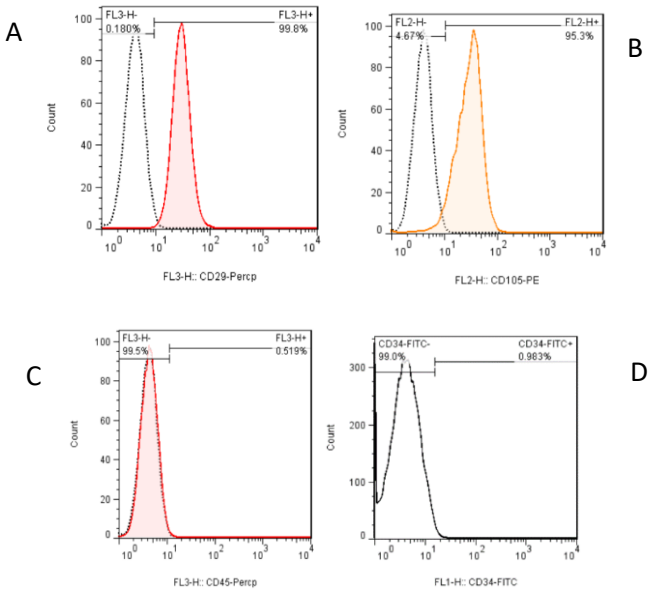

Figure 2. Flow cytometry patterns of AM-MSCs at passage 3 were positive for mesenchymal associated markers: CD29 (A), CD34 (B), and CD105 and negative for the hematopoietic cell antigens CD45. Mouse IgG1 conjugated with FITC, PE, and PerCP served as the controls (FL1: FITC, FL2: PE, and FL3: PerCP).

Osteogenic and adipogenic differentiation from AM-MSCs

AM-MSCs of passage 5 cultured in both osteogenic and adipogenic induction medium differentiated into osteogenic and adipogenic, whereas the AM-MSCs cultured in the basic culture medium did not show any differentiation (Fig. 3). After 14 days the osteogenic and adipogenic cells were identified by Alizarin Red and Oil-Red-O staining, respectively and observed by inverted microscopy. Alizarin Red staining could represent osteogenic differentiation from AM-MSCs with calcium deposition. In addition, Oil-Red-O staining could reveal adipocytes differentiated from AM-MSCs with red lipid vesicles.

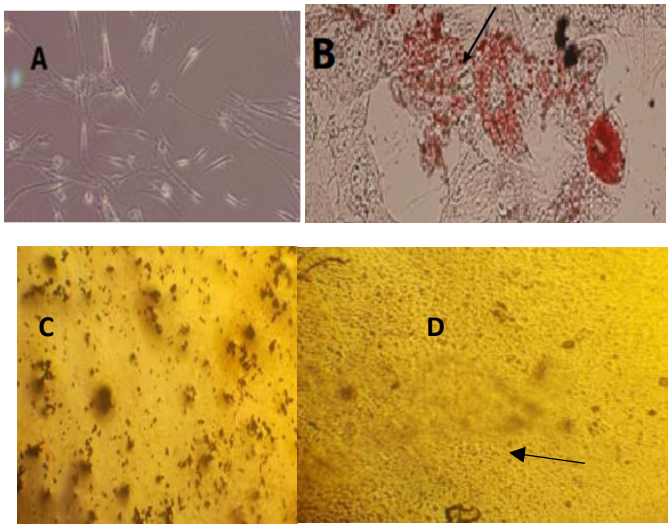

Figure 3. Osteogenic and adipogenic differentiation from AM-MSCs. A-AM-MSCs were considered control cells. B- Osteogenic differentiated from AM-MSCs were stained by Alizarin Red. Red points represented calcium deposition crystals. C- AM-MSCs were considered as control cells. D- Adipogenic differentiated from AM-MSCs was stained by Oil-Red-O. Red points represented lipid vesicles. These images were taken by a reverse microscope with 100x magnification.

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Co- Culture AM-MSC and NPC cells

Several AM-MSC cells were transferred to the flask and after 24 hours of incubation, a certain number of NPC cells were added to the flask containing AM-MSC. NPC cells grow rapidly and are elongated and sticky. After 72 hours, NPC cells were well placed next to AM-MSC cells (Fig. 4). Images were taken with a reverse microscope at 100x magnification.

