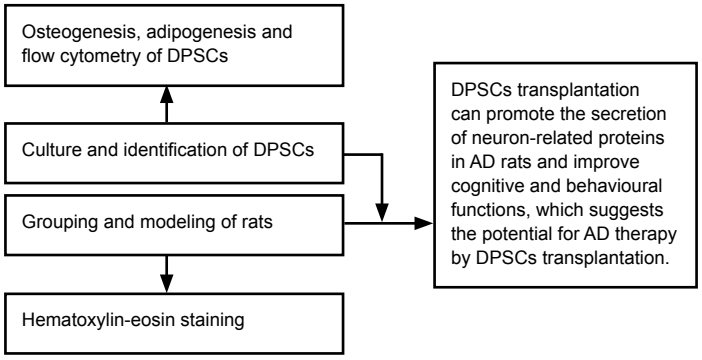
Research Article

Therapeutic potential of dental pulp stem cell transplantation in a rat model of Alzheimer's disease

https://doi.org/10.4103/1673-5374.297088
Date of submission: March 18, 2020
Date of decision: April 23, 2020
Date of acceptance: June 8, 2020
Date of web publication: November 16, 2020

Xue-Mei Zhang[1, #], Yuan-Jiao Ouyang[1, #], Bing-Qian Yu[2], Wei Li[1], Mei-Yu Yu[1], Jin-Yue Li[1], Zhuo-Min Jiao[1], Dan Yang[1], Na Li[3], Ying Shi[1], Yun-Yun Xu[1], Zhi-Jun He[1], Duo Wang[1], Hui Yue[1], Jin Fu[1, *]

Graphical Abstract *Dental pulp stem cells (DPSCs) transplantation improved the function of Alzheimer's disease (AD) rats as confirmed by histology and behavior tests*



Abstract

Dental pulp stem cells are dental pulp-derived mesenchymal stem cells that originate from the neural crest. They exhibit greater potential for the treatment of nervous system diseases than other types of stem cells because of their neurogenic differentiation capability and their ability to secrete multiple neurotrophic factors. Few studies have reported Alzheimer's disease treatment using dental pulp stem cells. Rat models of Alzheimer's disease were established by injecting amyloid-β1–42 into the hippocampus. Fourteen days later, 5×10^6 dental pulp stem cells were injected into the hippocampus. Immunohistochemistry and western blot assays showed that dental pulp stem cell transplantation increased the expression of neuron-related doublecortin, NeuN, and neurofilament 200 in the hippocampus, while the expression of amyloid-β was decreased. Moreover, cognitive and behavioral abilities were improved. These findings indicate that dental pulp stem cell transplantation in rats can improve cognitive function by regulating the secretion of neuron-related proteins, which indicates a potential therapeutic effect for Alzheimer's disease. This study was approved by the Animal Ethics Committee of Harbin Medical University, China (approval No. KY2017-132) on February 21, 2017.

Key Words: Alzheimer's disease; brain; central nervous system; dental pulp stem cell; *in vivo*; model; rat; stem cells; transplantation

Chinese Library Classification No. R456; R741; Q2

Introduction

Alzheimer's disease (AD) is a degenerative disease of the central nervous system and is the most common cause of dementia (Zhao et al., 2016; Doxtater et al., 2020; Sumbria, 2020). AD is associated with age and is characterized by irreversible, progressive cognitive decline and behavioral impairment. There are two main hallmarks of AD pathology: beta-amyloid (Aβ) deposition and neurofibrillary tangles (called tau tangles) (Holtzman et al., 2011). Aβ plaques and tau tangles interfere with neuron-to-neuron communication and neuronal nutrition, leading to neuronal death, memory loss and the abnormal behavior observed in dementia (Brier et al., 2016). With increased life expectancy, the number of people suffering from AD has also increased. In 2016, a patient was diagnosed with AD every 66 seconds in the USA, and AD was the fifth leading cause of death in people over the age of 65. In 2016, the cost of health care, long-term care and hospice care for dementia patients in the USA was estimated to be $236 billion. By 2050, the number of AD patients is expected to have doubled, and one new case of AD will occur every 33 seconds (Alzheimer's Association, 2016). However, only two types of drugs, cholinesterase inhibitors and the N-methyl-D-aspartate receptor antagonist, memantine, have been approved for AD by the U.S. Food and Drug Administration. These drugs can slightly improve dementia symptoms in AD patients, but they have no effect on the pathophysiology of AD and cannot reverse the progress of AD or prolong the survival of patients (Caselli et al., 2017). Therefore, finding other innovative therapeutic methods is necessary and urgent.

[1]Department of Neurology, The 2nd Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China; [2]Department of Neurology, Ningbo Hangzhou Bay Hospital, Ningbo, Zhejiang Province, China; [3]Department of Neurology, the First Hospital of Qiqihar, Qiqihar, Heilongjiang Province, China
*Correspondence to: Jin Fu, PhD, MD, fujin198@126.com.
https://orcid.org/0000-0001-8425-8129 (Jin Fu)
#Both authors contributed equally to this work.

