RESEARCH ARTICLE

Dental pulp stem cells promote regeneration of damaged neuron cells on the cellular model of Alzheimer’s disease

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Abstract

Alzheimer’s disease (AD) is an incurable neurodegenerative disease and many types of stem cells have been used in AD therapy with some favorable effects. In this study, we investigated the potential therapeutical effects of human dental pulp stem cells (hDPSCs) on AD cellular model which established by okadaic acid (OA)-induced damage to human neuroblastoma cell line, SH-SY5Y, in vitro for 24 h. After confirmed the AD cellular model, the cells were co-culture with hDPSCs by transwell co-culture system till 24 h for treatment. Then the cytomorphology of the hDPSCs-treated cells were found to restore gradually with re-elongation of retracted dendrites. Meanwhile, Cell Counting Kit-8 assay and Hoechst 33258 staining showed that hDPSCs caused significant increase in the viability and decrease in apoptosis of the model cells, respectively. Observation of Dil labeling also exhibited the prolongation dendrites in hDPSCs-treated cells which were obviously different from the retraction dendrites in AD model cells. Furthermore, specific staining of α-tubulin and F-actin demonstrated that the hDPSCs-treated cells had the morphology of restored neurons, with elongated dendrites, densely arranged microfilaments, and thickened microtubular fibrils. In addition, results from western blotting revealed that phosphorylation at Ser 396 of Tau protein was significantly suppressed by adding of hDPSCs. These results indicate that hDPSCs may promote regeneration of damaged neuron cells in vitro model of AD and may serve as a useful cell source for treatment of AD.

Keywords: Alzheimer’s disease; cell model; dental pulp stem cells; okadaic acid; therapeutical effects

Introduction

Alzheimer’s disease (AD) often occurs in the elderly (over 65 years old) and is a common neurodegenerative disease with clinical manifestations that include a gradual decline in memory, cognitive function, personality traits, and linguistic function. Neuropathological features of AD include neurofibrillary tangles (NFT) and extracellular senile plaques, which are accompanied by neuronal apoptosis and loss of synapses. NFT are markers for neuronal apoptosis due to the formation and phosphorylation of Tau protein (Gong et al., 1995).

To date, treatments of AD are based primarily on symptomatic approaches (Singh et al., 2012), which use drugs that target neurotransmitters and revitalize brain metabolism to control psychotic symptoms associated with AD. However, these treatments do not effectively control the development of the disease or improve cognitive impairment. Recently, stem cell therapy has become a prominent topic in life science research as a new therapeutic approach that brings hope for certain incurable diseases such as AD. Studies reporting stem cell therapy in both in vitro and in vivo AD models have demonstrated improvements in
AD pathologies and behaviors. Zhang et al. (2014) transplanted neural stem cells (NSCs) into the hippocampi of AD mice and observed improved recovery of neuronal function in the corresponding regions. They found that increased neuronal expression of proteins related to cognitive function significantly improved spatial learning and memory in AD mice. Salem et al. (2014) intravenously injected bone marrow mesenchymal stem cells (BMSCs) into AD mice and found significantly reduced amyloid plaques in the hippocampi 4 months after BMSC treatment. This therapeutic result of BMSCs was significantly better than conventional drug therapies. However, extraction of NSCs and BMSCs is difficult and invasive. Moreover, it is difficult to control the quality of these stem cell cultures, which limits their clinical application. In recent years, adipose-derived mesenchymal stem cells (ADSCs), human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs), and amniotic membrane-derived mesenchymal stem cells (AMSCs) have been reported as new cell sources for AD therapy. Injection of ADSCs into the cerebral cortex of AD mice led to recovery of spatial learning and memory (Ma et al., 2013), and intravenous injection of ADSCs effectively alleviated dementia in AD mice (Kim et al., 2012; Chang et al., 2014). Lee et al. (2010) demonstrated that after 24 h co-culture of hUCB-MSCs and amyloid beta (Aβ)-injured hippocampal neurons, the number of apoptotic neurons was reduced as assessed by TUNEL staining. In addition, transplantation of hUCB-MSCs into AD mice restored learning and memory. Kim et al. (2013) intravenously injected human AMSCs into AD transgenic mice to improve spatial learning, and the amyloid plaque deposition in the brain was reduced, which suggests that AMSCs transplantation has therapeutical effects on AD via immunomodulatory and paracrine mechanisms. These types of stem cells alleviated AD-related neuropathological disorders with no limitations in the cell preparation or ethical issues, thereby providing ideal sources for stem cell therapy in AD. However, these cells are derived primarily from the mesoderm, and adult stem cell functions are relatively tissue-specific. Mesoderm-derived mesenchymal stem cells may serve as a substitute and may promote regeneration and repair of neuroectodermal tissues. Nevertheless, this substitution is not likely to achieve the effects of germ layer-derived adult stem cells. Hence, an in-depth evaluation of better and more reliable primary cells remains an important topic for stem cell therapy in AD.

