

Therapeutic effect of placenta-derived mesenchymal stem cells on hypoxic-ischemic brain damage in rats

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Background: Oxidative stress is involved in the development of hypoxic-ischemic brain damage (HIBD). In this study, we investigated the therapeutic effects of placenta-derived mesenchymal stem cells (PD-MSCs) and explored the NF-E2-related factor-2/heme oxygenase-1 (Nrf2/HO-1) signaling pathway in treating HIBD.

Methods: P7 rats were subjected to hypoxic-ischemic brain injury and randomly divided into four groups (control, HIBD, HIBD+PD-MSCs, and HIBD+fibroblasts). Forty-eight hours after the induction of HIBD, 5×10^5 of PD-MSCs were injected into cerebral tissue in the HIBD+PD-MSCs group, while the same dose of fibroblasts were injected in the HIBD+fibroblasts group. Morris Water Maze, gross and pathological changes were tested at P28. The level of malondialdehyde (MDA) was detected in rats' hippocampus. RT-PCR and western blot analysis were used to evaluate the changes of Nrf2/HO-1.

Results: The HIBD group showed significantly longer escape latency and a lower frequency of original platform crossing in the Morris Water Maze compared with the control group. Rats receiving PD-MSCs showed significant improvement of HIBD. The pathological changes were evident after HIBD, but ameliorated in the PD-MSCs group. Compared with the control group, HO-1 and Nrf2 were up-regulated at gene and protein levels in the HI brain, beginning at 6 hours and peaking at 48 hours ($P < 0.05$). The expression of HO-1 and Nrf2 in the PD-MSCs treatment group was more pronounced than in the HIBD group ($P < 0.01$). PD-MSCs also decreased MDA production in the brain tissue.

Conclusion: These results demonstrate that PD-MSCs have neuroprotective effect during the treatment of HIBD and that the mechanism may be partly due to alleviating oxidative stress.

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Key words: hypoxia-ischemia;
mesenchymal stem cells;
neonatal rat;
oxidative stress

Introduction

Hypoxic-ischemic brain damage (HIBD) causes brain injury in infants and represents a major cause of cerebral palsy, cognitive impairment, learning disability, and epilepsy.^[1,2] Previous studies^[3,4] showed that blood brain barrier impairment, apoptosis, inflammatory and other mechanisms were involved in the pathogenesis of HIBD. However, there are no effective strategies to improve neonatal brain injury. It is crucial to identify new therapeutic targets for the performance of clinical trials to address the treatment of HIBD.

In recent years, oxidative stress, inflammatory cytokines, and multiple apoptosis have been identified in HIBD.^[5] It is well established that oxidative stress is significantly activated after hypoxic-ischemic injury in the neonatal brain and peripheral organs.^[6] NF-E2-related factor-2 (Nrf2), a transcription factor, is an important protective factor for central nervous system tissue and a master regulator of antioxidative defence responses.^[7] Nrf2 binds to the antioxidant/electrophilic response element and regulates the expression of multiple cytoprotective proteins, including antioxidant and glutathione generating enzymes.^[8] Nrf2 is a rapidly turned-over protein that is normally sequestered in the cytoplasm via an interaction with the actin-binding protein Keap1. Nrf2 can induce the expression of Nrf2-dependent phase enzymes such as heme oxygenase-1 (HO-1), nicotine adenine dinucleotide phosphate, quinone oxidoreductase 1, and glutathione peroxidase.^[9] HO-1 is an

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ubiquitous and redox-sensitive inducible stress protein that degrades heme to CO, iron and biliverdin.^[10] Up-regulation of HO-1 has been known to protect against HI injury and HO-1 has been shown to exert potent endogenous anti-oxidative, anti-apoptotic and anti-inflammatory properties.^[11]

Accumulating evidence has shown that mesenchymal stem cells (MSCs) therapy is a potential method in improving brain functions.^[12,13] MSCs are capable of differentiating into a variety of tissue specific cells.^[14] Growing data have indicated that MSCs promote neurological functional recovery in neonatal hypoxic-ischemic brain injury.^[15,16] Despite the availability of various cell sources for experimental investigations, the ethical problems of the source and the safety of transplantation and xenotransplantation have become important clinical concerns. Placenta-derived mesenchymal stem cells (PD-MSCs) have distinct advantages of being abundant, easy to obtain with minimal invasiveness, and readily cultured to a sufficient number for transplantation without ethical issues of allografting.^[17] However, the mechanism of PD-MSCs treatment for HIBD remains unknown.

Therefore, this study aimed to investigate the neuroprotective effect of PD-MSCs in a rat model of HIBD and the effect of PD-MSCs on the production of oxidative/antioxidative markers, and to test the hypothesis that PD-MSCs can reduce hypoxic-ischemic brain injury in rats via its powerful antioxidant activity.