Funding: *This study was supported by Yu Weihan Fund for Distinguished Young Scholars of Harbin Medical University of China, No. 002000013 (to XMZ).*
How to cite this article: *Zhang XM, Ouyang YJ, Yu BQ, Li W, Yu MY, Li JY, Jiao ZM, Yang D, Li N, Shi Y, Xu YY, He ZJ, Wang D, Yue H, Fu J (2021) Therapeutic potential of dental pulp stem cell transplantation in a rat model of Alzheimer's disease. Neural Regen Res 16(5):893-898.*

AD pathology involves damage to hippocampal neurons and neural circuits. The development of neuronal circuits includes cell proliferation, migration, differentiation and synaptic formation. Doublecortin (DCX) is a regulatory microtubule-associated protein that plays an important role in the migration and differentiation of neurons. DCX immunoreactivity is a marker of newborn neurons (Francis et al., 1999). In DCX knockout mice, the pyramidal cells in the hippocampal CA3 region are abnormal, which affects the neural network and function of the hippocampus (Khalaf-Nazzal et al., 2017). NeuN, also known as Fox3, is a member of the Fox1 gene family and is responsible for selective regulation of RNA splicing. NeuN exists only in neurons (Maxeiner et al., 2014) and is widely found in the nuclei of vertebrate neurons. NeuN immunoreactivity is a mature neuron marker and is widely used to detect nerve cells. NeuN plays an important role in synaptic formation and functional regulation in the hippocampus (Lin et al., 2016) and NeuN dysfunction is related to neurodevelopmental retardation, cognitive impairment and synaptic plasticity defects. Neurofilaments (NFs) consist of three main subunits: NF200, NF165 and NF68. NFs support the morphology of neurons as a cytoskeleton component, and also play a basic role in axonal transport. NF200 is mainly involved in the stabilization of newborn axons and plays an important role in the stability and maturation of axonal connections. In the pathological state, NFs in the hippocampus are destroyed and changes to NFs trigger further axonal damage (Lee et al., 2018). Our study investigates the levels of three neuron-related proteins, DCX, NeuN, and NF200, as indexes to explore AD damage and repair.

Several studies have shown that cognitive function in AD can be improved by the transplantation of stem cells, such as bone marrow stem cells (BMSCs), embryonic stem cells, and neural stem cells (Lee et al., 2015; Chakari-Khiavi et al., 2019; Staff et al., 2019). In particular, significant transplantation and therapeutic achievements have been made using mesenchymal stem cells (MSCs). MSCs, a type of pluripotent stem cell, have been widely used because they are easy to collect and have multi-lineage differentiation potential. MSCs reside in bone marrow, dental pulp, and adipose tissue (Ullah et al., 2015). Dental pulp stem cells (DPSCs) are widely used because of their easy availability. In addition, DPSCs originate from the neural crest, have the potential for neurogenic differentiation, and can secrete neurotrophic factors. They can therefore be used to treat nervous system diseases (Mead et al., 2017). Furthermore, DPSCs have better neural differentiation potential than BMSCs and adipose-derived MSCs (Mead et al., 2014). Therefore, treatment of AD by DPSC transplantation is worth exploring. In this study, the therapeutic effect of DPSCs on neurodegeneration was observed in a rat model of AD by evaluating the secretion of neuron-associated proteins and by assessing cognitive function.

Methods and Materials

Animals

Sprague-Dawley rats were bought from the Animal Experiment Center of the 2nd Affiliated Hospital of Harbin Medical University [license No. SCXK (Hei) 2019-001]. One hundred rats (clean grade, 6–8 weeks old, 190–200 g) were randomly divided into four groups (n = 25/group): control, Aβ + phosphate buffered saline (PBS), Aβ + DPSC, and PBS + PBS groups (named according to the substance injected into the hippocampus). The rats in the control group did not undergo any procedure or treatment. We established a rat model of AD by injecting human $A\beta_{1-42}$ (Cat# ab82795; Abcam, Cambridge, UK) diluted to 1 mg/mL into the hippocampus. Rats were anaesthetized with pentobarbital sodium (40 mg/kg, intraperitoneal injection; Sitiande; Chengdu, China) (Li et al., 2019) and secured in a stereotaxic instrument (Rwdls, Shenzhen, China). Each side of the hippocampus was then injected with 10 μL $A\beta_{1-42}$ solution with a microinjector (Rwdls) (Banik et al., 2015). The rats in the PBS + PBS group were injected with 10 μL PBS in the same manner. Fourteen days later, brains from one human $A\beta_{1-42}$-injected and one PBS-injected rat were hematoxylin-eosin stained. The remaining rats were then injected with 10 μL DPSCs or PBS. The DPSCs were initially isolated from two male rats (clean grade, 2–3 weeks old, 40–50 g) and 5×10^6 third-generation DPSCs were injected along the same needle track as the $A\beta_{1-42}$ injection (Ager et al., 2015). On the 29th day, we collected the brains of rats for analysis (**Figure 1A**). All animal experiments were approved by Ethics Committee for Laboratory Animals of Harbin Medical University of China (approval No. KY2017-132) on February 21, 2017.

Isolation of DPSCs

Dental pulp tissues were isolated from the pulpal cavity of rat incisors (Hata et al., 2015), digested with collagenase, and transferred to 25 mL flasks containing 5 mL medium comprising 20% fetal bovine serum, 1% penicillin and streptomycin and 79% Dulbecco's modified Eagle's medium. The DPSCs were cultured at 37°C in a 5% CO_2 atmosphere and the culture medium was changed every 2–3 days. When the cells reached 85–90% confluence, they were digested with trypsin. Third-generation DPSCs were used in this study. Osteogenic differentiation of DPSCs was performed using a BMSC osteogenic differentiation medium (Cat# RASMX-90021; Cyagen Biosciences, Santa Clara, CA, USA). After 2–4 weeks of induction, cells were fixed with 4% neutral formaldehyde and stained with alizarin red. BMSC adipogenic differentiation medium (Cat# RASMX-90031; Cyagen Biosciences) was used for adipogenic differentiation. After completion of adipogenic differentiation, cells were fixed with 4% neutral formaldehyde solution and stained with oil red O. There is no commercially available osteogenic or adipogenic differentiation medium made specifically for DPSCs. Both DPSCs and BMSCs are MSCs, therefore BMSC differentiation medium was used instead.