Dental pulp stem cells (DPSCs) are adult stem cells isolated from pulp tissues of extracted teeth. Extraction of DPSCs is simple without causing any secondary damage or ethical controversy. Compared with other adult stem cells, DPSCs have a high rate of proliferation, strong capacity for repair, low immunogenicity, and better plasticity (Gronthos et al., 2000). Most importantly, DPSCs are derived from the ectomesenchymal neural crest, which has biological features of mesenchymal stem cells (MSCs) and neural crest stem cells. Previous studies using both in vitro and in vivo models demonstrated that DPSCs could differentiate into glial cells and neurons (Gronthos et al., 2002; Miura et al., 2003). DPSCs have a greater capacity for neural differentiation than other mesoderm-derived stem cells (Sakai et al., 2012) and more potential for treatment of neurological diseases (De Almeida et al., 2011). Currently, many in vivo and in vitro studies have shown that DPSCs prevent and repair neuronal damage (Arthur et al., 2008; Kiraly et al., 2009; Kiraly et al., 2011; Ellis et al., 2014) and achieve therapeutical effects in treatments of brain and spinal cord injuries in animal models (Sakai et al., 2012; Inoue et al., 2013). Thus, these studies suggest that DPSCs may play a role in the treatment of central nervous system diseases.

Up to now, no reports have shown the therapeutical potential of human DPSCs (hDPSCs) in an in vitro AD model. Hence, to evaluate further the therapeutic potential of hDPSCs and lay the foundation for clinical application of hDPSCs in AD, we established an in vitro AD model using OA to induce neuronal damage in the human neuroblastoma cell line, SH-SY5Y.

Here we report, for the first time, that hDPSC-treatment is therapeutically effective in an OA-induced in vitro AD model, which may present promising prospects for clinical application in the prevention and treatment of AD.

Materials and methods

Culture and isolation of hDPSCs

Normal human third molar teeth indicated for extraction were collected from patients that aged 19–26 years in the Department of Oral and Maxillofacial Surgery at the General Hospital of the People’s Liberation Army (PLA) (Beijing, China) according to written informed consent signed by all patients involved in this study. All experiment protocols were approved by the Ethics Committee of the General Hospital of PLA. The pulp obtained from at least 3–4 teeth of different patients were gently removed for primary cell culture each time. Primary cultures of hDPSCs were prepared as described by Gronthos et al. (2000). Briefly, the extracted pulp tissue was dissected and digested in 3 mg/mL type I collagenase (Gibco) and 4 mg/mL dispase (Roche, Basel, Switzerland) for 1 h at 37°C. After terminated with PBS containing 10% fetal bovine serum (FBS; Gibco), the samples were filtered through a 70 µm strainer (Biolgix, Lenexa, KS, USA) to obtain single-cell suspensions. Then the cells (1.0–5.0 × 10^5/well) were cultured in Alpha Minimal Essential Medium (α-MEM; Gibco) supplemented with 20% FBS, 2 mM glutamine (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (both from Sigma, St. Louis, MO, US) containing 5 mg/mL ascorbic acid (Vitamin C solution; Sigma, St. Louis, MO, US) and 2 mM β-glycerophosphate (Sigma, St. Louis, MO, US). The cells were maintained in a humidified incubator at 37°C under 10% CO2, and the media were changed every 2 days for 3–4 days to form colonies. Then the colonies were detached with 0.05% trypsin–EDTA (Gibco) and sub-cultured at 1:2 ratio.
USA). Cells were incubated at 37°C in a 5% CO₂ humidified incubator (Thermo Fisher Scientific, Waltham, MA), and were isolated on the basis of their ability to form cell colony. Passages 2–4 of the isolated cells were used in subsequent experiments.

