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Original Research

Human Placenta-Derived Mesenchymal Stem Cells Improve Neurological Function in Rats with Intrauterine Hypoxic-Ischaemic Encephalopathy by Reducing Apoptosis and Inflammatory Reactions

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Abstract

Background: Hypoxic-ischaemic encephalopathy (HIE) is a major cause of neonatal disability and mortality. Although hypothermia therapy offers some neuroprotection, the recovery of neurological function is limited. Therefore, new synergistic therapies are necessary to improve the prognosis. Mesenchymal stem cell-based therapy is emerging as a promising treatment option for HIE. In this study, we studied the therapeutic efficacy of human placenta-derived mesenchymal stem cells (PD-MSCs) in the HIE rat model and analyzed the underlying therapeutic mechanisms. **Methods**: Rats were divided into 6 groups (n = 9 for each) as follows: control, HIE model, HIE + normal saline, and HIE + PD-MSC transplantation at days 7, 14 and 28 postpartum. Following PD-MSC transplantation, neurological behavior was evaluated using rotarod tests, traction tests, and the Morris water maze test. The degree of brain tissue damage was assessed by histological examination and Nissl staining. Expression levels of apoptosis-related proteins and inflammatory factors were quantified by Western blotting and enzyme-linked immunosorbent assays. Immunofluorescence was used to investigate the ability of PD-MSCs to repair the morphology and function of hippocampal neurons with hypoxic-ischaemic (HI) injury. **Results**: PD-MSC transplantation enhanced motor coordination and muscle strength in HIE rats. This treatment also improved spatial memory ability by repairing pathological damage and preventing the loss of neurons in the cerebral cortex. The most effective treatment was observed in the HIE + PD-MSC transplantation at day 7 group. Expression levels of microtubule-associated protein-2 (MAP-2), B-cell lymphoma-2 (BCL-2), interleukin (IL)-10, and transforming growth factor (TGF -β1) were significantly higher in the HIE + PD-MSC treatment groups compared to the HIE group, whereas the levels of BCL-2-associated X protein (BAX), BCL-2-associated agonist of cell death (BAD), IL-1β and tumour necrosis factor α (TNF-α) were significantly lower. **Conclusions**: We demonstrated that intravenous injection of PD-MSC at 7, 14 and 28 days after intrauterine HI damage in a rat model could improve learning, memory, and motor function, possibly by inhibiting apoptosis and inflammatory damage. These findings indicate that autologous PD-MSC therapy could have potential application for the treatment of HIE.

Keywords: human placenta-derived mesenchymal stem cells; hypoxic-ischaemic encephalopathy; treatment option; neuroprotection; therapeutic mechanisms

1. Introduction

Perinatal hypoxia-ischaemia (HI) is a major cause of newborn disability and mortality, especially hypoxic-ischaemic encephalopathy (HIE), which is the leading cause of cerebral palsy in neonates [1]. The worldwide incidence of HIE is approximately 5/1000 live births. Approximately 20–25% of affected individuals die during the neonatal period, and 25% of the survivors suffer lifelong neurological sequelae [2]. The major causes of HIE are intrapartum HI events, such as uterine rupture, placental abruption, cord prolapse, and amniotic fluid embolism [3]. The pathogenesis of HIE injury may be related to inflammation, oxidative stress and neuronal apoptosis, which are secondary to oxygen and blood shortage [4]. Hypothermia is the standard treatment for neonates with HIE and can reduce the risk of death and major neurodevelopmental disabilities [5]. Although therapeutic hypothermia has become the standard treatment for babies with moderate to severe HIE, the mortality rate after this treatment is still as high as 27%, and almost 28% of surviving babies develop

major neurodevelopmental disabilities [2]. However, some patients cannot receive hypothermia treatment if the gestational age is <35 weeks, the birth weight is <1800 g, the neonate is >6-hours old, or the patient has major congenital abnormalities [6]. Thus, it is necessary to find new ways to improve treatment outcomes for HIE.