Methods

Preparation of placenta-derived mesenchymal stem cells and dermal fibroblasts

PD-MSCs lines were obtained from the term placenta of healthy rats (16-18 days gestation). The rat term placentas ($n=10$) were thoroughly washed in 0.1 mol/L phosphate buffer (pH 7.4) to remove excess blood. The central placental cotyledons were minced. The remaining placental tissue was cut into small pieces (1-2 mm³) and cultured in standard medium. Standard medium contained minimum essential medium- α (Gibco Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μ g/mL streptomycin. Non-adherent cells were removed, and the medium was replaced. The adherent, spindle-shaped cells were propagated through four to five passages ($>5 \times 10^4$ cells/cm²). Cell culture was incubated at 37°C in 5% humidified CO₂.

Phenotypes of adherent cells were analyzed using flow cytometry (Cytometer 1.0, CytomicsTM FC500, Beckman Coulter), including CD29, CD44, CD90, CD105, and CD45. Cells were incubated for 20 minutes

at room temperature with hamster anti-rat CD29 monoclonal antibody (BD, Franklin Lakes, NJ, USA), mouse monoclonal antibodies against rat CD44 (AbD, Oxford, UK), CD45 (Biolegend, USA), CD105, and CD90 (BD, Franklin Lakes, NJ, USA).

The cultured cells were differentiated into adipogenic and osteogenic cells due to their differential characteristics. Adipogenic differentiation was induced by seeding the cells in medium supplemented with high glucose dulbecco's modified eagle medium (DMEM) with 0.25 mmol 3-isobutyl-1-methylxanthine, 0.1 μ mol dexamethasone, 0.1 mmol indomethacin (Sigma-Aldrich, St. Louis, MO, USA), 6.25 μ g/mL insulin (PeproTech, Jiangsu, China), and 10% FBS. On the 14th day, cultures were stained with oil red O (Sigma-Aldrich, St. Louis, MO, USA), and the dye content was quantified by isopropanol elution. Osteogenic differentiation was induced by culturing in medium containing low glucose DMEM supplemented with 10% FBS, 0.1 μ mol dexamethasone, 50 mmol β -glycerol phosphate, and 0.2 mmol ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) for 3 weeks. On the 14th day, the cultures were stained with Alizarin Red Solution (Sigma-Aldrich, St. Louis, MO, USA).

The dermal fibroblasts were cultured as described by Griffin et al.^[18] Briefly, 1 mm³ pieces from rat skin were adhered in a small volume of culture medium for several hours and then supplemented with another 5 mL of culture medium. In the first week it was replenished every 3 days. Cell culture was incubated at 37°C in 5% humidified CO₂.

Green fluorescent protein (GFP) transfected placenta-derived mesenchymal stem cells

PD-MSCs at passage 3 were transfected with a lentivirus carrying the *GFP* gene. The *GFP* gene was linked to a lentivirus vector and packaged by 293T cells. MSCs were placed into six well plates and cultured in a humidified atmosphere at 37°C with 5% CO₂. When the cells reached 80% confluency, the medium was removed and replaced with the culture medium supplemented with GFP lentivirus. The cells were cultured for 12 hours at 37°C with 5% CO₂, and confirmed for successful transfection under a fluorescence microscope after 24-48 hours. The transfected cells were passaged when they reached 80% confluency.

Animal care and hypoxic-ischemic brain damage rat model

Wistar rats weighing 12-15 g were obtained on postpartum day 7 (P7) from the Experimental Animal Center of Shandong University. All experimental protocols were approved by the Institutional Animal

Care and Use Committee of Shandong University. A modified Rice-Vannucci model was used.^[19] Briefly, P7 Wistar rat pups were anesthetized with 300 mg/kg chloral hydrate intraperitoneally. The left common carotid artery of each pup was isolated, double-ligated, and cut between the ligatures. After the surgical procedure, the pups recovered for 2 hours in a temperature-controlled incubator. Then the pups were placed in a jar perfused with warmed, humidified 8% oxygen (balanced with nitrogen) at 1.5 L/min for 2.5 hours. A constant temperature of 37°C was maintained throughout the procedures. After hypoxia, the animals returned to their dams and the ambient temperature was maintained at 37°C for 24 hours. Control animals underwent anesthesia but they were treated without the common carotid artery ligation and hypoxia. Body weight of the rats was monitored after HIBD injury.

Cell transplantation

After HIBD was induced, the newborn rats were randomly divided into three groups: HIBD group ($n=20$), HIBD+PD-MSCs group ($n=20$) and HIBD+fibroblasts group ($n=20$). For the HIBD+PD-MSCs group, the skull bregma (3 mm from the front of Herringbone stitch, 2 mm from the left of midline, depth of 2 mm) was determined and a single cell suspension (5×10^5 cell/ μ L) of PD-MSCs was transplanted at 48 hours after HIBD with stereotaxic apparatus. The injection rate was 1 μ L/min with total 2 μ L volume. The HIBD+fibroblasts group received the same volume of fibroblasts at the same time. Rats from the HIBD group did not receive any injections. The rats were reared by dams in the same environment.