Identification of hDPSC

Cell phenotype

Phenotypic identification of hDPSCs required at least 1 × 10⁵ cells per antibody. Appropriate numbers of cells were aliquoted into 12 Eppendorf tubes containing individual monoclonal antibodies as follows: CD166-PE, CD31-APC, CD44-APC-H7, CD34-PE, CD29-PE, CD73-PE, CD90-PE, CD105-FITC, CD45-PE (all from BD Biosciences, San Jose, CA, USA), STRO-1-PE (Santa Cruz, CA, USA), CD3-FITC, HLA-DR-Percp-cy5.5 (both from eBioscience, USA), and CD38-FITC (BioLegend, USA). The cells were then incubated at 4°C in the dark for 30 min. After repeated washes with PBS, cells were resuspended and cell surface markers were analyzed by flow cytometry (BD Biosciences).

Osteogenic differentiation

Osteogenic differentiation was induced by using the OriCell™ osteogenesis differentiation kit (Cyagen, Guangzhou, China). The cells were grown in osteogenic differentiation medium and refed every 3 days for 3 weeks. Alkaline phosphatase (ALP) staining was performed with an ALP staining kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer’s protocol. For alizarin red staining, the cells were fixed with 4% formaldehyde solution for 30 min, and stained with alizarin red working solution for 3–5 min.

Adipogenic differentiation

Adipogenic differentiation was induced by using OriCell™ adipogenic differentiation kit (Cyagen). The cells were grown in adipogenic differentiation medium. For oil red O stain analysis, cells were fixed with 4% formaldehyde solution, stained with oil red O working solution (3:2 dilution with distilled water, filtered) for 30 min, and visualized under light microscopy with image capture.

Establishment of the AD cell model

Human neuroblastoma SH-SY5Y cells (purchased from the Shanghai Institutes for Biological Sciences, China) were cultured in Dulbecco’s Modified Eagle’s Medium-high glucose (H-DMEM; Sigma) supplemented with 10% FBS. SH-SY5Y cells with 6 × 10⁴ cells per well were seeded on 6-well plates for establishment of AD cell model as described by Wang et al. (2004). In brief, the wells were randomly divided into control and model groups (six wells per group). Cells in the control group were treated with serum-free H-DMEM, while those in the model group were then incubated with varying concentrations OA for variable incubation times to determine the optimal concentration and incubation time. Thus, treatment with 20 nmol/L of okadaic acid (OA; Sigma) for 24 h was chosen for the subsequent experiments including analyses of cell morphology, cell viability, apoptosis, microtubule specificity in neurons, and microfilament observation using a laser scanning confocal microscope (Nikon Corporation, Tokyo, Japan). Methods used to assess the in vitro model are described in the following sections.

Assessment of therapeutical effects of hDPSCs in the AD cell model

Group division

Six-well plates with 6 × 10⁴ SH-SY5Y cells per well were randomly divided into three groups (six wells per group) as follows: a) Control group—neither OA induction nor hDPSC treatment, b) Model group—20 nmol/L OA induced for establishment of AD cell model as described above, and c) Treatment group—OA induced the same as b), and then dealt with hDPSCs. For DPSCs-treatment assay, a 1.0 μm porous transwell membrane (Millipore, USA) was inserted into each well of the six wells plate as described elsewhere (Kang et al., 2013). Briefly, the lower compartment of the transwell chamber was filled with 6 × 10⁴ OA-induced cells. Before treatment with DPSCs, the medium was removed and the cells were washed two times with PBS to remove trace amount of OA inducer. Subsequently the transwell insert was added to each well of the group by merging the bottom of the insert into the medium in the lower side. To the upper compartment, 6 × 10⁴ hDPSCs were gently seeded into each well and incubated for another 24 h. Cells in these groups were continuously incubated in α-MEM for 24 h and performed following analyses and assays.

