Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
30 (1): 5-14, 2024
DOI: 10.9775/kvfd.2023.30057

RESEARCH ARTICLE

Beclin-1 Improves the Cognitive Function of Mice with Alzheimer's Disease

Li SHAO [1] Qing HE 1 Xin DU [1] Qing LI [1] Siyuan YANG [1] Chen DONG [1] Pengju MA [2 (*)]

[1] Department of Neurology, The Municipal Hospital Affiliated to Xuzhou Medical University, Xuzhou First People's Hospital, Xuzhou 221116, Jiangsu Province, CHINA

[2] Linguistics and Applied Linguistics, School of Liberal Arts, Nanjing Normal University, Nanjing 210000, Jiangsu Province, CHINA

[(*)] **Corresponding author:** Pengju MA
Phone: +86-018630551036
E-mail: mapjnnu@nau-edu.cn

How to cite this article?

Shao L, He Q, Du X, Li Q, Yang S, Dong C, M Pa: Beclin-1 improves the cognitive function of mice with Alzheimer's disease. *Kafkas Univ Vet Fak Derg*, 30 (1): 5-14, 2024. DOI: 10.9775/kvfd.2023.30057

Article ID: KVFD-2023-30057
Received: 13.06.2023
Accepted: 13.11.2023
Published Online: 09.12.2023

Abstract

We aimed to investigate the improvement effect of Beclin-1 on the cognitive function of mice with Alzheimer's disease (AD). Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) were isolated from neonatal umbilical cord tissues. The cells stably overexpressing Beclin-1 were constructed, and those of passages 5, 10, and 15 were used. Forty-eight AD mice were randomly divided into AD, P5 hUC-MSCs, P15 hUC-MSCs (P15MSCs) and OE Beclin 1-P15 hUC-MSCs (OE Bec-P15MSCs) groups (n=12). Overexpressing Beclin-1 significantly enhanced the proliferation and migration of MSCs of different passages, and reduced G0/G1 arrest. The OE Bec-P15MSCs group had the longest total distance, the shortest time in forced swimming test, and the largest total food consumption in novelty suppressed feeding test. Transplanting hUC-MSCs and overexpressing Beclin-1 significantly reduced Aβ deposition. The expressions of P-tau (Ser396), P-tau (Ser231) and P-tau (Ser235) were significantly inhibited by hUC-MSCs transplantation and Beclin-1 overexpression, especially in the OE Bec-P15MSCs group. Double positive staining of EdU^+/DCX^+ cells, $EdU^+/NeuN^+$ cells and $EdU^+/Nestin^+$ cells significantly increased in the OE Bec-P15MSCs group compared to those in other groups ($P<0.05$). The activation degrees of astrocytes and microglia were lowest and the superoxide dismutase activity was highest in the OE Bec-P15MSCs group ($P<0.05$). The protein expression of nuclear factor E2-related factor 2 (Nrf2) in NAD(P)H quinone oxidoreductase 1 and superoxide dismutase 1 in brain tissues significantly rose, while that of Keap-1 was down-regulated in the OE Bec-P15MSCs group ($P<0.05$). Beclin-1 can partly restore the viability of hUC-MSCs by activating the Nrf2 signaling pathway, thereby enhancing the therapeutic effect of transplantation on AD mice.

Keywords: Aging, Alzheimer's disease, Beclin-1, Mesenchymal stem cell

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder clinically manifested as global dementia characterized by memory impairment, aphasia, apraxia, agnosia, impairment of visuospatial skills, executive dysfunction, and personality and behavioral changes [1]. Amyloid-β (Aβ) deposition and neurofibrillary tangles induced by hyperphosphorylated tau are major pathological hallmarks of AD, which can lead to progressive neuronal loss and memory loss [2]. Recently, embryonic stem cells, mesenchymal stem cells (MSCs), brain-derived neural stem cells and induced pluripotent stem cells have been most commonly used in AD-related studies [3]. MSCs can be extracted from pluripotent stem cells, so they are more easily accessible and abundant than other types of stem cells [4]. Besides, the autologous source of MSCs overcomes the ethical issues related to embryonic stem cells [5].

MSCs are a kind of adult stem cells with self-replication ability and multi-directional differentiation potential, which can be induced to differentiate into a variety of tissue cells under specific circumstances. The immunomodulatory and neuroprotective activities of MSCs have been well-documented [6]. The mice treated with bone marrow-derived MSCs undergo brain changes, including increased synaptic density, decreased ratio of M1/M2 type activated microglia, and elevated levels of anti-inflammatory and neuroprotective cytokines such as

CXCL5, MCP-1, β-NGF, TIMP-1, VEGF-A, TGF-β and IL-10 [7]. MSC-based replacement therapy has become a potential treatment strategy for AD [8]. Transplantation of human umbilical cord-derived MSCs (hUC-MSCs) can improve the cognitive function of AD animal models by reducing Aβ deposition, protecting neuronal integrity and promoting neurogenesis [9]. However, stem cells, like other cells, will gradually develop a senescent phenotype, leading to a gradual decline in cell function. With the increase of passage times, hUC-MSCs cultured *in vitro* are prone to decreased proliferative activity and differentiation capacity, seriously affecting the therapeutic effect [10,11]. Therefore, it is urgently needed to explore new strategies for delaying the aging process of hUC-MSCs and increasing their viability to ensure post-transplantation efficacy.

Beclin-1 is one of the key parts of class III phosphatidylinositol 3-kinase complex, and its abnormal level is closely associated with cancers and neurodegenerative diseases [12,13]. Additionally, the overexpression of Beclin-1 has been reported to protect MSCs from apoptosis and inflammation-induced membrane damage [14]. However, whether Beclin-1 can rejuvenate aged hUC-MSCs and enhance their neuroprotective effects in the chronic pathological state of AD remains to be further studied.

Material and Methods

A- *In vitro* Experiments

Isolation and Culture of hUC-MSCs and Construction of Cell Lines

Under aseptic conditions, the umbilical cord tissues of healthy full-term neonates born by cesarean section were harvested, the umbilical arteries and veins were carefully removed, and Wharton's jelly was separated, cut into small pieces (about 1 mm) and inoculated into DMEM/F12 complete medium (Gibco, USA), followed by culture in a 5% CO_2 incubator at 37°C. Upon reaching 90% confluency, the cells were passaged at 1:3, and those of passages 5, 10, and 15 (P5, P10, and P15) were used for further experiments [15]. Then the virus suspension containing pEGFP-N1-GFP-Beclin-1 was added to the complete medium, and after incubation at 37°C for 24 h, the medium containing virus particles was discarded and replaced with fresh complete medium. 48 h later, the successful construction of cell lines overexpressing Beclin-1 was confirmed by Western blotting.

Cell Counting Kit-8 (CCK-8) Assay

The viability of hUC-MSCs was detected by CCK-8 assay according to the kit's instructions (Suzhou Ribo Life Science Co., Ltd., China) [16]. Specifically, the cells were inoculated into a 96-well plate at a density of 1×10^5 cells/well, and cultured at 37°C for 12 h, 24 h, and 36 h. Then, 10 μL of CCK-8 solution was added into each well, and after incubation for 30 min, the optical density (OD) value was measured at 456 nm. Finally, the survival rate was calculated: survival rate = $[OD_{treated\ cells}/OD_{control\ cells}] \times 100\%$. The assay was independently repeated three times.