Morris Water Maze

Morris Water Maze was performed for all rat pups from P24 to P28.^[20] It consisted of a circular pool 110 cm in diameter and 60 cm in height, filled to a depth of 30 cm with water ($24 \pm 0.5^\circ\text{C}$). A hidden submerged platform was placed in one quadrant 2.5 cm below water surface. The time of locating the submerged platform (defined as the latency-cut off time 60 seconds) was measured. The longer the time required to locate the platform indicates the more impairment in spatial learning and memory. Each trial started from a different quadrant. Testing was consecutively repeated for 5 days. On testing day 6, the platform was removed and the animals were placed into the water maze for 60 seconds.

Histological examination

Five rats randomly selected from each group were anesthetized and perfused with 4% paraformaldehyde after the behavioral tests. Brains were collected, fixed,

and paraffin-embedded. Coronal brain sections were cut for 5 μ m thicknesses. Three slices of each specimen were randomly selected to be dewaxed, hydrated, counterstained with hematoxylin and eosin and Nissl stain. The number of neurons was counted under a light microscope. Five horizons were taken from each slice. The average neuron number of the five horizons was the neuron number of each slice. Finally, the mean number of three slices was the number of neurons in each specimen.

RNA extraction and quantitative real-time PCR

The expression of HO-1 and Nrf2 in brain tissue taken 6 hours, 24 hours, 48 hours, 72 hours, and 5 days after HI was detected using a quantitative real-time PCR. We also assessed the expressions of HO-1, Nrf2 at P28. Briefly, total RNA was extracted from tissue dissected from the ipsilateral hemisphere using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (5000 ng) was used to synthesize first-strand cDNA by SuperScript using the First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cDNA product was amplified by PCR. The expression of mRNA was quantified with SYBR Green two-step for HO-1, Nrf2 and Beta-actin. The PCR reaction mixture was prepared using the SYBER Green PCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Each sample was analyzed in duplicate using the conditions recommended by the manufacturer. The following primers were used: rat HO-1 sense primer, 5'-GGCCTGGCTTTTTTCACCTT-3', and anti-sense primer, 5'-TGCGAGCACGATAGAGCTGTT-3'; Nrf2 sense primer, 5'-CGGCATTTCACTGAACAAGT-3', and anti-sense primer, 5'-TGGGTCTCCGTAATGGGAAGA-3; beta-actin sense primer, 5'-TCTGTGTGGATTGGTGGCTCTA-3', and anti-sense primer, 5'-CTGCTTGCTGATCCACATCTG-3'. After initial denaturation at 95°C for 60 seconds, a two-cycle procedure was used (denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute) for 40 cycles. The expression of each genes relative to beta-actin was determined using the 2-dCT method, where $dCT = (CT_{\text{target gene}} - CT_{\text{actin}})$. The data were analyzed by Sequence Detection Software 1.4 (Applied Biosystems). Reported data were the means \pm SD of at least three independent experiments. The mRNA expression was presented as fold difference with respect to the control group, and the control group values were set as fold change=1.

Western blot analysis

Tissue samples were first washed with ice-cold phosphate buffered saline (PBS), and the homogenate

was lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). All samples were assayed for protein concentration using a bicinchoninic acid assay (Pierce Chemical Co, Rockford, IL, USA), after which the samples were stored at -80°C until use. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin in PBS/Tween 20 solution. The blots were incubated with HO-1, Nrf2 and GAPDH (Sigma-Aldrich, St. Louis, MO, USA) antibodies at 4°C . After washing with PBS/Tween 20 solution, the blots were incubated with appropriate horseradish peroxidase-(HRP-) conjugated secondary antibodies for 1 hour. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Pittsburgh, PA, USA) which was then exposed to Biomax L film (Kodak, Rochester, NY, USA). Relative densities of the bands were analyzed using the Kodak Digital Science Imaging System.

Malondialdehyde (MDA) analysis

MDA levels in the brain tissue were used as an indicator of lipid peroxidation, samples of the hippocampus were taken from the impaired brain hemispheres at 6 hours, 24 hours, 48 hours, 72 hours, and 5 days after hypoxia-ischemia induction. Furthermore, MDA levels were also measured 5 days after cell transplantation. Briefly, 10% (w/v) homogenate was centrifuged at $2000\times g$ for 10 minutes at 4°C . The supernatant was collected to measure the MDA activity. The MDA level was measured with the thiobarbituric acid method. The absorbance of the organic layer was measured via spectrophotometry at 532 nm. The MDA level was expressed as nmol/mg protein.

Statistical analysis

Statistical analysis was performed using SPSS software version 14.0 (SPSS Inc, Chicago, IL). All descriptive data were presented as means \pm SD. The means were compared by a two-way ANOVA. Tukey's honestly significant difference multiple comparisons were performed at a 0.05 significance level. A *P* value less than 0.05 was considered statistically significant.