Analysis of cell morphology

After incubated for 24 h, cells in these three groups were observed under an inverted microscope (Nikon Corporation), and ten images were chosen randomly from each group for image analysis. To compare the length of cell dendrites, eight cells with the longest dendrites from each group were selected and the length measurement and analysis was carried out using Image-Pro Plus 6.0 software. Meanwhile, membrane probe DiI (Beyotime Biotechnology) staining was undertaken to evaluate cell morphology. After hDPSC-treatment and transwell inserts being taken out, cells in the lower transwell were incubation with DiI staining solution in the dark at room temperature for 25 min, and then fixed by 4% paraformaldehyde solution for 30 min.
Finally, laser scanning confocal microscopy (Nikon Corporation) was used to observe cell morphology in each group.

**Cell viability assay**

Cells exposed to above three groups were harvested and re-suspended in 96-well plates (eight wells per group) at the concentration of \(4 \times 10^5\) cells per well. Cell viability was assessed using a Cell-Counting Kit-8 (CCK-8; Beyotime Biotechnology) at 12 h. Before assessment, the cells were washed and then 0.1 mL 10% CCK-8 basal medium was added to each well, followed by incubation for 2 h at 37°C. The absorbance at 490 nm was determined by a microplate reader (Thermo Scientific, Multimode). Experiments were repeated three times on different days using fresh cells to obtain the average absorbance.

**Cell apoptosis assay**

Cells were collected from the three groups as described in the preceding section and seeded at 6 \(\times\) 10^5 cells on each well of six-well plates (six wells per group). After removal of the medium, the three groups of cells were fixed in 4% paraformaldehyde solution for 30 min, followed by washing with PBS and staining with 1 mL Hoechst 33258 dye (Beyotime Biotechnology) in the dark for 5 min at 37°C. The features of apoptotic nuclei were visualized under laser scanning confocal microscopy. Ten images were chosen randomly from each group and approximately 200 cells per group were counted to determine the ratio of densely stained or broken nuclei to the total number of nuclei and to perform further statistical analysis.

**Immunofluorescent assay**

Cells were collected from the above three groups were seeded onto coverslips and washed in PBS twice. After fixation with 4% paraformaldehyde solution for 30 min, cell membranes were permeabilized in 0.1% Triton X-100 solution for 15 min. Non-specific antigens were blocked with 10% normal goat serum (Gibco) in a moist chamber for 30 min. Cells were then incubated in \(\alpha\)-tubulin (DM1A) primary mouse monoclonal antibody (1:4000 dilution; Cell Signaling Technology, Beverly, MA, USA) at 37°C for 2 h, and rinsed three times with PBS. Next, cells were incubated with goat anti-mouse fluorescein isothiocyanate (FITC) secondary antibody (1:400 dilution; ZSGB Biotechnology, Beijing, China) and tetramethylrhodamine (TRITC)-conjugated phalloidin (1:100 dilution; Thermo Fisher) in the dark for 30 min. Finally, cells were washed three times with PBS and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology). All coverslips were mounted in 80% glycerol buffer and observed under a laser scanning confocal microscope. The longest dentrite length of 20 cells in each group was measured, essentially as previously described, and the parameters of mean fluorescence intensity and fluorescent area per cell were analyzed using Volocity Demo 6.1.1 software.

**Transmission electron microscopy (TEM) analysis**

Cells from the above three groups were fixed with 2% glutaraldehyde for 2 h at 4°C, and then the samples were post-fixed in 1% Osmium tetroxide for 2 h, dehydrated with a graded ethanol series (50–100%), infiltrated with propylene oxide, embedded in fresh resin and polymerized at 60°C for 24 h for preparation of ultrathin sections. After stained with toluidine blue, the cell cytoskeleton in three groups was observed under transmission electron microscopy (JEM-1400, JEOL, Japan). Ten images were chosen randomly from each group (n = 10), and the longest microtubules of cells in each group were selected for length measurement and analyses using Image-Pro Plus 6.0 software.