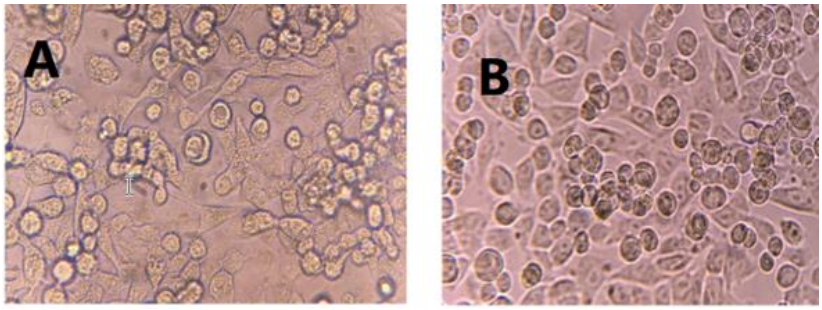

Figure 4. A: AM-MSC and NPC on the first day. B: AM-MSC and NPC on the third day (72 hours). These images were taken with a reverse microscope and 100 x magnifications. Arrows indicate the cell.

TREM2, Tau, Aβ and ABCA7 gene expression by RT-PCR analysis

After 72 hours of treatment of neural progenitor cells (NPC) with mesenchymal cells, RNA was extracted from selected samples (NPC, NPC with scopolamine, NPC with scopolamine with AM-MSC (co-culture)). Then cDNA was made and RT-PCR (Real-Time PCR) was applied to analyze the expression of TREM2, Tau, Aβ, and ABCA7 (Fig. 5).

The quality of extracted RNA from cells was presented in Fig. 5A. Expression of the Tau gene in NPC control samples with scopolamine was 2 times higher than in Co-culture samples (Fig 5B). In Co-culture of AM-MSC with NPC cells decreased expression of Tau and TREM2 genes ($p<0.05$). Also, ABCA7 gene expression was increased in co-culture cells compared to scopolamine-treated NPC cells ($p<0.05$). While Aβ expression was reduced in co-cultured cells ($p<0.05$).

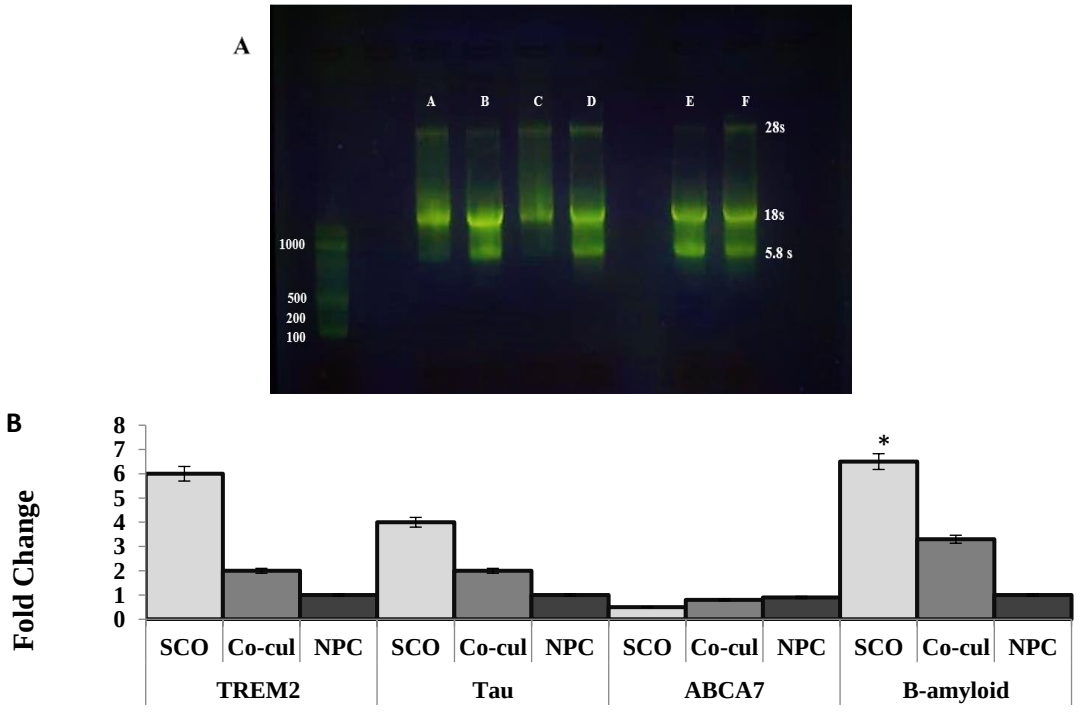

Figure 5. A: Quality control of RNA extracted on electrophoresis gel. **B**: RT-PCR analysis of TREM2, Tau, ABCA7, B-amyloid gene expression level in SCO, Co-culture, and NPC cells. All data are presented as the means ± SD. $P < 0.05$. The sign * indicates a significant difference.