Flow cytometry was performed to identify cell surface markers. The third-generation DPSCs were digested with trypsin to prepare a cell suspension, which was washed twice with PBS, and adjusted to 1×10^6 cells/mL. The cells were incubated at room temperature for 1 hour with a fluorescein isothiocyanate isomer-conjugated antibody against CD29, an allophycocyanin-conjugated antibody against CD90, a phycoerythrin-conjugated antibody against CD34, or a phycoerythrin-conjugated antibody against CD45. Staining was terminated with 1% bovine serum albumin, and the cells were then loaded onto the flow cytometer (Becton-Dickinson, Franklin, NJ, USA) (Huang et al., 2018). The same method was used for homotypic controls.

Immunofluorescence

DPSCs were fluorescently labeled with PKH67 (1:250; Sigma-Aldrich, St. Louis, MO, USA) and then immediately injected into the hippocampus. Fourteen days after DPSC transplantation, rats were anesthetized with pentobarbital sodium and transcardially perfused with normal saline followed by 4% paraformaldehyde solution. Brains were then dissected and further fixed in 4% formaldehyde, dehydrated in 20% then 30% sucrose, and then frozen sections were prepared. The sections were incubated with rabbit anti-rat DCX (1:100; Cat# ab18723; Abcam), rabbit anti-rat NeuN (1:100; Cat# ab177487; Abcam), or mouse anti-rat NF200 (1:100; Cat# ab82259; Abcam) as primary antibodies and then with a tetramethyl rhodamine isothiocynate-conjugated goat anti-mouse IgG (1:1000; Cat# ab6786; Abcam) or a tetramethyl rhodamine isothiocynate-conjugated goat anti-rabbit IgG (1:1000; Cat# ab6718; Abcam) as secondary antibodies. The primary antibody was incubated at 4°C overnight, and the secondary antibody was incubated at 37°C for 30 minutes. The nuclei were then labeled with 4′,6-diamidino-2-phenylindole fluorescent dye

(1:50; Beyotime, Shanghai, China). Fluorescence was observed under a confocal microscope (Olympus, Tokyo, Japan). All of the above steps were performed in the dark.

Immunohistochemistry

After rats were anesthetized, the thoracic cavity was exposed and transcardial perfusion with 0.9% physiological saline and then 4% paraformaldehyde performed. Brains were then dissected, embedded in paraffin and sectioned at a thickness of 10 μm. To observe the expression of neuron-associated proteins, we performed dewaxing, antigen retrieval, antibody incubation, staining, and sealing. The NeuN antigen was retrieved with ethylenediaminetetraacetic acid, while the other antigens were retrieved with sodium citrate. Prior to the addition of the primary antibodies, goat serum was used for blocking. Sections were incubated overnight at 4°C with the primary antibodies, rabbit anti-rat DCX (1:500), rabbit anti-rat NeuN (1:500), mouse anti-rat NF200 (1:200) or mouse anti-human $A\beta_{1-42}$ (1:200; Cat# ab11132; Abcam). Sections were then incubated with goat anti-mouse IgG (1:1000; Boster Biological Technology, Pleasanton, CA, USA) or goat anti-rabbit IgG (1:1000; Boster Biological Technology) at 37°C for 30 minutes. Then, 3,3′-diaminobezidin (Solarbio, Beijing, China) was added, and the tissues were counterstained with hematoxylin-eosin staining, dehydrated, sealed with neutral gum, and observed at 200× magnification.

Real-time polymerase chain reaction

Brain specimens for real-time polymerase chain reactions were stored at –80°C until use. RNA was extracted using the TRIzol method (Thermo Fisher, Waltham, MA, USA). The concentration of RNA in each specimen was determined using a NanoDrop 2000 UV spectrophotometer (Thermo Fisher). The RNA samples were reverse transcribed to produce cDNAs for downstream experiments. The expression of β-actin was used for quantification normalization. The primer sequences for the target genes are listed in **Table 1**. The reaction conditions were as follows: predenaturation at 94°C for 5 minutes; 40 cycles of denaturation at 94°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 30 seconds; incubation at 72°C for 2.5 minutes; then 40°C for 1.5 minutes; melting at every 1°C between 60°C to 94°C for 1 second; and incubation at 25°C for 1–2 minutes. An amplification curve and a dissolution curve were obtained, and the data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Western blot analysis

Fresh hippocampal tissues were dissected and protein extracted. A bicinchoninic acid protein concentration assay kit was used to measure the protein concentration of each specimen. Following sodium dodecyl sulfate polyacrylamide gel electrophoresis, polyvinylidene fluoride membrane transfer, and protein blocking steps, the membranes were incubated overnight at 4°C with primary antibodies, including rabbit anti-rat DCX (1:500), rabbit anti-rat NeuN (1:500), mouse anti-human $A\beta_{1-42}$ (1:1000) and mouse anti-rat NF200 (1:1000). The secondary antibody for NeuN and DCX was a goat anti-rabbit IgG-horseradish peroxidase (Wanleibio, Shenyang, Liaoning Province, China) and the secondary antibody for $A\beta_{1-42}$ and NF200 was a rabbit anti-mouse IgG-horseradish peroxidase (Wanleibio). The membranes were incubated with the secondary antibodies at a 1:2000 dilution at 37°C for 45 minutes. The internal control antibody was β-actin. The membranes were then incubated with an enhanced chemiluminescence luminophore for 5 minutes and exposed in a cassette. The grey level of the target bands was analyzed with a gel image processing system (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China).

Table 1 | **Target gene primer sequences used in real-time polymerase chain reactions**

Gene	Primer sequence (5′–3′)
DCX	Forward: TCC TCA TAG CCA CGC TCC C
	Reverse: CGT ACA GAT CAC GTT GCC CTA A
NeuN	Forward: CAC CGC CGT CGC CTA TC
	Reverse: CCA GTG CCG CTC CGT AAG
NF200	Forward: GGA GGA CCG TCA TCA GGT AGA C
	Reverse: TTT CTG TAA GCA GCG ATC TCA AT
β-Actin	Forward: GGA GAT TAC TGC CCT GGC TCC TAG C
	Reverse: GGC CGG ACT CAT CGT ACT CCT GCT T

DCX: Doublecortin; NF200: neurofilament 200.