**Western blot analysis**

Western blotting were performed and analyzed as previously described (Jia et al., 2011). Briefly, cells in above three groups were obtained and lysed in RIPA buffer supplemented with protease inhibitors on ice. Then the cell lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). After incubated with 5% BSA for 1 h, the membranes were incubated overnight at 4°C with primary antibodies (\(\alpha\)-tubulin, 1:1000, Cell Signaling Technology; total Tau, 1:800, Proteintech, Chicago, IL, USA; Ser 396, 1:1000, Invitrogen, USA) and then washed three times with TBST, incubated with horseradish peroxidase-conjugated secondary antibodies (ZSGB Biotechnology) for 1 h. Finally, the expression of protein was detected via enhanced chemiluminescence (Santa Cruz Biotechnology).

**Statistical analysis**

Data are presented as mean ± standard deviation. Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL) software. Apoptotic rates of different groups were using the Chi-square test. Other data were analyzed using ANOVA. \(P < 0.05\) was considered statistically significant.

**Results**

Characteristics and differentiation potential of hDPSCs

hDSPCs were successfully isolated from extracted human teeth. Under an inverted microscope (Figure 1A), cells in primary cultures of hDSPCs appeared triangular-, polygonal-, or spindle-shaped. After passage, hDSPCs grew radially or spirally, with even sizes and shapes and elongated neurites in some cells. To assess the trans-differentiation ability of DPSCs, the osteogenic and adipogenic differentiation
potentials were tested by culturing hDPSCs in osteogenic and adipogenic induction medium for 3 weeks, respectively. The results suggest that DPSCs had a potential for osteogenic differentiation according to formation of mineral deposits by ALP staining and alizarin red staining (Figure 1B). Meanwhile, the adipogenic differentiation potential of DPSCs was identified based on the presence of intracellular lipid droplets by Oil Red staining. Moreover, results of surface markers of hDPSCs by flow cytometry showed that the markers of MSC including CD29, CD73, CD90, CD166, CD105, CD44, and STRO-1 were expressed positively, whereas CD14, CD38, HLA-DR, CD31, CD45, CD3, and CD34 were expressed negatively (Figures 1C and 1D).

hDPSCs restore the morphological damage in OA-induced SH-SY5Y cells

Results of the SH-SY5Y cells incubated with 20 nmol/L OA for 24 h showed that the AD cell model was established successfully based on serials assessments of cellular morphological, subcellular structure, function detection as described in following sections of the model group. In

![Image of hDPSCs and Alizarin Red staining](image)

![Flow Cytometry Graphs](flow_cytometry_graphs)

**Fig. 1** Characteristics and differentiation potential of hDPSCs. (A) Representative images of hDPSCs displayed a spindle shape at passages 0 and 4; ×200. (B) After osteogenic induction for 3 weeks, the cells demonstrated strong ALP-positive staining, and many mineralized nodules formed in the extracellular matrix, which was determined by alizarin red staining; ×40. After adipogenic differentiation induction for 3 weeks, the cells demonstrated robust oil droplet deposition by staining with Oil Red O; ×400. (C) Flow cytometry analysis indicated that hDPSCs expressed standard ISCT-defined mesenchymal markers, including CD29, CD73, CD90, CD166, CD105, CD44, and STRO-1. The cells were negative for CD14, CD38, HLA-DR, CD31, CD45, CD3, and CD34. (D) Representative mean data of flow cytometry tests. The positive expression level of the hDPSC immunophenotype.
In this section, cell morphology in the control SH-SY5Y cells presented the characteristic of neuronal cell with spindle cell bodies and elongated dendrites. After induced by 20 nmol/L OA for 24 h, cells in the model group (OA) changed obviously with the rounder shape and the less number, the cytoplasm retracted gradually, and most of the cell bodies became shiny and round, with poor adherence. In the treatment group (OA + DPSC), the retracted cytoplasm of some OA-damaged cells recovered with increased cytoplasm, clear cell margins, elongated dendrites, strong refraction, and better cell morphology compared with the model group. In addition, cell densities increased distinctly (Figure 2A). Results of laser scanning confocal microscopy showed that in the control group, projections were branched and continuous, and dendrites were thick and strong with obvious crest synapses, while in the model group, dendrites of the OA-damaged cells were broken, shortened, or even absent. By comparison, in the treatment group, dendrites of some OA-damaged cells had recovered and elongated, and appeared similar to those in the control group (Figure 2B). Measurement of dendritic length (marked with red staff, Figure 2A) showed that dendrites in the treatment group (46.83 ± 5.94 μm) were significantly longer than those in the model group (34.14 ± 4.35 μm) (P < 0.001), but were not significantly different from those in the control group (53.32 ± 8.83 μm) (P > 0.05) (Figure 2C).