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Immunocytochemistry

After the Co-culture of AM-MSC and NPC cells for 72 hours, we evaluated the expression of BDNF and CHAT protein in Co-cultured NPC cells by immunocytochemical test. In addition, the DAPI-labeled nuclei of cells were assessed. The cells were stained using DAPI dye which binds to the nucleic acid present in the nucleus and is used to detect living cells (Fig 6A). The results showed that the expression of BNDF protein in co-culture cells was enhanced by 43% compared to cells treated with scopolamine ($p<0.005$). Also, CHAT expression notably reached 65% in Co-culture cells while it was only 25% in cells treated with scopolamine ($p<0.05$). (Fig 6B).

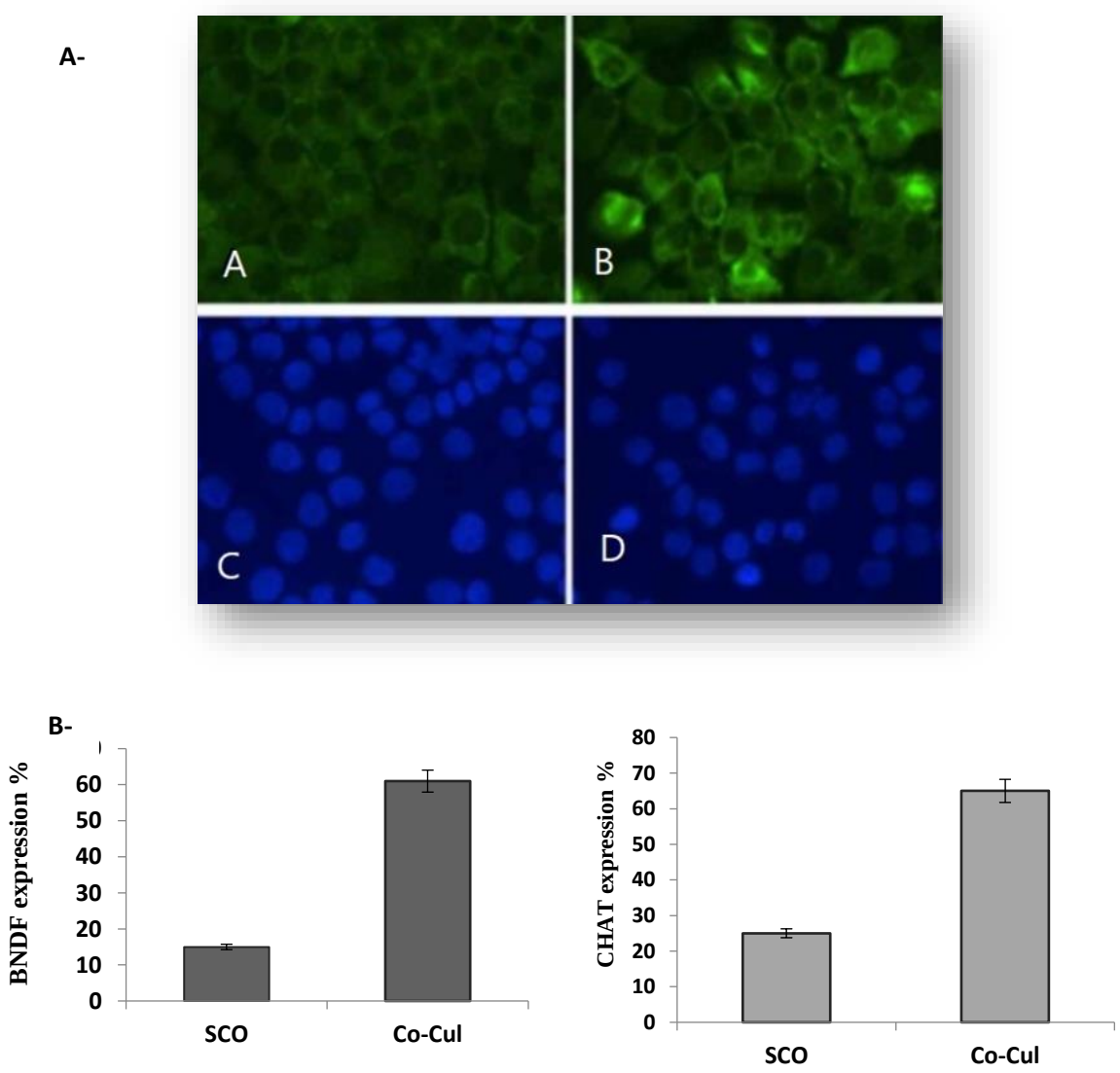

Figure 6. A - A: NPC- B: AM-MSC –NPC (Co-culture) - C: The nucleus of NPC cells is treated with DAPI dye-D: The nucleus of AM-MSC –NPC (Co-culture) is treated with DAPI dye. **B**-Comparison of expression of CHAT and BNDF proteins in Sco and Co-culture cells. All data are presented as the means ± SD and $P < 0.05$. The sign * indicates a significant difference.

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Discussion

Alzheimer's disease (AD) is an oncoming neurodegenerative disease defined by memory loss and cognitive destruction. To date, almost all advanced clinical trials on precise AD-related pathways have failed mostly because of a large number of neurons lost in the brain of patients with AD (30). AM-MSC mesenchymal stem cells can differentiate into bone, fat cells, cartilage, and nerve cells. Therefore, they are used to treat various diseases (34). These cells can be used for the treatment of several neurological diseases such as Parkinson's and Alzheimer's disease (6, 12). Neural progenitor cells (NPCs) exist throughout life and can proliferate and generate neurons, astrocytes, and oligodendrocytes in the central nervous system (CNS). Due to their regenerative potential, NPCs have been concerned with the treatment of neurodegenerative diseases (19). In the present study we investigated the inhibitory effect of mesenchymal stem cells isolated from the amniotic membrane on NPC cells treated with Scopolamine. We developed a model of Alzheimer's cells treated with scopolamine and measured the expression of Alzheimer's-related genes.

In addition, in this study, the identification of amniotic membrane stem cells was done by using flow cytometry. The result showed that MSCs were differentiated into osteoblasts and adipocytes. Many researchers have represented that special surface antigens, such as CD29, CD44, CD90, and CD105, are detected in MSCs that initiate from umbilical cord blood, bone marrow, and adipose tissue (8, 14, 35). In our study, AM-MSCs showed high-expression levels of CD29 and CD105. Also, calcium accumulation and adipose vesicles respectively displayed the differentiation of AM-MSCs to osteocyte and adipocyte.