EdU Proliferation Assay

The proliferation ability of hUC-MSCs was detected by EdU proliferation assay [17]. The cells in the logarithmic growth phase were inoculated into a 96-well plate at a density of 4×10^5 cells/well and incubated overnight. 100 μL of EdU solution diluted with medium at 1:1000 was added into each well for 2 h of incubation. After washing three times with PBS, the cells were added with Apollo staining solution, incubated for 30 min, and washed three times with PBS, followed by DAPI staining and methanol fixation. Finally, at least 8 randomly-selected fields were photographed under a fluorescence microscope (200×; Olympus, Japan).

Wound Healing Assay

The migration capacity of hUC-MSCs was evaluated by wound healing assay and Transwell assay [16]. The cells were inoculated into a 24-well plate and starved for 12 h after a cell monolayer was formed. The monolayer was scratched with a 10 μL pipette tip to create a wound, and the floating cell debris was removed by PBS. Finally, the cells were observed and photographed under a light microscope (Olympus, Japan) at 0 h and 24 h.

Transwell Assay

Specifically, 2.5×10^4 cells were prepared into cell suspension with serum-free medium and dropwise added to the upper Transwell chamber, while complete medium containing 10% fetal bovine serum (Gibco, USA) was added to the lower chamber. After 48 h of incubation, the cells were fixed with paraformaldehyde solution for 20 min and stained with crystal violet for 20 min. Then the cells in the upper Transwell chamber were removed with cotton swabs, and the number of cells passing through the membrane in the lower chamber was observed and counted under a microscope.

β-Galactosidase Activity Staining

The number of β-gal$^+$ cells in hUC-MSCs was observed by β-galactosidase activity staining [18]. The cells were inoculated into a 6-well plate. After complete adherence, the cells were washed twice with PBS, added with 1 mL of staining fixative, and incubated at room temperature for 20 min. After washing twice with PBS, the cells were added with 1 mL of SA-β-gal staining solution in each well, and incubated at 37°C overnight. Finally, they were observed and photographed under a light microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of interleukin-1α (IL-1α), IL-1β, IL-6, tumor necrosis factor-α (TNF-α) and IL-13 in hUC-MSCs were detected using ELISA according to the kits' instructions (Thermo Fisher Scientific, USA). To be specific, 100 μL of cell culture supernatant was added to the plate, incubated and washed, and then the corresponding antibodies (1:100) were added (Cell Signaling Technology, USA). After washing, 100 μL of horseradish peroxidase-labeled streptavidin (1:100) was added to each well. After washing, 100 μL of chromogenic substrate TMB was added to each well for incubation away from light for 10 min, and the reaction was terminated by 100 μL of stop buffer in each well. Finally, the OD values at 450 nm and 570 nm were measured with a microplate reader.

Detection of Oxidative Stress-related Factors

The reactive oxygen species (ROS) content in hUC-MSCs was measured using ROS assay kit (Beyotime Biotechnology, Shanghai, China). The cells were inoculated into a 12-well plate and cultured until reaching 85% confluency before modeling. After washing with PBS, 1 mL of 10 μM DCFH-DA working solution was added to each well, and the cells were incubated in an incubator at 37°C away from light for 30 min. Then the cells were washed twice with serum-free medium, and rapidly observed and photographed under a fluorescence microscope. After ultrasonication, hUC-MSCs were collected, and 300 μL of MDA working solution (Solarbio Biotech, Wuhan, China) and 100 μL of samples were mixed, heat-preserved in a 100°C water bath for 60 min, and cooled in an ice bath, followed by centrifugation at 10000×g and room temperature for 10 min. Finally, 200 μL of supernatant was added to a 96-well plate, and the OD values were measured at 532 nm and 600 nm.

B- *In vivo* Experiments

Ethical Approval

The human experiment regarding the collection of umbilical cord tissues has been approved by the ethics committee of Xuzhou First People's Hospital on January 4th, 2021 (Approval No. XFPH202101003). All animal experiments have been approved by the animal experiments ethics committee of Xuzhou First People's Hospital on March 8th, 2021 (Approval No. XFPH-dong202103004), and great efforts have been made to minimize the animals' suffering.

Animal Grouping and Treatment

To further verify the improvement effect of Beclin-1 on the cognitive function of AD mice, forty-eight 6-month-old APP/PS1 transgenic mice (AD model mice) purchased from Jackson Medical Technology (Shanghai) Co., Ltd. were randomly divided into AD group, P5 hUC-MSCs (P5MSCs) group, P15 hUC-MSCs (P15MSCs) group and OE Beclin 1-P15 hUC-MSCs (OE Bec-P15MSCs) group (n=12). For the AD group, the same volume of normal saline was intravenously injected. For P5MSCs, P15MSCs and OE Bec-P15MSCs groups, corresponding cells overexpressing Beclin-1 were injected intravenously once daily, respectively, with approximately 1×10^6 cells per injection for 3 d.

Behavioral Tests

Then the cognitive function, anxiety and depression behavior of mice were assessed by Morris water maze (MWM), new object recognition (NOR), open field testing (OFT), forced swimming test (FST), tail suspended test (TST) and sucrose preference test (SPT) [19].

Nissl Staining

Following the behavioral tests, the venous blood was drawn and centrifuged, and the upper serum was collected and stored at -80°C. After the end of the experiment, the mice were anesthetized and sacrificed, and the brain tissues were separated. Finally, the brain tissues were dehydrated, frozen, embedded with OCT and serially sectioned at a thickness of 10 μm with a cryostat. The brain sections were stained with cresol purple to assess neuronal loss and integrity of Nissl bodies. The frozen sections were subjected to gradient heating, placed in cresol purple dye, incubated in an incubator at 56°C for 1 h, washed twice and added with Nissl differentiation solution for 1 min. Finally, the sections were dehydrated, transparentized, and photographed under a microscope [20].

TUNEL Staining

The sections were deparaffinized, hydrated, treated with proteinase K (10 μg/mL) for 15 min, incubated with TUNEL mixture for 60 min, and washed twice with PBS, followed by nuclear staining with 0.1 μg/mL DAPI dye. Finally, the number of TUNEL-positive cells was counted in eight randomly-selected fields per sample under a fluorescence microscope [20].

Immunofluorescence Staining

The sections were placed in citric acid repair buffer, heated by microwave for 6 min, cooled naturally, washed twice with PBS, permeabilized with 0.2% Triton X-100 for 15 min, and washed twice with PBS. After PBS was gently wiped off, the sections were incubated with blocking serum for 1 h, and washed twice with PBS, followed by incubation with the corresponding primary antibodies (Cell Signaling Technology, USA) at 4°C overnight, washing three times with PBS, incubation with fluorescent secondary antibodies (Cell Signaling Technology, USA) at room temperature for 2 h, and washing twice with PBS.