hDPSCs increase cell viability in OA-induced SH-SY5Y cells

CCK-8 assay was performed to determine the effects of hDPSCs on cell viability of the OA-induced cells. After 24 h of 20 nmol/L OA-induced neuronal injury in the model group, the viability of the cells significantly decreased in comparison with the control group (OD 0.85 ± 0.01 vs. 0.92 ± 0.03; P < 0.001). However, there was a significant increased viability in cells treated with hDPSCs for 24 h in the treated group compared to the model group (OD 0.89 ± 0.04 vs. 0.85 ± 0.01; P < 0.05) (Figure 3), which suggests that hDPSCs improved cell viability after OA-induced neuronal injury.

![Fig. 2 Effects of hDPSCs on morphological changes of SH-SY5Y cells.](image-url)
hDPSCs reduce apoptosis levels in OA-induced SH-SY5Y cells

Results from Hoechst 33258 staining for identification of apoptotic cells by LSCM demonstrated that some nuclei in the model cells were shrunken, appearing as densely stained sheets, strips, or fragmented, which are less visible in control cells. In contrast, nuclei in the treatment group were similar to those in the control group, appearing as round, light blue stained, containing densely and evenly stained granules that were consistent in size (Figure 4A). A further experiment on the proportion of apoptotic cells among the total number of cells and the average value in each group displayed (Figure 4B) that the apoptotic rates of the control, model, and treatment groups were 6.71 ± 1.07%, 20.55 ± 4.91%, and 9.44 ± 1.56%, respectively. The rate for the model group was significantly different from those of the control and treatment groups (P < 0.05), while no significant difference was found between the control and treatment groups (P > 0.05). This suggests that hDPSCs reduced the levels of apoptosis after OA-neuronal injury.

hDPSCs restore cytoskeletal structure in OA-induced SH-SY5Y cells

A further observation of subcellular structure under LSCM showed that the cells in the control group extended well with strong dendrites. Microtubules and microfilaments in these cells were dense with strong fluorescence intensity. While in the model group, the dendrites of these cells retracted or disappeared, the cells became rounded, and cytoplasmic microtubules and actin was loose, disordered, and showed weak fluorescent staining. After 24 h treatment with hDPSCs, dendrites recovered with elongated, dendritic bundles of microtubules, and microtubules were relatively thick with strong fluorescent staining (Figure 5A). Quantification of dendritic length (Figure 5B), mean fluorescence intensity (Figure 5C), and fluorescence area (Figure 5D) showed that the cells in hDPSC-treatment group had significantly more intact structure than those in the model group (P < 0.001). These results suggest that hDPSCs co-culture protects SH-SY5Y cells from cytoskeletal damage.

hDPSCs protect OA-induced microtubule damage in SH-SY5Y cells

Morphometric ultrastructural analyses from TEM (Figure 6A) showed that cells in the control group displayed thick cytoplasmic bundles of microtubules, whereas cytoplasmic microtubules were rarely noted in the model group. The structures of microtubules were collapsed and loose, and bundles of microtubules were smaller after OA-induced neuronal injury in the model group. By contrast, hDPSCs co-culture with OA-damaged cells resulted in a large portion of microtubules being recovered and relatively thick dendritic bundles of microtubules. Statistical analyses (Figures 6B and 6C) showed that the length and number of microtubules for the model group were significantly different from those for the treatment groups (P < 0.01). These results suggest that hDPSCs co-culture protects SH-SY5Y cells from microtubule damage.
hDPSCs decrease the level of OA-induced tau phosphorylation

To determine whether hDPSCs regulate the phosphorylation of Tau upon OA injury of SH-SY5Y cells, we analyzed the protein level of phosphorylated and total Tau by western blotting. The results showed that OA-induced phosphorylation at Ser 396 of Tau protein and total Tau was increased. After a 24 h-treatment with hDPSCs, protein expression of OA-induced Tau phosphorylation was significantly reduced compared with total Tau (Figures 7A–7C). These results indicated the protective effect of DPSCs on the OA-induced cells against Tau phosphorylation.