Results

Characteristics of placenta-derived mesenchymal stem cells and their distribution in the brain

Adherent cells with fibroblastic morphology could be observed as early as 48 hours by the tissue explants adherent method (Fig. 1A). Cells formed a monolayer of homogeneous bipolar spindle-like cells with a

whirl pool like array within 2 weeks (Fig. 1B). Flow cytometry results showed that placenta-derived cells shared most of their immunophenotype with mesenchymal stem cells, namely positive expression for stromal markers CD29 (79.5%), CD44 (93.2%), CD90 (98.7%), and CD105 (95.4%) and lack expression of CD45 (0.4%) (Fig. 1C).

Differentiation of MSCs was assessed using P4 cells derived from placenta ($n=6$). Cells kept in the regular growth medium served as the control. The spindle shape of the MSCs flattened and broadened after 1 week of adipogenic induction. Small oil droplets were observed to gradually appear in the cytoplasm, and almost all of the cells contained numerous oil-red-O-positive lipid droplets on day 14 (Fig. 1D&E). When induced to differentiate under osteogenic conditions, MSCs congregation was increased and mineralized matrix formed. Most of the MSCs-like cells became alkaline-phosphatase positive (Fig. 1F&G). GFP-positive PD-MSCs were found primarily around the location of injection at 6 hours. Six days after MSCs implantation, green fluorescent signal was significantly attenuated (Fig. 1H&I).

Motor function

The HIBD group showed a significantly longer escape latency ($P<0.05$) and a lower frequency of original platform crossing ($P<0.05$) in contrast to the control group. The average time of escape latency was shorter in the PD-MSCs transplantation group than in the HIBD group ($P<0.01$). In addition, the frequency of platform passing was significantly greater in the PD-MSCs transplantation group than in the HIBD group ($P<0.01$) and the fibroblasts transplantation group ($P<0.05$) (Table).

General observations and histopathologic results

Rats in the control group were well-groomed, whereas rats in the HIBD group were insufficient-groomed with

Table. Morris Water Maze performed on day 28 (means \pm SD)

Group	Swimming time (s)	Frequency platform passing (times/60 s)
Control	10.28 \pm 1.94*	9.75 \pm 2.25*
HIBD	32.29 \pm 2.88†	4.38 \pm 2.39†
HIBD+PD-MSCs	15.58 \pm 1.98	6.30 \pm 1.64
HIBD+fibroblasts	30.12 \pm 2.84‡	5.01 \pm 1.71‡

Table showing the mean time taken to reach the platform for the 5 trial days. A significant increase in the latency to reach the platform was found in the hypoxic-ischemic brain damage (HIBD) group. Placenta-derived mesenchymal stem cells (PD-MSCs) treating rats had a significantly lower latency to locate the platform than hypoxic-ischemic rats. The HIBD group showed a significantly lower frequency of original platform crossing compared with the PD-MSCs treatment group. *: $P<0.05$, vs. the control group; †: $P<0.01$, vs. the HIBD group; ‡: $P<0.05$, vs. the fibroblasts group ($n=5$ in each group).

yellow, gray, and sticky fur. The gross morphology of the brains of the HIBD group showed significant atrophy of the left cerebral hemisphere. Brains from the PD-MSCs group were similar to those from the control group, without obvious cerebral atrophy and edema (Fig. 2A1-A3). Microscopic examination with hematoxylin-eosin revealed shrunken neurons and eosinophilic cell bodies in the HIBD group. Nissl staining showed that there were many abnormal neurons with shrunken cytoplasm, pyknotic nuclei, and reduction of Nissl substance after HIBD, but not in the control group. These neurons were dark stained or triangular in shape. Intact neurons were also significantly less in the fibroblasts treatment group. Pathological changes were significantly improved

after PD-MSCs treatment (Fig. 2A2-D3). Moreover, the number of intact neurons was significantly greater in the PD-MSCs group than in the fibroblasts group (Fig. 2E).

Before HIBD treatment, no apparent behavioral changes were observed in these groups. Compared with the control group, the HIBD group showed a lower body weight. PD-MSCs treatment significantly improved the HIBD weight reduction in rats (Fig. 2F). The levels of the body weight were similar among the four groups before the induction of HIBD ($P>0.05$). After induction of HIBD, the weight gain of the rats in the HIBD group and the HIBD+fibroblasts group was slower than that in the control group ($P>0.05$). The body weight of the rats was significantly improved