Preclinical studies have confirmed that mesenchymal stem cells (MSCs) may have neurotherapeutic effects, and MSC-based therapy is emerging as a promising treatment option for HIE [7]. MSCs are undifferentiated, pluripotent stem cells with self-renewal properties and immunomodulatory capacity. These cells have been investigated as a potential treatment for HIE, as they reduce inflammatory and reactive oxygen species (ROS) damage, and promote the restoration of tissue function [7]. The mechanism by which MSC transplantation improves central injury could involve mitochondrial transfer, modulation of the immune-inflammatory response, and regulation of ROS production [7]. MSCs originate from the bone marrow, peripheral blood, muscle, adipose tissue, umbilical cord, placenta, *etc*. Placenta-derived mesenchymal stem cells (PD-MSCs) are an ideal source for such treatment, as they are easy to access and have self-renewal capabilities. Compared to bone marrow-derived MSCs (BM-MSCs), PD-MSCs have high proliferative activity and are strongly immunosuppressed [8]. Moreover, BM-MSCs are difficult to obtain due to the very invasive nature of bone marrow collection. The characteristics of adipose-derived MSCs (AD-MSCs) can vary considerably between patients according to biological factors such as age, sex, body mass index and disease [9]. Although some methods have been described for maintaining the stemness and proliferative potential of AD-MSCs, there are still some limitations with their potential clinical application [10]. The neuroprotective effect of MSCs has been observed in postnatal rat models created by blocking the unilateral carotid arteries, followed by exposure to a hypoxic environment [11–13]. However, this model does not precisely match the clinical conditions of HIE, which is often due to poor intrauterine blood perfusion and oxygen supplementation during the peripartum period. To investigate the neuroprotective effect of MSCs during the acute phase of HIE, MSC transplantation was performed within 6 hours after HI. Other research that focused on the secondary phase (lasting hours to days after HI) mostly used a transplantation time of between 1 to 10 days post-insult [14]. Few studies to date have focused on the tertiary phase, lasting from days to years after HI. Since HIE involves progressive neuronal cell loss over a period of days and months after HI, the neuro-restorative effect of MSC transplantation is worthy of further investigation. In the present study we developed an intrauterine HI rat model to investigate the neuroprotective effect and optimal treatment timing of PD-MSCs in neonatal rats with HIE. This new model was based on the rabbit intrauterine HI model of cerebral palsy [15,16].

2. Materials and Methods

2.1 Isolation and Culture of Human PD-MSCs

All experiments on human tissues were approved by the Ethics Committee of the Zhejiang Provincial People's Hospital, Zhejiang, China. Written informed consent was obtained from all subjects. PD-MSCs were isolated from placental villous tissues procured from healthy women during labor. Briefly, placental villous tissues were cut into approximately 1 mm^3 pieces and treated with Collagenase type I (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. After passing through a 100-µm strainer (Falcon, BD Biosciences, Franklin Lakes, NJ, USA), the cell suspension was seeded into cell culture plates with serum-free MSC culture medium (SC2013-G, TBD Science, Tianjin, China) at a density of $1 \times 10^5/cm^2$. The plates were then incubated at 37 °C in a humidified incubator with 5% CO_2 and passaged after digestion with 0.25% trypsin-ethylenediaminetetraacetic acid (1:5 ratio) (15400054, Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Characterization of PD-MSCs and Flow Cytometric Analysis

PD-MSCs were characterized by their morphology, surface marker expression, and mesenchymal lineage differentiation, as previously described [17]. Osteogenic, adipogenic and chondrogenic inductions were performed to evaluate the multi-lineage differentiation capacity of PD-MSCs. To evaluate mineralization, the cultures were stained with Alizarin Red S according to the manufacturer's instructions (HUXXC-90021, Cyagen Biosciences Inc, Santa Clara, CA, USA). Briefly, cells were fixed with 4% paraformaldehyde (P0099, Beyotime Institute of Biotechnology, Shanghai, China) for 30 minutes and then stained with Alizarin Red S for 5 minutes at room temperature. The lipid droplets formed following induction of adipogenesis were visualized using Oil Red O staining (HUXXC-90031, Cyagen Biosciences Inc). Successful chondrogenic differentiation was tested by Alcian blue staining (HUXXC-90041, Cyagen Biosciences Inc).