Discussion

This is the first study to demonstrate hDPSC-treatment efficacy in an OA-induced in vitro AD model. Results showed that hDPSCs restored the cytomorphology of injured neurons, increased cell viability, relieved the rate of apoptosis, and restored cytoskeletal structure. hDPSCs rescued SH-SY5Y cells from OA-induced injury, possibly through the inhibition of Tau phosphorylation.

A previous study reported that during stem cell transplantation therapy, more than 90% of implanted stem cells were immediately lost, and the ultimate therapeutic effectiveness was most likely due to the paracrine action of MSCs (Caplan, 2009). DPSCs have strong paracrine action and can secrete a variety of cytokines, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF) (Tatullo et al., 2015), and these cytokines may induce the endogenous cell differentiation to replace the damaged cells or cause endogenous cells to secrete other neurotrophic factors to improve tissue regeneration (Martens et al., 2013). Apel et al. (2009) preincubated primary rat hippocampal neurons and rat dental pulp cells (rDPCs) for 2 days and then added Aβ1–42 protein.
for 24 h to induce neuronal injury. MTT assay results showed that cell viability of primary hippocampal neurons after preincubation was significantly higher than that of primary hippocampal neurons without preincubation with rDPCs. Another study reported that DPSCs improved survival rates of sensory neurons of trigeminal ganglion and motor neurons in the spinal cord through paracrine action (Nosrat et al., 2001). In addition, neurotrophic factors secreted by DPSCs protected dopamine neurons from damage by 6-hydroxy-dopamine (6-OHDA) and reduced the rate of neuronal apoptosis (Nosrat et al., 2004; Jarmalaviciute et al., 2015). This evidence suggests that hDPSCs may play potential roles in improving nerve cell viability and reducing apoptosis. In this study, we used a transwell system to co-culture hDPSCs and in vitro AD model cells for 24 h. Using a CCK-8 assay and Hoechst 33258 staining, we found that cell viability in the hDPSC-treatment group was significantly higher than in the model group, and the proportion of apoptotic cells in the hDPSC-treatment group was significantly reduced compared with the model group.

Fig. 6 hDPSCs protected OA-induced microtubules damage in SH-SY5Y cells. (A) Transmission electron microscopy results showed that microtubules were rarely noted in OA-damaged SH-SY5Y cells, and the bundles of microtubules were smaller, collapsed and loose. In contrast, hDPSC co-culture in OA-damaged cells resulted in a large portion of microtubules being recovered, and dendritic bundles of microtubules were relatively thick. (B) Quantitation of the maximum length of microtubules per group. (C) Average number of microtubules per cell. At least 10 cells were assessed in each group. Values are presented as means ± SD; statistical significance was analyzed by the ANOVA, **P < 0.01, ***P < 0.001. n = 10 in each group. Red arrows indicate the cytoskeleton. Scale bars: 0.5 and 1 μm.

Tau proteins are important elements for maintaining stable neuronal cytoskeletons. The main function of tau protein is to promote stability of microtubule assembly and maintenance of microtubules. A previous study showed that Aβ toxicity was mediated by Tau protein (Ittner and Gotz, 2011). Other large-scale studies showed that abnormal phosphorylation of Tau protein played an important role in the incidence and development of nerve cell degeneration as well as the impairment of learning and memory in AD patients (Grundke-Iqbal et al., 1986; Wang et al., 2010). Tau protein hyperphosphorylation results from either increased protein kinase activity or reduced phosphatase activity. Of the numerous protein kinases and phosphatases, glycogen synthase kinase-3 beta (GSK-3β) and protein phosphatase 2A (PP2A) play important roles in Tau protein hyperphosphorylation (Tian and Wang, 2002; Wang and Liu, 2008; Wang et al., 2013).