Radial arm maze

Behavioral experiments were performed with an 8-arm radial arm maze. Rats were fasted for 24 hours before the experiment. Before the test, rats were adapted to the environment once a day for 3 days by being allowed to freely explore the maze for 10 minutes each day. Training occurred over the next two days (**Figure 1A**). Four arms were selected and food was placed in a food tray at the end of each arm. Food was not placed in the food trays of the remaining four arms. The activity of each rat in the maze was observed over 600 seconds. At the beginning of the experiment, the rats were placed on the central platform of the maze. At the end of 600 seconds or once the food in all of the arms was foraged, the trial was completed. Testing was conducted on the sixth day, and two indicators were measured: the time taken for rats to complete the radial arm maze and the total number of errors. If the test was not completed in 600 seconds, the time was recorded as 600 seconds. The number of errors was determined by counting the number of entries into the wrong arms and the number of repeated entries into an arm (Postu et al., 2018).

Statistical analysis

SPSS 23.0 statistical software (IBM, Armonk, NY, USA) was used to process the data. All data are expressed as the mean ± standard deviation (SD). One-way analysis of variance was used for comparisons among all groups and Student's *t*-test was used for comparisons between two groups. When $P < 0.05$, the difference was considered to be significant.

Results

Successful construction of a rat model of AD

We successfully injected Aβ or PBS into the hippocampus in the different groups. After 14 days, hematoxylin-eosin staining of the hippocampus showed that, unlike normal tissue, the AD model tissue exhibited nerve cell death. Cell damage manifested as a widened interstitial space, shrunken cells, and vacuolar degeneration (**Figure 1B** and **C**). The changes in hippocampal tissue after hematoxylin-eosin staining indicated successful establishment of the AD model.

Successful isolation of DPSCs

DPSCs were isolated from pulpal cavities and the cells were cultured to the third generation (**Figure 2A**). After induction of osteogenic differentiation, DPSCs differentiated into osteoblasts, which were positively stained with alizarin red (**Figure 2B**). DPSCs were also able to differentiate into adipocytes, and the lipid droplets appeared orange after staining with oil red O dye (**Figure 2C**). The two experiments demonstrated the multidirectional differentiation ability of DPSCs. Flow cytometry showed that cell surface markers of MSCs, such as CD29 (**Figure 2D**) and CD90 (**Figure 2E**), were highly expressed, whereas the hematopoietic cell surface markers CD34 (**Figure 2F**) and CD45 (**Figure 2G**) were expressed at low levels.

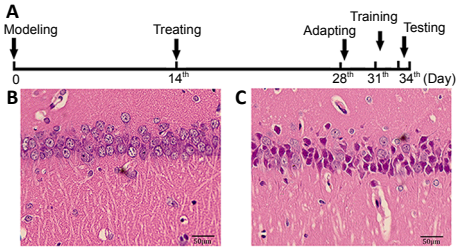


Figure 1 | Experimental flow chart and AD model characterization.
(A) The schedule for preparing the Sprague-Dawley rat model, treatment and behavior testing. First, $A\beta_{1-42}$ or PBS was injected into the hippocampus of rats in different groups. Fourteen days later, DPSCs or PBS was injected into the hippocampus along the same needle track. On day 29, rats were adapted to the environment of the radial arm maze for 3 days. Then, behavioral tests were carried out for a total of 3 days. (B) Hematoxylin-eosin staining of normal hippocampal tissue: the cytoplasm of nerve cells was rich and light stained, nuclei were in the middle of cells, and the nucleoli were clear. (C) Hematoxylin-eosin staining of AD rat model hippocampal tissue: tissue spaces were expanded, cytoplasm was condensed, and nuclei were shrunken. Scale bars: 50 μm. $A\beta_{1-42}$: β-Amyloid 1–42; AD: Alzheimer's disease; DPSC: dental pulp stem cell; PBS: phosphate buffered saline.

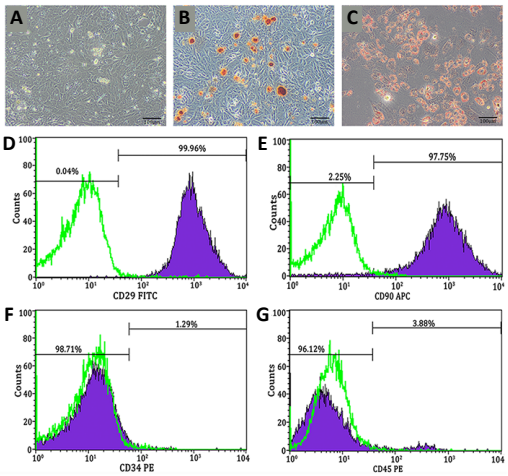


Figure 2 | Culture and identification of DPSCs.
(A) Morphology of third-generation DPSCs. (B) DPSCs were induced to differentiate into osteoblasts (arrow) and stained with alizarin red. (C) DPSCs were induced to differentiate into adipocytes (arrow), and the lipid droplets were stained with oil red O. Scale bars: 100 μm. (D) Flow cytometry showed that CD29, labeled by FITC, was expressed by 99.96% of the cells. (E) The surface marker, CD90-APC, was expressed by 97.75% of DPSCs. (F) CD34, which was PE-conjugated, was expressed by only 1.29% of DPSCs. (G) CD45, which was PE-conjugated, was expressed by 3.88% of DPSCs. All of the homotypic controls were negative. APC: Allophycocyanin; DPSCs: dental pulp stem cells; FITC: fluorescein isothiocyanate isomer; PE: phycoerythrin.