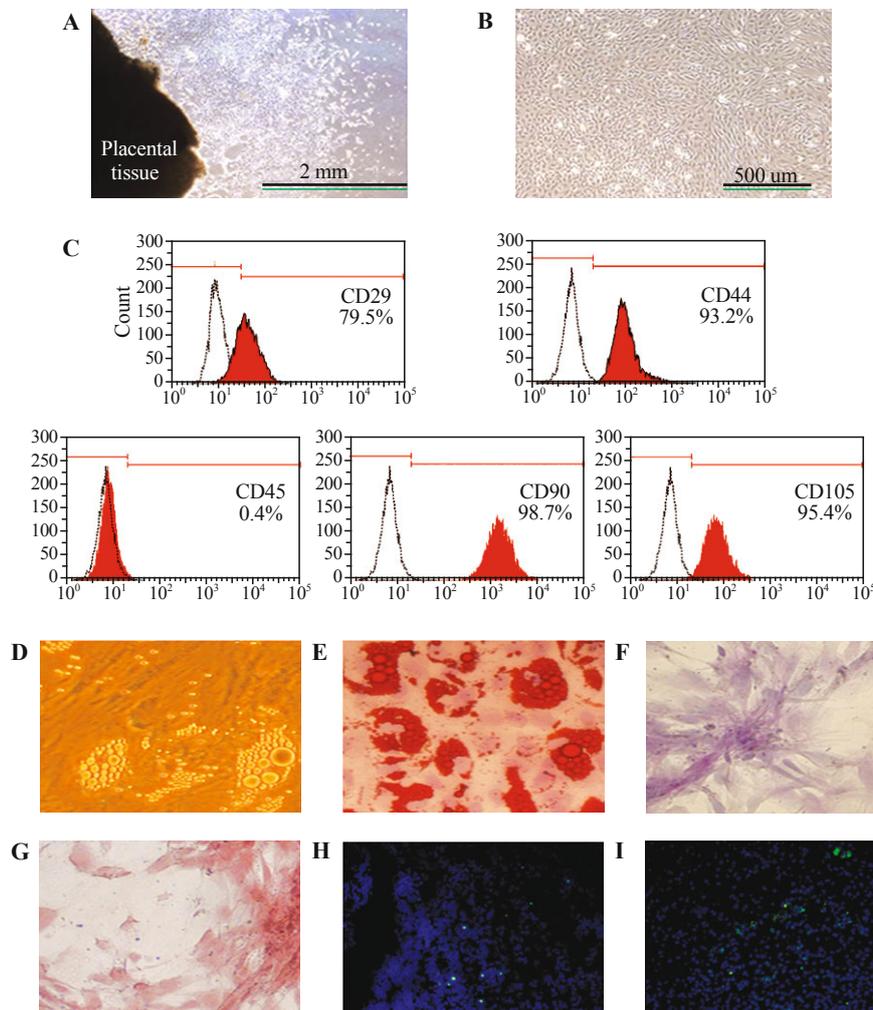


Fig. 1. Characterization and differentiation capacity of placenta derived adherent cells and distribution of the transplanted cells in the brain. **A:** The primary adherent cells with fibroblastic morphology could be observed to climb out from the edge of the placental tissue pieces as early as 48 hours; **B:** Two weeks later the cells formed a monolayer of homogeneous bipolar spindle-like cells; **C:** Immunophenotype of placenta derived cells; **D&E:** Small oil droplets appeared in the cytoplasm (original magnification $\times 200$); **F&G:** MSCs-like cells became alkaline-phosphatase positive and stained with alizarin red stain (original magnification $\times 200$); **H&I:** GFP-positive placenta-derived-MSCs were found primarily around the location of injection 48 hours after transplantation. Six days after MSCs implantation, the green fluorescent signal was significantly attenuated. The scale bar represents 200 μm . MSCs: mesenchymal stem cells; GFP: green fluorescent protein.

in the HIBD+PD-MSCs group (control group vs. HIBD+PD-MSCs group, $P>0.05$; HIBD group vs. HIBD+PD-MSCs group, $P>0.01$; HIBD+fibroblasts group vs. HIBD+PD-MSCs group, $P>0.01$).

PD-MSCs treatment regulating the generation of reactive oxygen species after HIBD injury-assessment at gene and protein level

The mRNA expression of HO-1 and Nrf2 was up-regulated in the HIBD group compared with the control group ($P>0.05$), beginning at 6 hours and peaking at 48 hours after HI. The mRNA expression remained at a high level, though gradually decreased at 72 hours and 5 days (Fig. 3A). It was higher in the three experimental

groups than in the control group (all $P>0.01$), and notably higher in the PD-MSCs group than in the HIBD and fibroblasts groups (all $P>0.05$) (Fig. 3B). Western blot analysis demonstrated that the expression of HO-1 and Nrf2 was significantly higher in the three experimental groups than that in the control group (all $P>0.01$). In agreement with the results of PCR, the expression of HO-1 and Nrf2 in the PD-MSCs group was significantly higher than that in the HIBD group and fibroblasts group (all $P>0.01$) (Fig. 3C & D).