OA, a specific inhibitor of PP2A, inhibits Tau protein dephosphorylation and promotes hyperphosphorylation. This leads to the formation of NFT within neurons, which is an important factor in the pathogenesis of AD. Abnormal
and hyperphosphorylation of Tau protein in the brains of AD patients significantly reduces the capacity for microtubule assembly, which results in microtubule depolymerization and cytoskeletal damage. This in turn leads to decreased activity of neurons and, eventually, to degeneration of peripheral nerve cells and apoptosis (Gotz et al., 2013; Kamat et al., 2013; Kamat and Nath, 2015). Therefore, changes in microtubules and microfilaments are valuable and key indicators for impairment and treatment status of Tau protein function in nerve cells. In this study, cells of the model group became round with retracted neurites and collapsed and loose microtubules. After hDPSC-treatment, structures of microtubules and microfilaments became denser, with normal quantities and areas of distribution. These results indicate that hDPSCs repaired OA-induced cytoskeletal injury in nerve cells. In this study, cells of the model group became round with retracted neurites and collapsed and loose microtubules. After hDPSC-treatment, structures of microtubules and microfilaments became denser, with normal quantities and areas of distribution. These results indicate that hDPSCs repaired OA-induced cytoskeletal injury in nerve cells. In contrast to these studies, we first elicited cell injury and then implemented hDPSC treatment, which confirmed that hDPSCs have therapeutical potential for damaged nerve cells. The approach used in this study better simulates features of clinical therapy compared with previous studies and provides evidence that is more convincing. In addition, results from flow cytometry indicated that the isolated hDPSCs in this study had relatively low expression of STRO-1, which might be associated with the isolation method and the cell states of the hDPSCs. MSCs are not homogeneous cell populations, and the very small numbers of STRO-1-positive cells belong to more primitive cell subsets. Shi and Gronthos (2003) used magnetic-activated cell sorting to demonstrate that the STRO-1-positive cells account for about 6% of the total pulp cells. A previous study confirmed that STRO-1-positive cells had a more powerful homing ability in tissues and organs (Bensidhoum et al., 2004). The hDPSCs used in this study were multipotent and had a mixed cell population. We will direct our future attention to subsets (e.g., STRO-1 or other potential markers) of hDPSCs, which may have more therapeutical potency in AD treatment.

As noted earlier, in most current studies testing stem cell therapy in in vitro models of AD, the protective incubation period often precedes the injury (Apel et al., 2009; Lee et al., 2010; Kim et al., 2013). In contrast to these studies, we first elicited cell injury and then implemented hDPSC treatment, which confirmed that hDPSCs have therapeutical potential for damaged nerve cells. The approach used in this study better simulates features of clinical therapy compared with previous studies and provides evidence that is more convincing. In addition, results from flow cytometry indicated that the isolated hDPSCs in this study had relatively low expression of STRO-1, which might be associated with the isolation method and the cell states of the hDPSCs. MSCs are not homogeneous cell populations, and the very small numbers of STRO-1-positive cells belong to more primitive cell subsets. Shi and Gronthos (2003) used magnetic-activated cell sorting to demonstrate that the STRO-1-positive cells account for about 6% of the total pulp cells. A previous study confirmed that STRO-1-positive cells had a more powerful homing ability in tissues and organs (Bensidhoum et al., 2004). The hDPSCs used in this study were multipotent and had a mixed cell population. We will direct our future attention to subsets (e.g., STRO-1 or other potential markers) of hDPSCs, which may have more therapeutical potency in AD treatment.
Conclusions

In summary, hDPSCs significantly promoted repair in the OA-induced in vitro AD model, which might be due to the various growth factors secreted by hDPSCs that antagonized phosphorylation of Tau protein or promoted neural stem cells proliferation. Our results suggest that hDPSCs have potential benefits and relatively promising prospects for clinical application in the prevention and treatment of AD and other neurodegenerative diseases.

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Authors’ contribution

He HX and Pei XT conceived and designed the study, and drafted the manuscript together; Wang FX and Jia YL also designed the study, performed all experiments, and wrote the paper. Liu JJ, Zhai JL, Cao N and Yue W contributed to the establishment of the AD cell model, and to the data analysis. All authors approved the final version of the manuscript.

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