Co-expression of cell membrane, nuclear and neuron-associated proteins in the hippocampus

Sections were observed by confocal microscopy. All nuclei were stained blue by 4′,6-diamidino-2-phenylindole, the membranes of DPSCs were stained green by PKH67, and the target proteins were stained red by tetramethyl rhodamine isothiocynate-conjugated fluorescence secondary antibodies. After DPSCs transplantation, co-expression of cell membrane, nuclear and neuron-associated proteins was observed in the hippocampus, likely because the neuronal proteins, DCX, NeuN and NF200, were expressed by the transplanted DPSCs (**Figure 3**).

DPSC transplantation promotes the secretion of neuron-associated proteins

The immunopositivities of DCX, NeuN and NF200 in the Aβ + DPSC group were higher than those in the Aβ + PBS group ($P < 0.001$), while the immunopositivities of Aβ protein in the Aβ + DPSC group were lower than those in the Aβ + PBS group

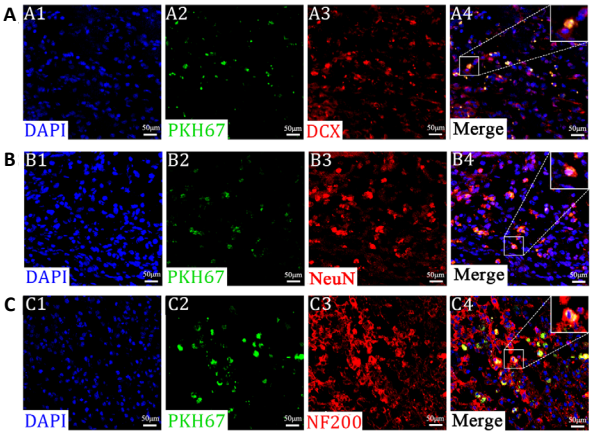


Figure 3 | Immunohistofluorescence analysis of DCX, NeuN, and NF200 after transplantation of DPSCs into the hippocampus of AD rats.
(A–C) The co-expression of PKH67 with DCX, NeuN, and NF200. (A1–C1) The blue fluorescence represents nuclei stained by DAPI. (A2–C2) The green fluorescence (PKH67) represents DPSCs, which were stained with PKH67 before being injected into the hippocampus. (A3–C3) The red fluorescence (tetramethyl rhodamine isothiocynate) is from the secondary fluorescent antibodies against the anti-DCX, anti-NeuN, and anti-NF200 primary antibodies. (A4–C4) The merged images show the co-expression of the nuclear, cell membrane and target proteins. Scale bars: 50 μm. DAPI: 4′,6-Diamidino-2-phenylindole; DCX: doublecortin; DPSCs: dental pulp stem cells; NF200: neurofilament 200; PBS: phosphate buffered saline.

($P < 0.001$; **Figure 4**). The mRNA levels of the three neuron-associated proteins (DCX, NeuN and NF200) were increased in the Aβ + DPSC group compared with those in the Aβ + PBS group ($P < 0.001$; **Figure 5**). The same results were obtained by western blot assays (**Figure 6**). There were no significant differences in the levels of neuron-associated proteins between PBS + PBS and control groups ($P > 0.05$). These experiments indicated an improvement in nerve cell function after DPSC transplantation

DPSC transplantation significantly improves the cognitive and behavioral abilities of AD rats

In the radial arm maze test, the total time required to complete the maze and the number of errors were significantly increased in the Aβ + PBS group compared with those in the control group. Compared to the PBS group, DPSC transplantation in Aβ-treated rats decreased the total time required to accomplish the maze and the number of errors. There was no distinct difference between control and PBS + PBS groups (**Figure 7**).

Discussion

In this study, a rat model of AD was constructed by injecting $A\beta_{1-42}$ into the hippocampus. $A\beta_{1-42}$ deposition is a characteristic manifestation of AD. Hippocampal injection of $A\beta_{1-42}$ is a commonly used method to establish models of AD in rats, which can be used to evaluate the effects of treatment by histological evaluation of pathological changes. However, this model only recapitulates one pathological feature of AD, Aβ deposition, and does not simulate all features of AD, such as tau tangles. There are also no AD-related changes in the internal environment of this model, so the experimental results should be considered objectively. MSCs are cells with multidirectional differentiation potential that migrate to sites of tissue injury. These cells also inhibit oxidative stress, reduce inflammatory factor secretion, and play anti-inflammatory, anti-apoptotic and immune regulatory roles in disease (Staff et al., 2019). DPSCs are a type of MSC and demonstrate all functions of MSCs. In addition, DPSCs are derived from the neural crest, and they have greater potential for treating neurological disorders compared with other MSCs (Mead et al., 2014). DPSCs can also secrete growth factors, including brain-derived neurotrophic