MDA levels

In brain tissue, MDA levels were measured at 6 hours, 24 hours, 48 hours, 72 hours, and 5 days after hypoxia-

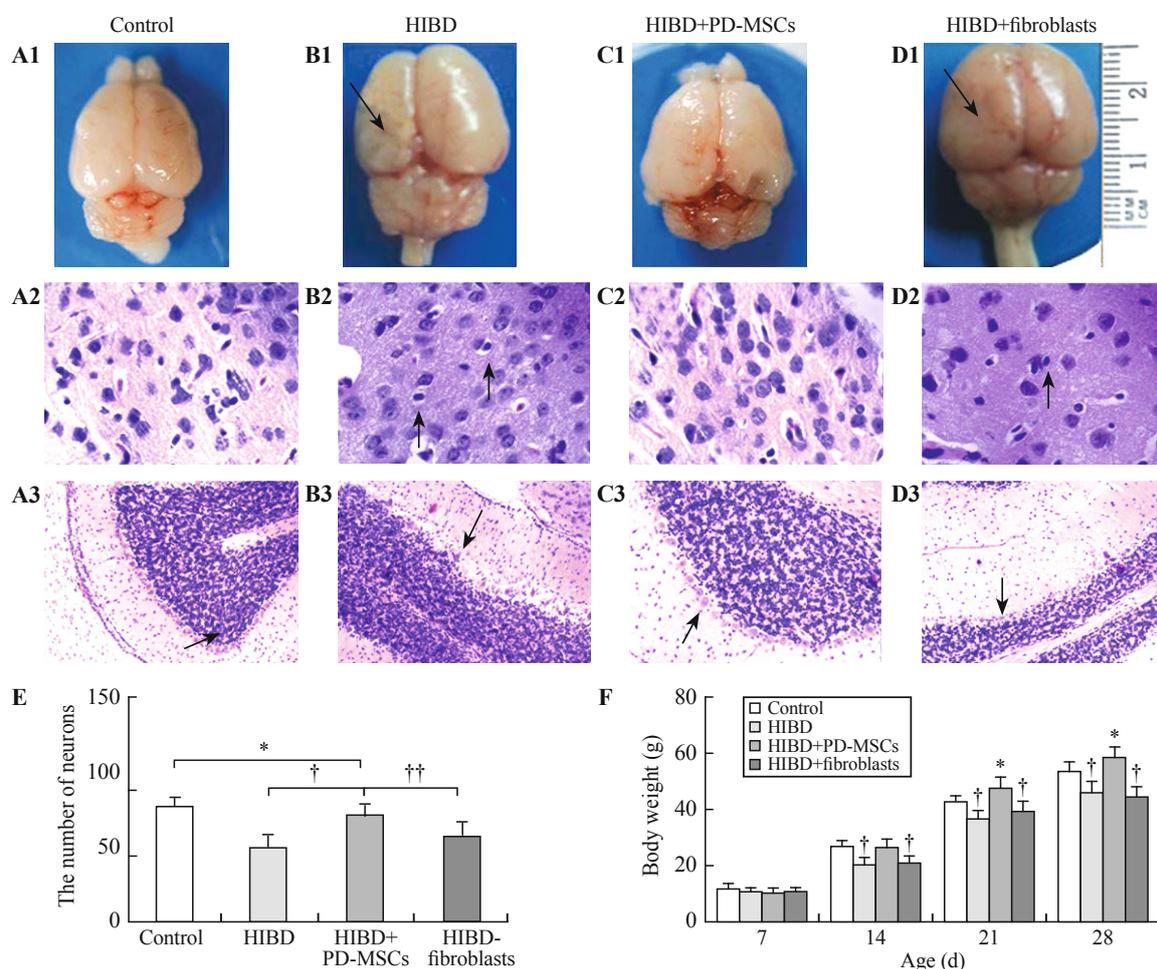


Fig. 2. Changes of brain morphology and body weight in the four groups after cell treatment: control group (A1-A3), HIBD group (B1-B3), HIBD+PD-MSCs group (C1-C3) and HIBD+fibroblasts group (D1-D3). A1-D1: The gross morphology of the brains of the HIBD group showed significant atrophy of the left cerebral hemisphere (Black arrow indicates atrophied brain); brains from the PD-MSCs group were similar to those from the control group, without obvious cerebral atrophy and edema. Brains of the fibroblasts group showed cerebral collapse (Black arrow indicates collapsed brain) (original magnification $\times 400$); A2-D2: Hematoxylin-eosin staining of the cortex (Black arrow indicates shrunken neuron) (original magnification $\times 400$); A3-D3: Hematoxylin-eosin staining of the Purkinje cells (Black arrow indicates unordered arrangement); E: The number of intact neurons was significantly greater in the PD-MSCs group than in the HIBD and fibroblasts groups; F: The body weight of the rats in the HIBD group was obviously decreased than that in the control group. On day 14 after PD-MSCs transplantation, the body weight of the rats in the PD-MSCs group was significantly improved, even notably higher than that in the control group. *: $P>0.05$, vs. the control group; †: $P>0.01$, vs. the HIBD and fibroblasts groups. HIBD: hypoxic-ischemic brain damage; PD-MSCs: placenta-derived mesenchymal stem cells.