Table 1. *Primer sequences*

Gene	Forward	Reverse
p16	5'-AACACTATGAGGAGCACC-3'	5'-AACGGTAAGCCACGTAGT-3'
p21	5'-CTTTGCCGACCTCGCTCC-3'	5'-CTAACGGTGATTCATGGT-3'
p53	5'-AACTATTAAGGCGACTCG-3'	5'-AGGTTTACGACTATATAG-3'
GAPDH	5'- CAAAT AAACCATCTC GA-3'	5'-AAGAATCGCACC TAGCG-3'

Then the nuclei were stained with DAPI for 15 min, and the sections were washed with PBS, mounted, observed and photographed under a fluorescence microscope [20].

Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cells or tissues using RNA extraction kit (TaKaRa, Japan). According to the manufacturer's instructions, the total RNA was reversely transcribed into cDNA and amplified using PrimeScript RT Master Mix (TaKaRa, Osaka, Japan) and SYBR Premix Ex Taq Kit (TaKaRa, Osaka, Japan). With GAPDH as an internal reference gene, the relative expression of mRNA was calculated by $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in *Table 1*.

Western Blotting

Total protein was extracted from cells or tissues using cell total protein extraction kit (Sangon Biotech (Shanghai) Co., Ltd., China). After separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Beijing Solarbio Biotechnology Co., Ltd., China), the protein was transferred onto a polyvinylidene fluoride membrane, and incubated with primary antibodies against p16, p21, p53, Tau, P-Tau (Ser396), P-Tau (Ser231), nuclear factor E2-related factor 2 (Nrf2), superoxide dismutase 1 (SOD1) and Keap-1 and horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (Cell Signaling Technology, USA). GAPDH and histone 3 were used as internal references for expressions of cytoplasmic and nuclear proteins, respectively.

Statistical Analysis

All assays were independently repeated at least three times, and SPSS20.0 software (IBM Inc., USA) was used for statistical analysis. The measurement data were described by mean ± standard deviation. One-way analysis of variance was used for comparisons among groups, and the LSD-*t* test was conducted for pairwise comparison. $P<0.05$ was considered statistically significant.

Results

Beclin-1 Promoted the Viability, Proliferation and Migration of hUC-MSCs

It was confirmed by Western blotting that hUC-MSC lacked the endogenous expression of Beclin-1, and the protein expression of Beclin-1 was significantly increased after lentivirus infection, indicating the successful construction of cell lines (*Fig. 1-A*). The effects of Beclin-1 on the proliferation and migration of hUC-MSCs of different passages were detected. The results of CCK-8 assay showed that the viability of P10 and P15 MSCs was significantly decreased compared with that of P5 MSCs *(Fig. 1-B)*. In addition, the proportion of P10 and P15 MSCs in G0/G1 phase gradually rose and the proportion of those in S phase declined with the increase of passage times *(Fig. 1-C)*. The results of Transwell migration assay *(Fig. 1-D)*, wound healing assay *(Fig. 1-E)* and EdU proliferation assay *(Fig. 1-F)* revealed that MSCs had gradually weakened migration and proliferation with the increase of passage times. Compared with those of control cells, overexpression of Beclin-1 significantly enhanced the proliferation and migration of hUC-MSCs of different passages, and reduced G0/G1 arrest.

Beclin-1 Inhibited the Senescence of hUC-MSCs

Furthermore, the effects of Beclin-1 on the senescent phenotype of P5, P10, and P15 hUC-MSCs were detected. It was observed by β-galactosidase activity staining that the number of β-gal$^+$ cells in hUC-MSCs gradually rose with the increase of passage times *(Fig. 2-A)*. Meanwhile, immunofluorescence staining also showed that the expression level of Lamin B1, an anti-aging marker, declined gradually with the increase of passage times. Overexpression of Beclin-1 significantly reduced the number of β-gal$^+$ cells and increased the Lamin B1 expression compared with those in untreated cells *(Fig. 2-A)*. As shown by RT-qPCR and Western blotting, the mRNA and protein expressions of p16, p21 and p53 in hUC-MSCs rose with the increase of passage times, while overexpression of Beclin-1 significantly reduced the mRNA and protein expressions of p16, p21, and p53 *(Fig. 2-B,C,D)*. Besides, the results of ELISA showed that overexpression of Beclin-1 significantly inhibited the secretion of IL-1α, IL-1β and TNF-α, and enhanced the release of anti-inflammatory cytokines IL-4, IL-10 and IL-13 in hUC-MSCs *(Fig. 2-E)*. These results suggest that overexpression of Beclin-1 can delay the aging process of hUC-MSCs and rejuvenate aged hUC-MSCs.

Beclin-1 Attenuated Oxidative Stress in Aged hUC-MSCs by Activating Nrf2 Signaling Pathway

Compared with P5 hUC-MSCs, the ROS production significantly rose, while the MDA level and SOD activity

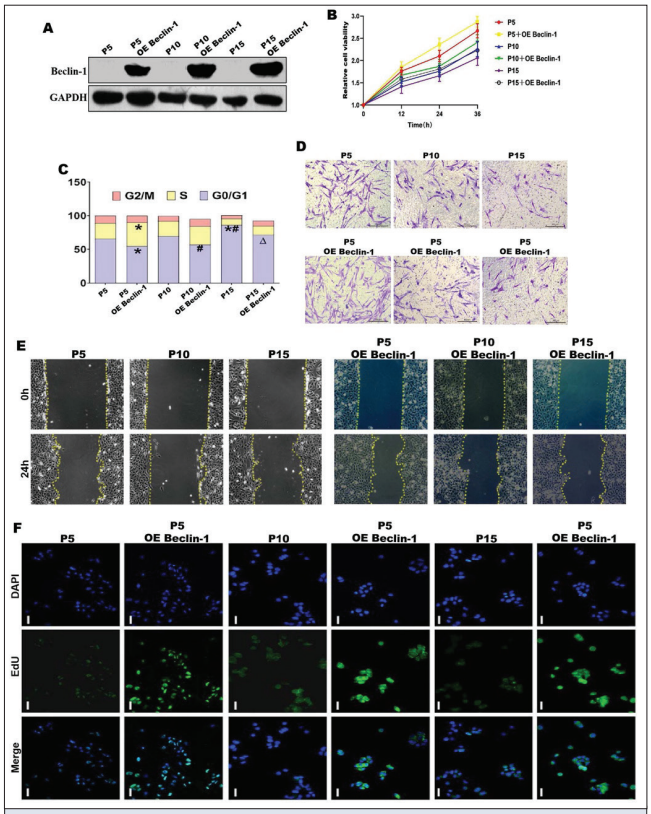

Fig 1. Beclin-1 promoted the viability, proliferation and migration of hUC-MSCs. **A:** Protein expression of Beclin-1detected by Western blotting, **B:** CCK-8 assay for cell viability, **C:** Histogram of cell cycle distribution, **D:** Transwell migration assay, **E:** Wound healing assay, **F:** EdU proliferation assay. The data were described by mean ± standard deviation. *P<0.05 vs. P5 group; #P<0.05 vs. P10 group; ΔP<0.05 vs. P15 group

gradually declined in P10 and P15 hUC-MSCs, which were significantly reversed by overexpression of Beclin-1 *(Fig. 3-A)*. In addition, overexpression of Beclin-1 significantly reduced the expression of Keap-1 in aged hUC-MSCs and enhanced the expressions of Nrf2, NAD(P)H quinone oxidoreductase 1 (NQO1), and SOD1 in the nucleus *(Fig. 3-B,C)*. These results demonstrate that overexpression of Beclin-1 can inhibit oxidative stress in aged hUC-MSCs by activating the Nrf2 signaling pathway.