The PD-MSC phenotype after the third passage was analysed by flow cytometry (BD FACSVerse, BD Biosciences, Franklin Lakes, NJ, USA) using the following antibodies: anti-CD14 (BD Biosciences, Franklin Lakes, NJ, USA, cat. no. 555397, 1:5 dilution), anti-CD45 (BD Biosciences, cat. no. 560973, 1:5 dilution), anti-CD19 (BD Biosciences, cat. no. 563326, 1:5 dilution), anti-CD34 (BD Biosciences, cat. no. 555822, 1:5 dilution), anti-CD73 (BioLegend, San Diego, CA, USA, cat. no. 344004, 1:20 dilution), anti-CD105 (BioGems, Westlake Village, CA, USA, cat. no. 17111-60, 1:20 dilution), anti-HLA-DR (BD Biosciences, Cat. no. 555811, 1:5 dilution) and anti-CD90 (BioLegend, cat. no. 328110, 1:5 dilution). Incubation with each of these antibodies was at room temperature for 15 minutes according to the manufacturer's instructions.

A suitable isotype-matched antibody (PE; BD Biosciences, cat. no. 555749; FITC, BD Biosciences, cat. no. 555573, 1:5 dilution) incubated at room temperature for 15 minutes was utilized as a negative control. The data were analyzed using BD FACSuite software (version 1.0.5.3841, BD Biosciences, Franklin Lakes, NJ, USA).

2.3 Isolation and Culture of Hippocampal Neurons

All animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang Provincial People's Hospital. Pregnant Sprague Dawley rats (Shanghai SLAC Laboratory Animal Co. Ltd, Shanghai, China) at day 18 of gestation were used to isolate hippocampal neurons from the brains of late rat embryos. Isolation and culture of the hippocampal neurons was carried out using a previously described protocol [18]. Briefly, rat embryos were harvested from pregnant rats at 18 days gestation. They were then dissected under a stereomicroscope and in an ice-cold and sterile environment. After exposing the surface of the cerebral cortex, the hippocampus was gently separated, cut into 1 mm^3 pieces, digested with 2 mL of 0.25% pancreatic enzyme solution (25200-072, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for 20 minutes at 37 °C, and then filtered through a 150-mesh stainless steel filter (352360, Corning, Somerville, MA, USA). A suspension containing 1×10^6 cells was added to a 35 mm diameter sterile plastic petri dish soaked overnight in 0.01% poly-L-lysine hydrobromide (P8140, Solarbio, Beijing, China). The cells were cultured in an incubator at 37 °C under 5% CO_2. All medium was replaced the next day with DMEM containing 10% FBS and a 1% penicillin/streptomycin (P/S) solution. Half the medium was replaced every three days thereafter. Cell growth was observed each day with a stereomicroscope.

2.4 In Vitro Hypoxic-Reoxygenated Cell Model and Co-Culture with PD-MSCs

To construct an *in vitro* hypoxic-reoxygenated cell model, hippocampal neurons were grown for six days in normal culture (37 °C, 5% CO_2, 95% atmosphere) and then placed in a hypoxic tank (37 °C, 5% CO_2, 95% N_2) for 2.5 hours. Third-generation PD-MSCs were obtained using the isolation and identification method established earlier by our research group. Three groups were set up for further experiments: (a) H/R group: hippocampal neurons with hypoxic-reoxygenated injury (H/R cells) were cultured in Transwell double-layer plates (28419067, CoStar, Suzhou, Jiangsu, China). (b) Co-culture group: H/R cells were cultured in the lower Transwell chamber, and PD-MSCs were cultured in the upper chamber without direct contact to H/R cells. (c) Control group: normal hippocampal neurons were cultured continually until the eleventh day. All three groups were incubated in a 37 °C incubator with 5% CO_2 for 5 days, with the cell culture medium changed every second day.