ischemia insult. The MDA level was 3.17 ± 0.30 nmol/mg protein in the control group. The results showed that the MDA level was significantly increased at 6 hours (6.49 ± 0.51 nmol/mg protein) after HI injury. The MDA level peaked at 72 hours (11.56 ± 1.09 nmol/mg protein). Subsequently, the MDA level decreased significantly on day 5 (10.67 ± 0.98 nmol/mg protein), but still higher than the normal level. PD-MSCs (4.36 ± 0.37 nmol/mg protein) decreased the MDA level compared to the hypoxia-ischemia injury group (6.13 ± 0.41 nmol/mg protein). There was a significant difference in MDA level between the PD-MSCs group and the fibroblasts group (6.09 ± 0.32 nmol/mg protein) ($P > 0.05$).

Discussion

In this study, we demonstrated that PD-MSCs can significantly ameliorate movement disorders and increase the body weight of the rats after hypoxic-ischemic injury. Furthermore, PD-MSCs initiated anti-oxidative effects through the Nrf2/HO-1 signaling pathway after hypoxic-ischemic injury.

Our study corroborated that PD-MSCs could improve the development and functional recovery

of rats after experimental hypoxic-ischemic brain damage. First, we succeeded in isolating and culturing mesenchymal stem cells from the placenta. Adherent cells generated from placenta tissues were able to expand and express characteristics of MSCs (CD29, CD44, CD90, and CD105) in terms of mesenchymal and functional properties.^[21] After cell transplantation, rats in the PD-MSCs group had no apparent brain atrophy. Moreover, their body weight was even higher than that in the control group. Compared with BM-MSCs, PD-MSCs may be an ideal and practical source because of their accessibility, painless procurement from donors, lower risk of viral contamination, and lack of any ethical concerns. Thus, it indicates that placenta tissues have the potential to serve as an alternative to bone marrow as a source of MSCs for future experimental use and clinical application in treating HIBD brain injury.

PD-MSCs can significantly ameliorate movement disorders and pathological changes in rats after HI injury. The cortex, striatum, hippocampus, and Purkinje cells were particularly sensitive to hypoxic-ischemic exposure. In our study, hematoxylin-eosin microscopic examination revealed shrunken neurons, eosinophilic cell bodies, disruption in microvessels, meningeal hemorrhage, and glial nodules in the HIBD group.