Overexpression of Beclin-1 Enhanced the Therapeutic Effect of hUC-MSCs in AD Mice

To determine whether overexpression of Beclin-1 can enhance the therapeutic effect of hUC-MSCs, Beclin-1-overexpressing P15 hUC-MSCs were injected into AD mice *via* the tail vein, and the spatial learning and memory function of the mice were evaluated by MWM and NOR tests. As shown in *Fig. 4-A,B,C*, the mice in P5MSCs group had shorter escape latency, more platform-crossing times, longer residence time in the target quadrant and a higher discrimination index than those in AD group (P<0.05). The improvement degree of behaviors was significantly decreased in P15MSCs group, and the behavioral score in OE Bec-P15MSCs group

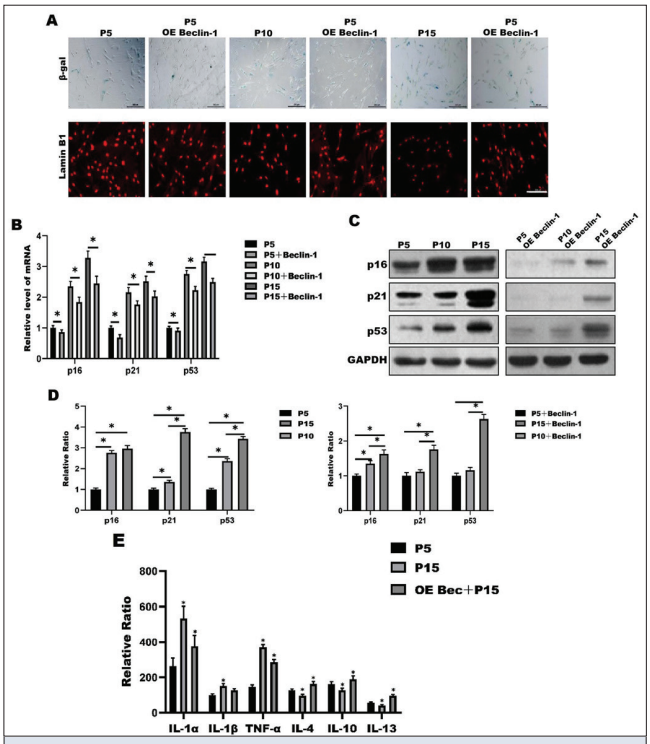

Fig 2. Beclin-1 rejuvenated aged hUC-MSCs. **A:** β-galactosidase activity staining and Lamin B1 immunofluorescence staining, **B:** RT-qPCR. C&D: Western blotting bands and histograms, **E:** Histograms of ELISA results. The data were described by mean ± standard deviation

was significantly better than that in P15MSCs group, indicating that Beclin-1-overexpressing P15 MSCs are superior to P15 MSCs in improving the memory of AD mice. The results of immunofluorescence staining showed that the survival rates of neuronal cells and NeuN$^+$ cells in P15MSCs group were significantly lower than those in P5MSCs group, and they were significantly higher in OE Bec-P15MSCs group than those in P15MSCs group (P<0.05) *(Fig. 4-D)*. The number of TUNEL$^+$ cells in brain tissues was smaller in OE Bec-P15MSCs group than that

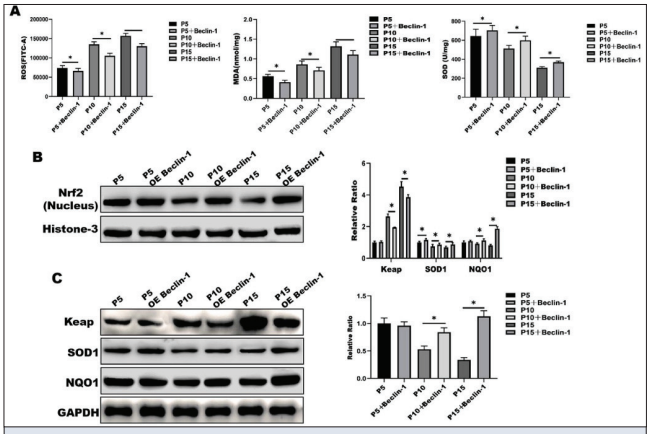

Fig 3. Beclin-1 attenuated oxidative stress in aged hUC-MSCs by activating Nrf2 signaling pathway. **A:** Levels of ROS, MDA and SOD, **B&C:** Western blotting bands and histograms. The data were described by mean ± standard deviation

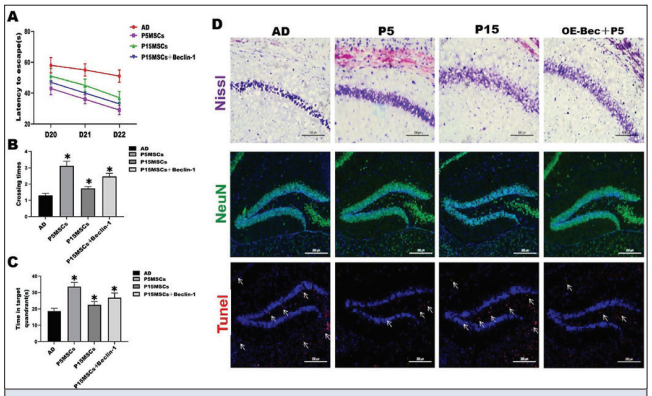

Fig 4. Overexpression of Beclin-1 enhanced the therapeutic effect of hUC-MSCs in AD mice. **A-C:** Histograms of statistical analysis of MWM and NOR test results, **D:** Immunofluorescence staining results of brain tissue sections. The data were described by mean ± standard deviation. *P<0.05 vs. AD group

in P15MSCs group (P<0.05). Beclin-1-overexpressing P15 MSCs promoted the expression of NeuN and reduced the number of TUNEL$^+$ cells in the hippocampus (P<0.05). To sum up, the overexpression of Beclin-1 can improve the therapeutic effect of hUC-MSCs in AD mice.