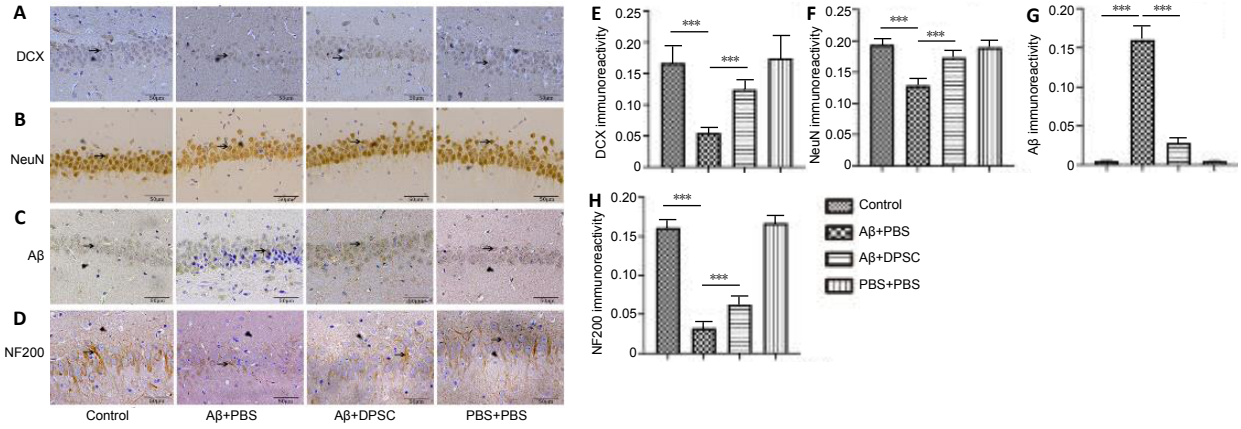


Figure 4 | **Effect of DPSC transplantation on the immunopositivity of neuron-associated proteins in the hippocampus of Alzheimer's disease rats.**
(A) The immunoreactivity of DCX was decreased in the Aβ + PBS group and increased in the Aβ + DPSC group. (B) The immunoreactivity of NeuN was decreased in the Aβ + PBS group and increased in the Aβ + DPSC group. (C) After DPSC transplantation, the immunoreactivity of Aβ was decreased. (D) The immunoreactivity of NF200 was decreased in the Aβ + PBS group and increased in the Aβ + DPSC group. Arrows indicate immunopositive cells. Scale bars: 50 μm. (E–H) The immunopositivities of DCX, NeuN, Aβ and NF200. Data are expressed as the mean ± SD (n = 5). ***P < 0.001 (one-way analysis of variance and Student's t-test). Aβ: β-Amyloid; DCX: doublecortin; DPSCs: dental pulp stem cells; NF200: neurofilament 200; PBS: phosphate buffered saline.

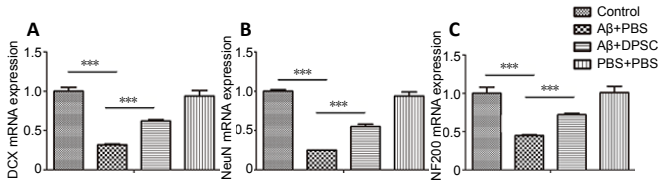


Figure 5 | **Effect of DPSC transplantation on the mRNA levels of neuron-associated proteins in the hippocampus of Alzheimer's disease rats.**
(A–C) Relative mRNA levels of DCX, NeuN, and NF200 detected by real-time polymerase chain reaction. Data are expressed as the mean ± SD (n = 5). ***P < 0.001 (one-way analysis of variance and Student's t-test). Aβ: β-Amyloid; DCX: doublecortin; DPSC: dental pulp stem cell; NF200: neurofilament 200; PBS: phosphate buffered saline.

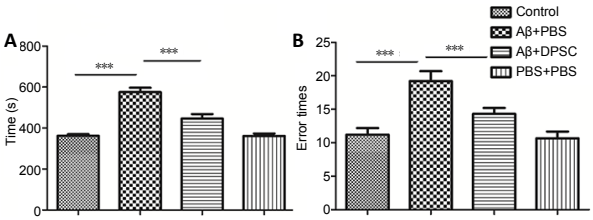


Figure 7 | **Effect of DPSC transplantation on the performance of Alzheimer's disease rats in the radial arm maze.**
(A) The total time to finish the maze. (B) The number of errors. Data are expressed as the mean ± SD (n = 5). ***P < 0.001 (one-way analysis of variance and Student's t-test). Aβ: β-Amyloid; DPSC: dental pulp stem cell; PBS: phosphate buffered saline.

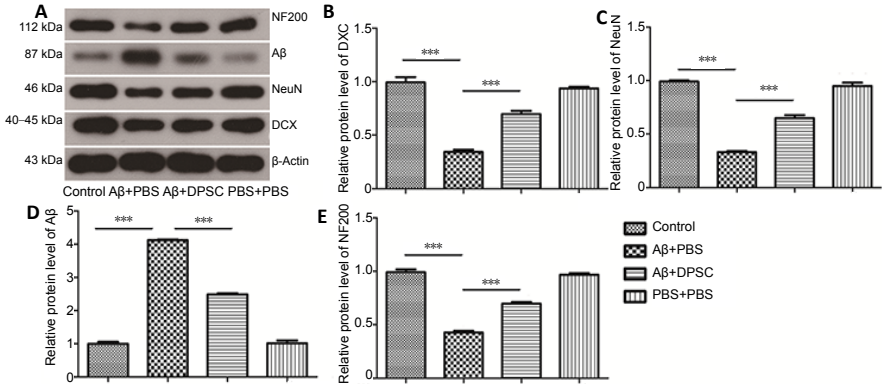


Figure 6 | **Effect of DPSC transplantation on the relative expression levels of neuron-associated proteins, DCX, NeuN, Aβ, and NF200 in the hippocampus of Alzheimer's disease rats.**
(A) Western blots of the target proteins. (B–E) The relative levels of DCX, NeuN, Aβ, and NF200. Data are expressed as the mean ± SD (n = 5). ***P < 0.001 (one-way analysis of variance and Student's t-test). Aβ: β-Amyloid; DCX: doublecortin; DPSC: dental pulp stem cell; NF200: neurofilament 200; PBS: phosphate buffered saline.

factor, nerve growth factor and neurotrophin-3, to promote neurological function recovery (Mead et al., 2013). In addition, the neural plasticity of differentiated DPSCs promotes axonal regeneration through the action of the chemokine C-X-C motif chemokine ligand 12 and its receptor CXCR4 (Arthur et al., 2009; Mead et al., 2013). Furthermore, DPSCs transplanted into the brain can express neuron-specific markers and exhibit characteristics of neurons through the establishment of voltage-dependent sodium and potassium channels (Király et al., 2011). Although this study shows the therapeutic ability of DPSCs, it is unclear which mechanism achieves the therapeutic effect. The results of this study provide clues as to the mechanisms underlying the therapeutic effects of DPSC transplantation and the basis by which DPSCs improve the memory and executive ability of AD rats.