Genetic factors such as rare variants of TREM2 (triggering receptor expressed on myeloid cells-2) strongly enhance the risk of developing AD, verifying the role of microglia in AD pathogenesis (7, 2). TREM2 expression is upregulated in pathological conditions such as Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), stroke, traumatic brain injury, and AD. In AD, increased expression of TREM2 has been definite in patients (13, 20, 23). Our results showed that the expression of the TREM2 gene decreased with the Co-culture of NPC and AM-MSC cells compared to the control group ($p<0.05$). In NPC cells alone, no increase in TREM2 expression was observed.

AM-MSC cells can be effective in inhibiting NPC cell degradation by reducing TREM2 gene expression. In this regard, bone marrow-derived mesenchymal stem cells co-culture with neurons extracted from the hippocampus of mice decreased apoptosis induced by Alzheimer's in these neurons (17). The two histopathological markers of AD are amyloid plaques composed of the amyloid-β (Aβ) peptide, and neurofibrillary tangles of condensed, abnormally hyperphosphorylated tau protein. Tau, the microtubule-associated protein, forms insoluble filaments that accumulate as neurofibrillary tangles in AD. In the diseased brain, however, tau becomes abnormally hyperphosphorylated, which eventually causes the microtubules to detach, and the free tau molecules are combined into paired helical filaments (21). Our results showed that the expression of the Tau gene decreased with the Co-culture of NPC and AM-MSC cells compared to the other groups ($p<0.05$). Zilka et al. reported that the expression of truncated tau in AD cells induced caspase-3–independent cell death, and tau apoptosis. Mesenchymal stem cells and their secretome rescued the AD cells from cell death. Also MSC induced cell differentiation and led to the formation of neurites in AD cells (33). MSCs have the capability of immune regulation, regeneration, and neuroprotection. The main mechanisms of MSCs in the treatment of AD are as follows, secrete growth factors: MSCs secrete a variety of pro-cytokines that could play a positive function in AD and secrete exosomes. Exosomes refer to extracellular vesicles, which are biocompatible nanoparticles with lipid membranes. These vesicles can transmit messages across biological barriers. Also, foreign proteins conveyed by MSCs can regulate microglia function and recover neurogenesis, thus as to improve early memory defects in AD (18, 24).