Beclin-1 Ameliorated Memory Impairment and Depression-like Behavior in AD Mice

OE Bec-P15MSCs group and MSCs groups had more trajectory crossings in the MWM test and longer residence time in the target quadrant than AD group, with the behavioral improvement being the most significant in OE Bec-P15MSCs group. Since most AD patients are usually accompanied by mental symptoms such as anxiety and depression, FST, OFT and NSF tests were also performed to evaluate the mood and behavior of mice. It was found that OE Bec-P15MSCs group had significantly longer

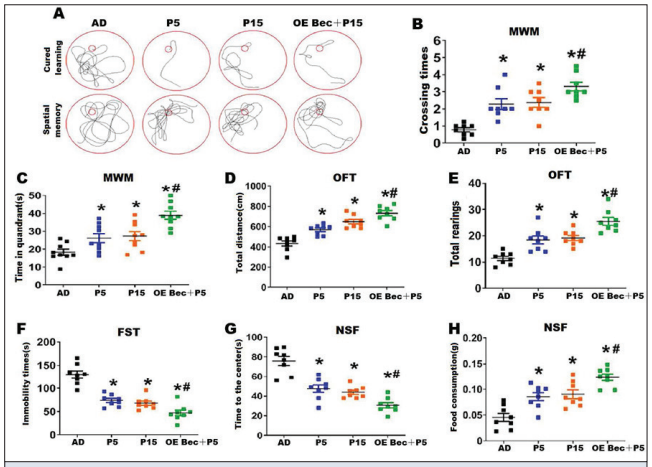

Fig 5. Beclin-1 ameliorated memory impairment and depression-like behavior in AD mice. **A:** MWM test trajectory chart, **B-H:** Histograms of statistical analysis of mouse behavioral test results. The data were described by mean ± standard deviation. *P<0.05 vs. AD group; #P<0.05 vs. P5MSCs group

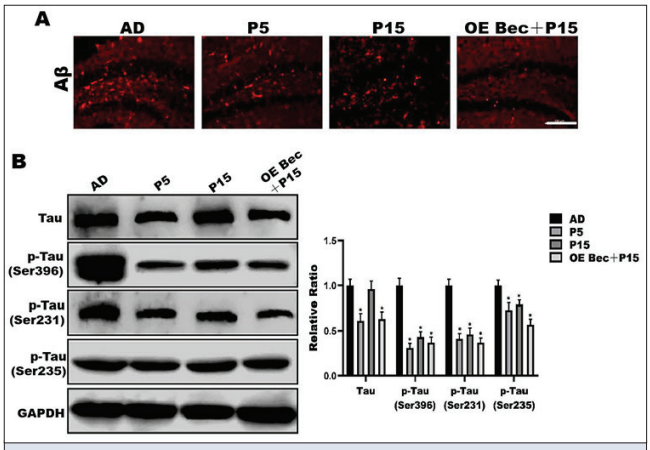

Fig 6. Overexpression of Beclin-1 reduced Aβ deposition and Tau phosphorylation in AD mice. **A:** Immunofluorescence staining of Aβ, **B:** Western blotting bands and statistical analysis histograms. The data were described by mean ± standard deviation. *P<0.05 vs. AD group; #P<0.05 vs. P5MSCs group

swimming time, shorter immobility time and the largest total food consumption than AD and MSCs groups *(Fig. 5)*. It can be inferred that overexpression of Beclin-1 and transplantation of hUC-MSCs can synergistically alleviate memory impairment and depression-like behavior in AD mice.

Overexpression of Beclin-1 Reduced Aβ Deposition and Tau Phosphorylation in AD Mice

Aβ deposition and neurofibrillary tangles induced by hyperphosphorylated tau are two major pathological hallmarks of AD. Therefore, the effects of Beclin-1 overexpression and hUC-MSCs transplantation on Aβ deposition and Tau phosphorylation in mouse brain tissues were detected by immunofluorescence and Western blotting, respectively. As shown in *Fig. 6-A*, obvious Aβ plaques were formed in the brains of AD mice, and transplantation of hUC-MSCs and overexpression of Beclin-1 significantly reduced Aβ deposition. The results of Western blotting revealed that the expression levels of P-tau (Ser396), P-tau (Ser231) and P-tau (Ser235) were all significantly inhibited by transplantation of hUC-MSCs and overexpression of Beclin-1, especially in OE Bec-P15MSCs group *(Fig. 6-B)*. These results suggest that overexpression of Beclin-1 combined with transplantation of hUC-MSCs can effectively reduce Aβ load and Tau hyperphosphorylation in AD mice.

Overexpression of Beclin-1 Combined with Transplantation of hUC-MSCs Promoted Neurogenesis in the Brain of AD Mice

Neuronal loss and neurogenesis in the hippocampus play a critical role in maintaining cognitive function. Therefore, neurogenesis was detected by double immunofluorescence staining of neuron-specific markers. The results showed that double positive staining of EdU$^+$/DCX$^+$ cells and

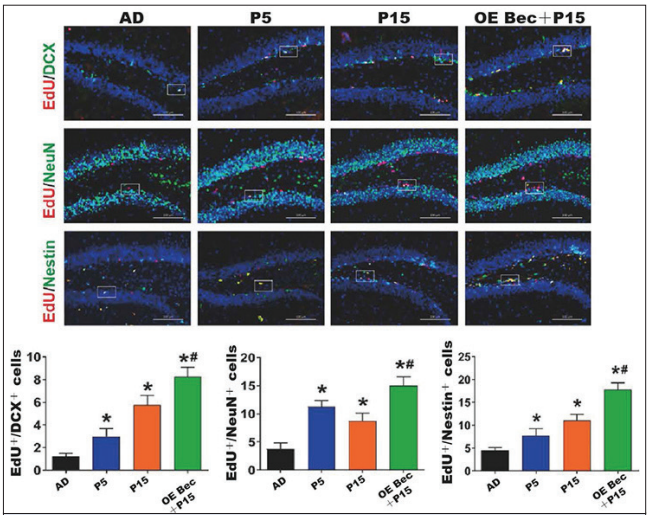

Fig 7. Overexpression of Beclin-1 combined with transplantation of hUC-MSCs promoted neurogenesis in the brain of AD mice. The data were described by mean ± standard deviation. *P<0.05 vs. AD group; #P<0.05 vs. P5MSCs group

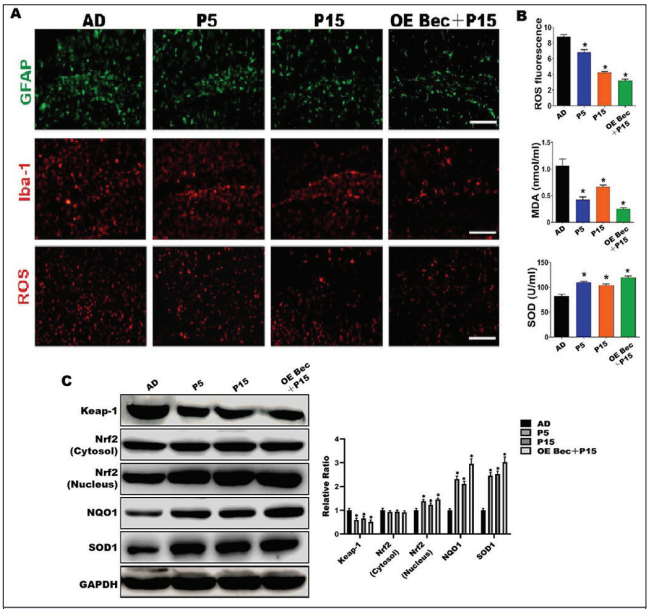

Fig 8. Overexpression of Beclin-1 combined with transplantation of hUC-MSCs activated the Nrf2 signaling pathway. **A:** Immunofluorescence staining of GFAP, Iba-1 and ROS, **B:** Levels of ROS, MDA, and SOD, **C:** Western blotting bands and statistical analysis histograms. The data were described by mean ± standard deviation. *P<0.05 vs. AD group; #P<0.05 vs. P5MSCs group