DPSC transplantation increased the expression of neuron-associated proteins in the brain compared with that in the Aβ + PBS group, which reflects the potential therapeutic effect of DPSCs. As a microtubule-associated protein, DCX is indispensable in the generation and migration of neurons. DCX coordinates microtubule functions, such as cell movement, transport, polarity and mitosis, and is considered the gold standard marker of neurogenesis (Ayanlaja et al., 2017). NeuN is widely used to detect the nuclei of neurons and is a reliable marker of mature neurons. NeuN plays a dominant role in the development of nerve tissue and the regulation of nerve function (Dent et al., 2010; Duan et al., 2016). NF200 is also known as NF-H and is a subunit of NF. NFs are members of the intermediate filament protein family and contact other neural proteins to coordinate brain function. NFs are also associated with the formation of axons and are involved in repair of the nervous system (Yuan et al., 2017). The proteins evaluated in this experiment represent different stages of neurons, from infancy to maturity. Higher levels of DCX, NeuN and NF200 proteins were detected in the Aβ + DPSC group than in the Aβ

+ PBS group, demonstrating the differentiation and migration abilities of DPSCs in AD. Immunofluorescence enabled us to co-localize cell membrane, nuclear and target proteins in the hippocampus, which showed that exogenous DPSCs have the ability to secrete neuron-related proteins.

Short-term memory impairment is considered to be the earliest sign of AD. Here, the radial arm maze was used to observe the memory capacity of rats (Templer et al., 2019). The time required to complete the maze and the number of errors were substantially better in the DPSCs treatment group than in the Aβ + PBS group. The PBS + PBS group confirmed that injecting fluid into the hippocampus with a needle had no effect on the cognitive or behavioral functions of the rats. Injecting DPSCs into the hippocampus allowed the cells to reach the site of injury directly, thus ensuring their therapeutic effect. Although DPSCs have the ability to find an injury site, their administration to an elderly patient with AD, who often suffer from multiple diseases, may limit the number of stem cells that reach the target area, greatly weakening the therapeutic effect. Gaining acceptance for this approach is therefore a tremendous challenge. This study is the first exploration of the therapeutic potential of DPSCs in an AD animal model. It is not clear how DPSCs may improve cognitive ability in AD and further research to determine the underlying mechanism is still needed.

This study also has some shortcomings and limitations. In our model, Aβ was injected into the brain, which is different from the natural process of deposition in AD. Also there was an absence of tau tangles in our model, which may lead to less severe brain damage, making the efficacy of DPSCs more effective in the short term. Moreover, it is not clear how long DPSCs can survive in rats or what the long-term therapeutic effect of DPSCs is after transplantation.

Each part of this study confirmed the therapeutic potential of DPSCs in an AD model. We predict that further research will continue to demonstrate stem cell transplantation to be an effective way of treating AD in humans. However, even if direct stem cell transplantation works in humans, further research will be required to bring this therapy to the clinic.

Acknowledgments: *We appreciate those researchers in the Research Center of the 2nd Affiliated Hospital of Harbin Medical University, China for their assistance in the experimental research.*
Author contributions: *Study design: XMZ, YJO, JF; experimental implementation: YJO, BQY, WL, MYY, JYL; data collection: ZMJ, DY, NL; data analysis: YS, YYX, ZJH; literature search: DW, HY; manuscript writing: XMZ, YJO. All authors read and approved the final manuscript.*
Conflicts of interest: *The authors declare that they have no conflict of interest.*
Financial support: *This study was supported by Yu Weihan Fund for Distinguished Young Scholars of Harbin Medical University of China, No. 002000013 (to XMZ). The funder had no roles in the study design, conduction of experiment, data collection and analysis, decision to publish, or preparation of the manuscript.*
Institutional review board statement: *The study was approved by Ethics Committee for Laboratory Animals of the 2nd Affiliated Hospital of Harbin Medical University of China (approval No. KY2017-132) on February 21, 2017.*
Copyright license agreement: *The Copyright License Agreement has been signed by all authors before publication.*
Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer reviewer: *Willian Orlando Castillo, Universidade de Sao Paulo, Brazil.*
Additional file: *Open peer reviewer report 1.*