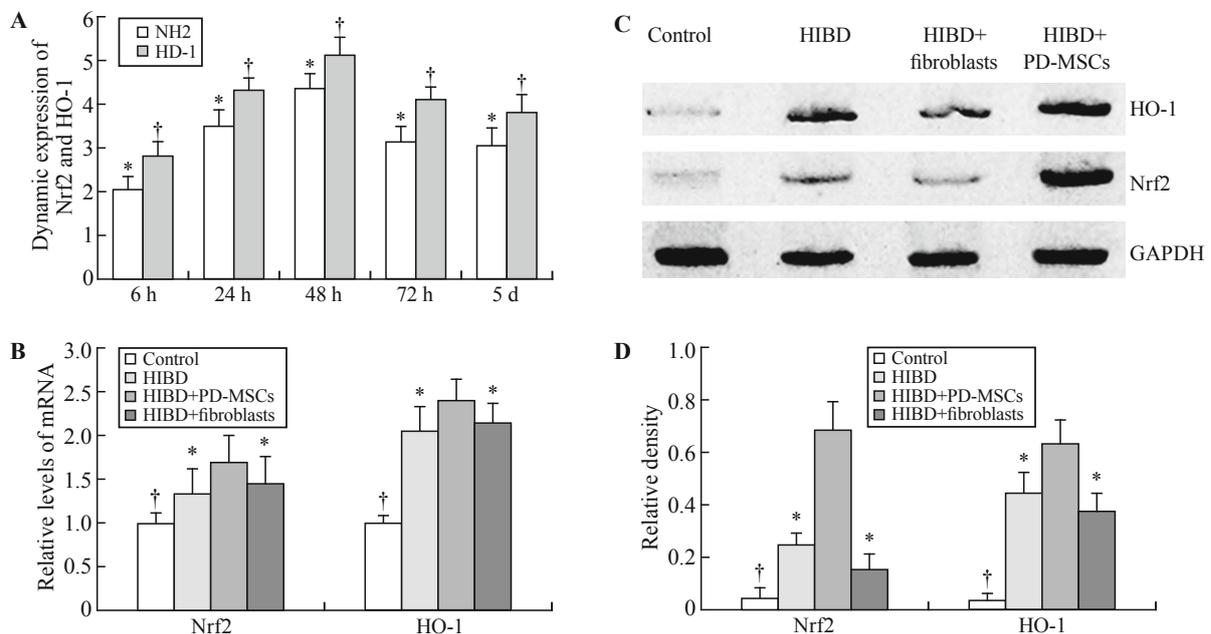


Fig. 3. The cerebral expression of HO-1 and Nrf2 in the four groups after cell treatment. **A:** Illustration of the dynamic mRNA expression of HO-1 and Nrf2 (*: $P > 0.05$ for Nrf2 vs. control; †: $P < 0.05$ for HO-1 vs. control); **B:** The mRNA expression of HO-1 and Nrf2 14 days after treatment. The mRNA expression of HO-1 in the PD-MSCs group was notably higher than that in the HIBD group. The Nrf2 mRNA expression in the HIBD and PD-MSCs groups was notably higher than that in the control group, and the expression in the PD-MSCs group was highest (*: $P > 0.05$ for the PD-MSCs group vs. the HIBD and fibroblasts groups; †: $P < 0.01$ for the PD-MSCs group vs. the control group); **C&D:** Western blot analysis of HO-1 and Nrf2 expression on day 5 after PD-MSCs treatment, representative photographs of the expression of HO-1 and Nrf2; **C:** The bar graph showing the relative expression of HO-1 and Nrf2 proteins normalized to GAPDH expression; **D:** HO-1 expression was significantly higher in the HIBD group than in the control group, and the protein expression of HO-1 in the PD-MSCs group was much higher than that in the HIBD group. The Nrf2 protein levels were more significantly enhanced in the PD-MSCs group than that in the HIBD and fibroblasts groups (*: $P > 0.01$ for the PD-MSCs group vs. the HIBD and fibroblasts groups; †: $P > 0.01$ for the PD-MSCs group vs. the control group). HO-1: heme oxygenase-1; Nrf2: NF-E2-related factor-2; PD-MSCs: placenta-derived mesenchymal stem cells; HIBD: hypoxic-ischemic brain damage.

The degree of injury in the lesioned hemisphere was more obvious after HI, but was restored after PD-MSCs transplantation. Moreover, neurobehavioral tests showed that the group with PD-MSCs transplantation had the best recovery from HIBD injury.

PD-MSCs delivered to ischemic brain tissue have the capacity to restore nervous tissue. However, the mechanisms by which PD-MSCs enhance functional recovery are more complex than the multilineage differentiation potential and proliferative capacity. We focused on the anti-oxidant activities associated with PD-MSCs. It has been demonstrated that oxidative stress is a mediator of cell death following cerebral ischemia. Although some studies^[22-24] have confirmed that MSCs can increase the expression of HO-1 in a variety of ischemic tissues, such as the heart, liver, kidney, and the exact mechanism is still not clear, which is the focus of our study. When cells are subjected to oxidative stress, Nrf2 traverses to the nucleus and activates the expression of phase II enzymes such as HO-1.^[25] HO-1 can be induced by the activation of Nrf2. In our study, we found that the mRNA expression as well as the protein expression of HO-1 and Nrf2 was remarkably increased in the HIBD group compared with the control group after hypoxic-ischemic brain injury. As compared with the hypoxic-ischemic injured rats without treatment, the expression of these biomarkers at both gene and protein levels was significantly increased in rats following PD-MSCs treatment. Taken together, the data demonstrated that the cerebral protection from PD-MSCs treatment is partly mediated by the Nrf2 signaling pathway via the up-regulation of HO-1.

To assess the oxygen free radical level, brain MDA level was simultaneously determined in our study. MDA is a stable lipid peroxide end product produced during the oxidation of membrane lipid unsaturated fatty acid by oxygen free radicals.^[26,27] The central nervous system is rich in unsaturated fatty acids that interact with oxygen free radicals after ischemia, thus generating a large amount of MDA. Therefore, MDA level indirectly reflects the level of oxygen free radicals and the degree of lipid peroxidation in brain tissue. The high MDA level in the HIBD group suggested the occurrence of oxidative stress and the production of a large amount of lipid peroxide. The PD-MSCs group had a lower MDA level, indicating that PD-MSCs induced a significant suppression on the generation of free radicals and provided an antioxidative protection to the cell and organelle membranes.

Taken together, PD-MSCs treatment can markedly restore the brain tissue and improve the functional outcomes. The key mechanisms underlying the positive therapeutic impact of PD-MSCs treatment on brain function could be due to the suppression of oxidative stress.

PD-MSCs transplantation in cerebral hypoxic-ischemic infarction may help prevent the progression of tissue damage and open up a possible new therapeutic way.

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Ethical approval: This study was approved by the Ethical Committee of Shandong University.

Competing interest: No conflict of interest.

Contributors: JXL designed and supervised the experiments, revised the paper, and approved the final version of the paper. DHF, ZH conceived and designed the experiments, provided financial support on reagents and materials, performed the experiments, analyzed the data, wrote the draft, and approved the final version of the paper. LD, YXH, GXY, and MWW designed and guided the experiments, revised the paper, and approved the final version of the paper. All authors contributed to the design and interpretation of the study and to further drafts. JXL is the guarantor.

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