EdU$^+$/NeuN$^+$ cells was significantly increased in OE Bec-P15MSCs group as compared to that in other groups (P<0.05), and the proportion of EdU$^+$/Nestin$^+$ cells was also the highest in OE Bec-P15MSCs group (P<0.05). Beclin-1 overexpression promoted the survival of hUC-MSCs *in vivo* and activated the generation of endogenous neural stem cells (NSCs) *(Fig. 7)*. These results suggest that overexpression of Beclin-1 combined with transplantation of hUC-MSCs can ameliorate neuronal loss and promote neurogenesis in the brain of AD mice.

Overexpression of Beclin-1 Combined with Transplantation of hUC-MSCs Activated the Nrf2 Signaling Pathway

To determine whether overexpression of Beclin-1 can regulate the neuroinflammatory microenvironment in the brain of AD mice, the activation status of hippocampal astrocytes and microglia was detected by GFAP and Iba-1 immunofluorescence staining. Compared with that in AD group, the number of GFAP$^+$ or Iba-1$^+$ cells significantly declined in other groups, and the activation degrees of astrocytes and microglia were the lowest in OE Bec-P15MSCs group (P<0.05). Meanwhile, OE Bec-P15MSCs group had the lowest ROS production and MDA content and the highest SOD activity (P<0.05), suggesting that overexpression of Beclin-1 combined with transplantation of hUC-MSCs can alleviate the activation and oxidative stress of glial cells in AD mice. Moreover, the protein expression of Nrf2 in NQO1 and SOD1 in brain tissues significantly rose, while the expression of Keap-1 was down-regulated in OE Bec-P15MSCs group (P<0.05) *(Fig. 8)*. To sum up, overexpression of Beclin-1 combined with transplantation of hUC-MSCs can reduce the activation and oxidative stress of glial cells in AD mice through activating the Nrf2 signaling pathway.

Discussion

There are many close links between cellular senescence and oxidative stress. Oxidative stress responses can induce apoptosis and cell cycle arrest, thus accelerating cell death or depriving cells of normal function [21]. HUC-MSCs are the most widely used stem cells in clinical practice, but they are prone to senescent phenotype after culture over several passages *in vitro* [22], thus limiting their further practical application. In this study, compared with P5 hUC-MSCs, P15 hUC-MSCs exhibited signs of cellular senescence such as significant morphological changes, swelling, decreased proliferation and migration, and enhanced oxidative stress. Rejuvenating aged hUC-MSCs to increase the therapeutic time window and efficacy in regenerative medicine is of clinical application value [23]. In this study, the overexpression of Beclin-1 reduced the proportion of SA-β-gal$^+$ cells, inhibited the expression of senescence-related proteins, promoted the proliferation and migration of hUC-MSCs, and rejuvenated aged hUC-MSCs.

Nrf2 is a key transcription factor that regulates cellular oxidative stress and inflammatory response, and maintains stem cell function and nerve regeneration [24]. Under normal circumstances, Nrf2 is continuously ubiquitinated by Keap-1 and degraded *via* the ubiquitin-proteasome pathway. Under oxidative stress, however, Nrf2 translocates to the nucleus and binds to antioxidant response element sites to activate the expression of downstream genes

such as HO-1, SOD-1 and NQO-1 [25]. In this study, the overexpression of Beclin-1 significantly reduced the expression of Keap-1 in aged hUC-MSCs and enhanced the expression of Nrf2, NQO1 and SOD1 in the nucleus, indicating that Beclin-1 activated the activity of Nrf2 signaling pathway. In addition, Beclin-1-overexpressing hUC-MSCs were superior to simple hUC-MSCs in improving the cognitive function, reducing neuronal loss, and promoting neurogenesis in AD mice. Pickford et al.[26] demonstrated the protective role of Beclin-1 in AD. Additionally, Beclin-1 depletion in APP transgenic mice disrupts autophagy, aggravates Aβ pathology, and promotes neurodegeneration [27]. These findings verify the ability of Beclin-1 to rejuvenate aged hUC-MSCs both *in vivo* and *in vitro*, but the exact underlying mechanism still needs further investigation.

Despite growing evidence that stem cell therapy is a promising strategy for treating AD, the chronic pathological microenvironment in the brain is not conducive to the survival of transplanted stem cells [28]. Therefore, it is necessary to develop new approaches to improve the efficacy of stem cell therapy. Wang et al. found that the neuroprotective effect of hUC-MSCs plus resveratrol on AD mice was better than that of single treatment, and MG53 protected hUC-MSCs from LPS-induced inflammatory injury and enhanced their effect on LPS-treated C57/BL6 mice [29]. In this study, the overexpression of Beclin-1 combined with transplantation of hUC-MSCs significantly alleviated the cognitive impairment and depression-like behavior in AD mice, indicating that this combination therapy had a better therapeutic effect than the transplantation of hUC-MSCs alone.

Aβ deposition and Tau phosphorylation are two main pathological features of AD. Esmaeilzade et al.[30]reported that Aβ plaques formed in the brain of APP/PS1 mice at 3 months of age. In this study, the overexpression of Beclin-1 combined with transplantation of hUC-MSCs significantly reduced Aβ deposition and Tau hyperphosphorylation in AD mice. Likewise, the reduced levels of Aβ plaques and Tau phosphorylation have been reported to benefit both young and aged AD-like animals [31,32]. These results suggest that Beclin-1 and hUC-MSCs can effectively alleviate the pathological changes in AD through a synergistic effect. Aβ-induced neuronal loss is the main cause of cognitive impairment in AD [33]. This study showed that the combination therapy relieved the neuronal loss and preserved the neuronal viability in AD mice.

In addition, exogenous stem cell transplantation or endogenous NSC activation can promote neurogenesis [34]. In this study, the overexpression of Beclin-1 raised the survival rate of hUC-MSCs in the brain of AD mice. The overexpression of Beclin-1 combined with transplantation of hUC-MSCs increased the number of EdU^+/DCX^+, $EdU^+/NeuN^+$, and $EdU^+/Nestin^+$ cells in the hippocampus, suggesting that this combination therapy can also promote neurogenesis by activating endogenous NSCs. Besides, activated glial cells and oxidative stress can damage the structure and function of neuronal cells, and overactivated microglia and astrocytes can induce persistent chronic neuroinflammation, leading to neurodegeneration [35]. Oxidative stress induced by excessive ROS contributes to the senescence of MSCs. Nrf2 is one of the key molecular pathways regulating senescence, inflammation and oxidative stress [36]. In this study, the overexpression of Beclin-1 combined with transplantation of hUC-MSCs inhibited glial cell activation and oxidative stress in AD mice by increasing the expressions of Nrf2, NQO1 and SOD1, demonstrating that Beclin-1 promoted the neuroprotective effect of hUC-MSCs on AD mice at least partly by targeting the Nrf2 signaling pathway.