References

Ager RR, Davis JL, Agazaryan A, Benavente F, Poon WW, LaFerla FM, Blurton-Jones M (2015) Human neural stem cells improve cognition and promote synaptic growth in two complementary transgenic models of Alzheimer's disease and neuronal loss. Hippocampus 25:813-826.
Alzheimer's Association (2016) 2016 Alzheimer's disease facts and figures. Alzheimers Dement 12:459-509.
Arthur A, Shi S, Zannettino AC, Fujii N, Gronthos S, Koblar SA (2009) Implanted adult human dental pulp stem cells induce endogenous axon guidance. Stem Cells 27:2229-2237.
Ayanlaja AA, Xiong Y, Gao Y, Ji G, Tang C, Abdikani Abdullah Z, Gao D (2017) Distinct features of doublecortin as a marker of neuronal migration and its implications in cancer cell mobility. Front Mol Neurosci 10:199.
Banik A, Prabhakar S, Kalra J, Anand A (2015) Effect of human umbilical cord blood derived lineage negative stem cells transplanted in amyloid-β induced cognitive impaired mice. Behav Brain Res 291:46-59.
Brier MR, Gordon B, Friedrichsen K, McCarthy J, Stern A, Christensen J, Owen C, Aldea P, Su Y, Hassenstab J, Cairns NJ, Holtzman DM, Fagan AM, Morris JC, Benzinger TL, Ances BM (2016) Tau and Aβ imaging, CSF measures, and cognition in Alzheimer's disease. Sci Transl Med 8:338ra366.
Caselli RJ, Beach TG, Knopman DS, Graff-Radford NR (2017) Alzheimer disease: Scientific breakthroughs and translational challenges. Mayo Clin Proc 92:978-994.
Chakari-Khiavi F, Dolati S, Chakari-Khiavi A, Abbaszadeh H, Aghebati-Maleki L, Pourlak T, Mehdizadeh A, Yousefi M (2019) Prospects for the application of mesenchymal stem cells in Alzheimer's disease treatment. Life Sci 231:116564.
Dent MA, Segura-Anaya E, Alva-Medina J, Aranda-Anzaldo A (2010) NeuN/Fox-3 is an intrinsic component of the neuronal nuclear matrix. FEBS Lett 584:2767-2771.
Doxtater K, Tripathi MK, Khan MM (2020) Recent advances on the role of long non-coding RNAs in Alzheimer's disease. Neural Regen Res 15:2253-2254.
Duan W, Zhang YP, Hou Z, Huang C, Zhu H, Zhang CQ, Yin Q (2016) Novel insights into NeuN: from neuronal marker to splicing regulator. Mol Neurobiol 53:1637-1647.
Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J (1999) Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron 23:247-256.
Hata M, Omi M, Kobayashi Y, Nakamura N, Tosaki T, Miyabe M, Kojima N, Kubo K, Ozawa S, Maeda H, Tanaka Y, Matsubara T, Naruse K (2015) Transplantation of cultured dental pulp stem cells into the skeletal muscles ameliorated diabetic polyneuropathy: therapeutic plausibility of freshly isolated and cryopreserved dental pulp stem cells. Stem Cell Res Ther 6:162.
Holtzman DM, Morris JC, Goate AM (2011) Alzheimer's disease: the challenge of the second century. Sci Transl Med 3:77sr71.
Huang X, Chen X, Chen H, Xu D, Lin C, Peng B (2018) Rho/Rho-associated protein kinase signaling pathway-mediated downregulation of runt-related transcription factor 2 expression promotes the differentiation of dental pulp stem cells into odontoblasts. Exp Ther Med 15:4457-4464.
Khalaf-Nazzal R, Stouffer MA, Olaso R, Muresan L, Roumegous A, Lavilla V, Carpentier W, Moutkine I, Dumont S, Albaud B, Cagnard N, Roest Crollius H, Francis F (2017) Early born neurons are abnormally positioned in the doublecortin knockout hippocampus. Hum Mol Genet 26:90-108.
Király M, Kádár K, Horváthy DB, Nardai P, Rácz GZ, Lacza Z, Varga G, Gerber G (2011) Integration of neuronally predifferentiated human dental pulp stem cells into rat brain in vivo. Neurochem Int 59:371-381.
Lee IS, Jung K, Kim IS, Lee H, Kim M, Yun S, Hwang K, Shin JE, Park KI (2015) Human neural stem cells alleviate Alzheimer-like pathology in a mouse model. Mol Neurodegener 10:38.
Lee JC, Park JH, Ahn JH, Park J, Kim IH, Cho JH, Shin BN, Lee TK, Kim H, Song M, Cho GS, Kim DW, Kang IJ, Kim YM, Won MH, Choi SY (2018) Effects of chronic scopolamine treatment on cognitive impairment and neurofilament expression in the mouse hippocampus. Mol Med Rep 17:1625-1632.
Li Q, Che HX, Wang CC, Zhang LY, Ding L, Xue CH, Zhang TT, Wang YM (2019) Cerebrosides from sea cucumber improved Aβ(1-42)-induced cognitive deficiency in a rat model of Alzheimer's disease. Mol Nutr Food Res 63:e1800707.
Lin YS, Wang HY, Huang DF, Hsieh PF, Lin MY, Chou CH, Wu IJ, Huang GJ, Gau SS, Huang HS (2016) Neuronal splicing regulator RBFOX3 (NeuN) regulates adult hippocampal neurogenesis and synaptogenesis. PLoS One 11:e0164164.
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.
Maxeiner S, Glassmann A, Kao HT, Schilling K (2014) The molecular basis of the specificity and cross-reactivity of the NeuN epitope of the neuron-specific splicing regulator, Rbfox3. Histochem Cell Biol 141:43-55.
Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2013) Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. Invest Ophthalmol Vis Sci 54:7544-7556.
Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2014) Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells. PLoS One 9:e109305.
Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2017) Concise review: dental pulp stem cells: A novel cell therapy for retinal and central nervous system repair. Stem Cells 35:61-67.
Postu PA, Noumedem JAK, Cioanca O, Hancianu M, Mihasan M, Ciorpac M, Gorgan DL, Petre BA, Hritcu L (2018) Lactuca capensis reverses memory deficits in Aβ1-42-induced an animal model of Alzheimer's disease. J Cell Mol Med 22:111-122.
Staff NP, Jones DT, Singer W (2019) Mesenchymal stromal cell therapies for neurodegenerative diseases. Mayo Clin Proc 94:892-905.
Sumbria RK (2020) Targeting the transferrin receptor to develop erythropoietin for Alzheimer's disease. Neural Regen Res 15:2251-2252.
Templer VL, Wise TB, Heimer-McGinn VR (2019) Social housing protects against age-related working memory decline independently of physical enrichment in rats. Neurobiol Aging 75:117-125.
Ullah I, Subbarao RB, Rho GJ (2015) Human mesenchymal stem cells- current trends and future prospective. Biosci Rep 35:e00191.
Yuan A, Rao MV, Veeranna, Nixon RA (2017) Neurofilaments and neurofilament proteins in health and disease. Cold Spring Harb Perspect Biol 9:a018309.
Zhao H (2016) Embryonic neural stem cell transplantation for Alzheimer's disease. Embryonic neural stem cell transplantation for Alzheimer's disease. Zhongguo Zuzhi Gongcheng Yanjiu 20:4805-4810.

P-Reviewer: Castillo WO; C-Editor: Zhao M; S-Editors: Yu J, Li CH; L-Editors: Yu J, Song LP; T-Editor: Jia Y