Genome-wide association studies (GWAS) originally recognized ATP-binding cassette, sub-family A, member 7 (ABCA7), as a novel risk gene of AD (5). A study of ABCA7 protein levels in the hippocampus or parietal cortex of 123 individuals with or without AD neuropathology showed that individuals with low ABCA7 developed AD neuropathology at a younger age, those with intermediate ABCA7 developed it later, and individuals who developed it very late had high ABCA7, the same as the youngest controls (31). In our study, the expression of the ABCA7 gene in scopolamine-treated NPCs is about half that of AM-MSCs with NPC. It seems AM-MSC cells can be effective in inhibiting NPC cell degradation by increasing ABCA7 gene expression.

should lower the risk for or prevent AD. Converging data from animal models and clinical studies have confirmed that abnormal Aβ accumulation in the brain causes neurodegeneration, neuroinflammation, impaired neuronal function, and finally cognitive decline (4). In our study, Aβ gene expression was decreased in AM-MSCs with NPC co-cultured cells. Previous studies suggested that the Co-culture of human MSCs with AD in an *in vitro* model reduced the expression of amyloid-beta 42 (Aβ42) in the medium as well as the overexpression of amyloid-beta (Aβ) degrading enzymes such as neprilysin (27).

Another important hypothesis about the pathogenesis of AD is the acetylcholine hypothesis. According to this theory, AD is due to a decrease in the neurotransmitter synthesis of acetylcholine (Ach). The CHAT (Choline O-acetyltransferase) gene encodes an enzyme that catalyzes ACh biosynthesis (11). In this study, the expression of CHAT protein was investigated by immunocytochemistry and it was observed that in the Co-culture of AM-MSC and NPC-treated cells, the expression of CHAT protein increased compared to the control. Levels of brain-derived neurotrophic factor (BDNF) are reduced in specific brain regions in AD and BDNF gene polymorphisms have been suggested to influence AD risk, hippocampal function, and memory (16). Also, according to our findings, the expression of BDNF protein in the NPC Co-culture with AM-MSC was increased compared to the control. There is evidence to suggest that BDNF is a critical role, involved in the maintenance of the hippocampal volume in the adult hippocampus (3). Generally, working with a suitable cell model can be very functional, and also it seems that the co-culture method with mesenchymal cells is a very useful method for the study of AD. However, many steps still require to be taken before stem cell therapy becomes a clinically possible treatment for human AD and related diseases.

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Conclusion

In 2019, Alzheimer's disease (AD) and other forms of dementia ranked as the 7th leading cause of death (WHO). The disease is defined by the accumulation of beta-amyloid plaques and tau protein tangles in the brain, leading to brain cell death and progressive refuse in cognitive function. Currently, there is no cure for AD, but good treatments are accessible to manage symptoms and slow the progression of the disease. MSCs have several beneficial effects. Studies have shown that MSCs can help to decrease the buildup of Aβ plaques in the brain in animal models of Alzheimer's disease. MSCs can do this by secreting anti-inflammatory and anti-oxidants that can reduce inflammation and oxidative stress in the brain. In our research, the expression of AD risk genes was evaluated using AM-MSC cells and a cellular model treated with optimum concentrations of scopolamine. According to the results of this study, working with a suitable cell model can be very functional, and also it seems that the co-culture method with mesenchymal cells is a very useful method for the study of AD.

Disclosure of conflicting interests

The authors declare that there is no conflict of interest to disclose.

Author contributions

Kasraee and Tavakoli carried out the experiment (Both have an equal share in writing the article). Barikrow was involved in planning and supervised the work.

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