In conclusion, the overexpression of Beclin-1 delays the aging process of hUC-MSCs *in vitro* and enhances the therapeutic effect of hUC-MSCs on AD mice in improving cognition, reducing Aβ deposition and Tau hyperphosphorylation, and increasing neurogenesis.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (P. Ma) on reasonable request.

Financial Support

This study was financially supported by Scientific Research Project of Jiangsu Provincial Health Commission (No. Z2022051), Xuzhou Science and Technology Plan Project (No. KC22143), Xuzhou Pengcheng Elite Medical Key Talent Training Program, Jiangsu Senile Health Research Project (No. LKM2022045), and Science and Technology Development Fund Project of Xuzhou Medical University Affiliated Hospital (No. XYFM202239).

Ethical Approval

The human experiment regarding the collection of umbilical cord tissues has been approved by the ethics committee of Xuzhou First People's Hospital on January 4th, 2021 (approval No. XFPH202101003). All animal experiments have been approved by the animal experiments ethics committee of Xuzhou First People's Hospital on March 8th, 2021 (approval No. XFPH-dong202103004).

Competing Interests

There is no conflict of interest.

Authors' Contributions

Li Shao and Pengju Ma designed this study and significantly revised the manuscript; Qing He, Xin Du, Qing Li, Siyuan Yang and Chen Dong performed this study and prepared this manuscript. All authors have approved the submission and publication of this manuscript.

References

1. Abdi S, Javanmehr N, Ghasemi-Kasman M, Bali HY, Pirzadeh M: Stem

cell-based therapeutic and diagnostic approaches in Alzheimer's disease. *Curr Neuropharmacol,* 20, 1093-1115, 2022. DOI: 10.2174/1570159X20666211231090659

2. Wang X, Ma S, Yang B, Huang T, Meng N, Xu L, Xing Q, Zhang Y, Zhang K, Li Q, Zhang T, Wu J, Yang GL, Guan F, Wang J: Resveratrol promotes hUC-MSCs engraftment and neural repair in a mouse model of Alzheimer's disease. *Behav Brain Res,* 339, 297-304, 2018. DOI: 10.1016/j.bbr.2017.10.032

3. Duncan T, Valenzuela M: Alzheimer's disease, dementia, and stem cell therapy. *Stem Cell Res Ther,* 8:111, 2017. DOI: 10.1186/s13287-017-0567-5

4. Sadatpoor SO, Salehi Z, Rahban D, Salimi A: Manipulated mesenchymal stem cells applications in neurodegenerative diseases. *Int J Stem Cells,* 13, 24-45, 2020. DOI: 10.15283/ijsc19031

5. Ra JC, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, Kim YJ, Jo JY, Yoon EJ, Choi HJ, Kwon E: Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev,* 20, 1297-1308, 2011. DOI: 10.1089/scd.2010.0466

6. Reboussin É, Buffault J, Brignole-Baudouin F, Réaux-Le Goazigo A, Riancho L, Olmiere C, Sahel JA, Mélik Parsadaniantz S, Baudouin C: Evaluation of neuroprotective and immunomodulatory properties of mesenchymal stem cells in an ex vivo retinal explant model. *J Neuroinflammation,* 19:63, 2022. DOI: 10.1186/s12974-022-02418-w

7. Nakano M, Kubota K, Kobayashi E, Chikenji TS, Saito Y, Konari N, Fujimiya M: Bone marrow-derived mesenchymal stem cells improve cognitive impairment in an Alzheimer's disease model by increasing the expression of microRNA-146a in hippocampus. *Sci Rep,* 10:10772, 2020. DOI: 10.1038/s41598-020-67460-1

8. Cui Y, Ma S, Zhang C, Cao W, Liu M, Li D, Lv P, Xing Q, Qu R, Yao N, Yang B, Guan F: Human umbilical cord mesenchymal stem cells transplantation improves cognitive function in Alzheimer's disease mice by decreasing oxidative stress and promoting hippocampal neurogenesis. *Behav Brain Res,* 320, 291-301, 2017. DOI: 10.1016/j.bbr.2016.12.021

9. Jeong H, Kim OJ, Oh SH, Lee S, Reum Lee HA, Lee KO, Lee BY, Kim NK: Extracellular vesicles released from neprilysin gene-modified human umbilical cord-derived mesenchymal stem cell enhance therapeutic effects in an Alzheimer's disease animal model. *Stem Cells Int,* 2021:5548630, 2021. DOI: 10.1155/2021/5548630

10. Zhang T, Wang P, Liu Y, Zhou J, Shi Z, Cheng K, Huang T, Wang X, Yang GL, Yang B, Ma S, Guan F: Overexpression of FOXQ1 enhances anti-senescence and migration effects of human umbilical cord mesenchymal stem cells *in vitro* and *in vivo. Cell Tissue Res,* 373, 379-393, 2018. DOI: 10.1007/s00441-018-2815-0

11. Bagheri-Mohammadi S: Stem cell-based therapy as a promising approach in Alzheimer's disease: Current perspectives on novel treatment. *Cell Tissue Bank,* 22, 339-353, 2021. DOI: 10.1007/s10561-020-09896-3

12. Ren RJ, Huang Q, Xu G, Gu K, Dammer EB, Wang CF, Xie XY, Chen W, Shao ZY, Chen SD, Wang G: Association between Alzheimer's disease and risk of cancer: A retrospective cohort study in Shanghai, China. *Alzheimers Dement,* 18, 924-933, 2022. DOI: 10.1002/alz.12436

13. Fellner L, Gabassi E, Haybaeck J, Edenhofer F: Autophagy in α-synucleinopathies - An overstrained system. *Cells,* 10:3143, 2021. DOI: 10.3390/cells10113143

14. Qin X, Fei J, Duan Y, Ceylan AF, Zhang F, Ren J: Beclin1 haploinsufficiency compromises mesenchymal stem cell-offered cardioprotection against myocardial infarction. *Cell Regen,* 11:21, 2022. DOI: 10.1186/s13619-022-00121-y

15. Peng C, Li Y, Lu L, Zhu J, Li H, Hu J: Efficient one-step induction of human umbilical cord-derived mesenchymal stem cells (UC-MSCs) produces MSC-derived neurospheres (MSC-NS) with unique transcriptional profile and enhanced neurogenic and angiogenic secretomes. *Stem Cell Int,* 2019:9208173, 2019. DOI: 10.1155/2019/9208173

16. Tang S, Chen P, Zhang H, Weng H, Fang Z, Chen C, Peng G, Gao H, Hu K, Chen J, Chen L: Comparison of curative effect of human umbilical cord-derived mesenchymal stem cells and their small extracellular vesicles in treating osteoarthritis. *Int J Nanomed,* 16, 8185-8202, 2021. DOI: 10.2147/IJN.S336062

17. Xie Y, Liu S, Wang L, Yang H, Tai C, Ling L, Chen L, Liu S, Wang B: Individual heterogeneity screened umbilical cord-derived mesenchymal stromal cells with high Treg promotion demonstrate improved recovery of mouse liver fibrosis. *Stem Cell Res Ther,* 12:359, 2021. DOI: 10.1186/s13287-021-02430-6

18. Li X, Guo L, Chen J, Liang H, Liu Y, Chen W, Zhou L, Shan L, Wang H: Intravenous injection of human umbilical cord-derived mesenchymal stem cells ameliorates not only blood glucose but also nephrotic complication of diabetic rats through autophagy-mediated anti-senescent mechanism. *Stem Cell Res Ther,* 14:146, 2023. DOI: 10.1186/s13287-023-03354-z

19. Kotagale N, Deshmukh R, Dixit M, Fating R, Umekar M, Taksande B: Agmatine ameliorates manifestation of depression-like behavior and hippocampal neuroinflammation in mouse model of Alzheimer's disease. *Brain Res Bull,* 160, 56-64, 2020. DOI: 10.1016/j.brainresbull.2020.04.013

20. Shao P: MiR-216a-5p ameliorates learning-memory deficits and neuroinflammatory response of Alzheimer's disease mice via regulation of HMGB1/NF-κB signaling. *Brain Res,* 1766:147511, 2021. DOI: 10.1016/j.brainres.2021.147511

21. Guan F, Huang T, Wang X, Xing Q, Gumpper K, Li P, Song J, Tan T, Yang GL, Zang X, Zhang J, Wang Y, Yang Y, Liu Y, Zhang Y, Yang B, Ma J, Ma S: The TRIM protein Mitsugumin 53 enhances survival and therapeutic efficacy of stem cells in murine traumatic brain injury. *Stem Cell Res Ther,* 12:522, 2019. DOI: 10.1186/s13287-021-02598-x

22. Yang M, Lin J, Tang J, Chen Z, Qian X, Gao WQ, Xu H: Decreased immunomodulatory and secretory capability of aging human umbilical cord mesenchymal stem cells *in vitro. Biochem Biophys Res Commun,* 525, 633-638, 2020. DOI: 10.1016/j.bbrc.2020.02.125

23. Jiang X, Li W, Ge L, Lu M: Mesenchymal stem cell senescence during aging: From mechanisms to rejuvenation strategies. *Aging Dis,* 14, 1651-1676, 2023. DOI: 10.14336/AD.2023.0208

24. Shi W, Huang CJ, Xu XD, Jin GH, Huang RQ, Huang JF, Chen YN, Ju SQ, Wang Y, Shi YW, Qin JB, Zhang YQ, Liu QQ, Wang XB, Zhang XH, Chen J: Transplantation of RADA16-BDNF peptide scaffold with human umbilical cord mesenchymal stem cells forced with CXCR4 and activated astrocytes for repair of traumatic brain injury. *Acta Biomater,* 45, 247-261, 2016. DOI: 10.1016/j.actbio.2016.09.001

25. Reyhani S, Abbaspanah B, Mousavi SH: Umbilical cord-derived mesenchymal stem cells in neurodegenerative disorders: From literature to clinical practice. *Regen Med,* 15, 1561-1578, 2020. DOI: 10.2217/rme-2019-0119

26. Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, Small S, Spencer B, Rockenstein E, Levine B, Wyss-Coray T: The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid β accumulation in mice. *J Clin Invest,* 118, 2190-2199, 2008. DOI: 10.1172/JCI33585

27. Rohn TT, Wirawan E, Brown RJ, Harris JR, Masliah E, Vandenabeele P: Depletion of beclin-1 due to proteolytic cleavage by caspases in the Alzheimer's disease brain. *Neurobiol Dis,* 43, 68-78, 2011. DOI: 10.1016/j.nbd.2010.11.003

28. Kim J, Lee Y, Lee S, Kim K, Song M, Lee J: Mesenchymal stem cell therapy and Alzheimer's disease: Current status and future perspectives. *J Alzheimers Dis,* 77, 1-14, 2020. DOI: 10.3233/JAD-200219

29. Wang GH, Liu Y, Wu XB, Lu Y, Liu J, Qin YR, Li T, Duan HF: Neuroprotective effects of human umbilical cord-derived mesenchymal stromal cells combined with nimodipine against radiation-induced brain injury through inhibition of apoptosis. *Cytotherapy,* 18, 53-64, 2016. DOI: 10.1016/j.jcyt.2015.10.006

30. Esmaeilzade B, Artimani T, Amiri I, Najafi R, Shahidi S, Sabec M, Farzadinia P, Zare M, Zahiri M, Soleimani Asl S: Dimethyloxalylglycine preconditioning enhances protective effects of bone marrow-derived mesenchymal stem cells in Aβ-induced Alzheimer disease. *Physiol Behav,* 199, 265-272, 2019. DOI: 10.1016/j.physbeh.2018.11.034

31. Lee IS, Jung K, Kim IS, Lee H, Kim M, Yun S, Hwang K, Shin JE, Park KI: Human neural stem cells alleviate Alzheimer-like pathology in a mouse model. *Mol Neurodegener,* 21:38, 2015. DOI: 10.1186/s13024-015-0035-6

32. Lee JK, Jin HK, Endo S, Schuchman EH, Carter JE, Bae JS: Intracerebral

transplantation of bone marrow-derived mesenchymal stem cells reduces amyloid-beta deposition and rescues memory deficits in Alzheimer's disease mice by modulation of immune responses. *Stem Cells,* 28, 329-343, 2010. DOI: 10.1002/stem.277

33. Ma C, Feng Y, Yang L, Wang S, Sun X, Tai S, Guan X, Wang D, Yu Y: *In vitro* immunomodulatory effects of human umbilical cord-derived mesenchymal stem cells on peripheral blood cells from warm autoimmune hemolytic anemia patients. *Acta Haematol,* 145, 63-71, 2022. DOI: 10.1159/000506759

34. Zhao J, Liu S, Xiang X, Zhu X: Versatile strategies for adult neurogenesis: Avenues to repair the injured brain. *Neural Regen Res,* 19, 774-780, 2024. DOI: 10.4103/1673-5374.382224

35. Gao L, Zhao M, Li P, Kong J, Liu Z, Chen Y, Huang R, Chu J, Quan J, Zeng R: Glycogen synthase kinase 3 (GSK3)-inhibitor SB216763 promotes the conversion of human umbilical cord mesenchymal stem cells into neural precursors in adherent culture. *Hum Cell,* 30, 11-22, 2017. DOI: 10.1007/s13577-016-0146-6

36. Cao N, Liao T, Liu J, Fan Z, Zeng Q, Zhou J, Pei H, Xi J, He L, Chen L, Nan X, Jia Y, Yue W, Pei X: Clinical-grade human umbilical cord-derived mesenchymal stem cells reverse cognitive aging via improving synaptic plasticity and endogenous neurogenesis. *Cell Death Dis,* 8:e2996, 2017. DOI: 10.1038/cddis.2017.316