2.5 Immunofluorescence Analysis

Cell cultures were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes each, incubated with 5% bovine serum albumin (BSA) for 1 hour, and then stained overnight at 4 °C with mouse anti-neuron-specific enolase (anti-NSE; ab218388, Abcam, Waltham, MA, USA) or rabbit anti-growth-associated protein 43 (anti-GAP-43; AF0150, Beyotime Institute of Biotechnology). Subsequently, the cells were washed with PBS and incubated for 30 minutes at room temperature with Alexa Fluor 488/594-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). After further washes with PBS and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) solution, the cells were placed onto microslides and mounted with Dako Fluorescent Mounting Medium (Dako, Glostrup, Denmark). Cells were visualized and microphotographed using an FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

2.6 HIE Rat Model and Animal Care

The HIE model was established based on previous studies by clipping the bilateral uterine arteries for 5 minutes and delaying caesarean delivery [15,19]. Pregnant Sprague-Dawley rats (21 days gestation) underwent general anaesthesia using 3% isoflurane in a 30% O_2/70% N_2 atmosphere. Once unconscious, each rat was placed in the supine position on the operating table with monitoring of vital signs. An incision was made along the midline of the abdomen, and the superficialis was isolated to expose the uterus. Uterine ischaemia was induced by blocking four uterine arteries with 30 g aneurysm clips for 5 minutes. A Caesarean section was then performed after removing the clips and recovering the blood supply. The newborn pups were carefully rescued and raised manually.

2.7 PD-MSC Transplantation

The newborn pups were randomly assigned to six groups (9 pups per group) as follows: control group, HIE model group, HIE + normal saline (NS) group, and three HIE + PD-MSC treatment groups (transplantation at days 7, 14 and 28 postpartum, giving the P7, P14 and P28 groups, respectively). Five microlitres of PD-MSC cell suspension (5×10^5 cells/μL) was transplanted through the jugular vein (injection speed of 1 μL/min) in the P7, P14 and P28 groups. Pups from the control and HIE + NS groups received the same dose of saline injection on postpartum day 14.

2.8 Neurological Evaluation

Rats were evaluated for neurological function at 56–60 days postpartum.

2.8.1 Rotarod Test

The rotarod test was conducted to evaluate motor performance. The rats were placed on a rotarod apparatus (ZH-ZQ, Zhenghua, Huaibei, Anhui, China) with a starting speed of 0 rpm and accelerating 2 rpm every 5 seconds until reaching 45 rpm. The time that each rat spent on the rotary was recorded. Rats that remained on the apparatus after 60 seconds were given a score of 60 seconds.

2.8.2 Traction Test

The traction test was performed to evaluate muscle strength and equilibrium. The front paws of the rats were placed on a straight rope hung in the air. Scoring was determined as follows: if both paws held on, the score was 3 points; if one paw held on, the score was 2 points, and if neither paw held on the score was 0 points. The test was repeated three times, with a 3 min rest between trials. The average score was calculated for data analysis.

2.8.3 Morris Water Maze Test

The Morris water maze (MWM) test was performed to evaluate the spatial learning and memory of rats. The rats were trained to find a platform in a large open circular tank filled with tap water (150 cm in diameter, 45 cm in height, and filled to a depth of 30 cm with water at 28 °C). A 10 cm diameter white platform was placed in the center of target quadrants of the pool. On day 1, the rats were trained to find the platform 0.5 cm above the water. Rats were then trained for four more days to find the submerged platform placed 0.5 cm below the surface of opaque water. Each rat performed four consecutive trials from a different quadrant each day, and with a 20-second interval between two trials. The rats were placed in the water between quadrants, facing the wall of the pool with a different location for each trial. If the rats failed to locate the platform within 90 seconds, they were gently guided onto the platform where they remained for 20 seconds. The platform position was kept unaltered during the training session. On day 6, the platform was removed, and each rat was allowed to explore the pool for 90 seconds. The mean escape latency time to locate the hidden platform in all four quadrants and the number of crossings of the platform were measured using an image analyzer (ZH0065, Zhenghua).

2.9 Histological Examination and Nissl Staining

After neurological evaluation, five rats from each group were chosen randomly and the brains collected under anaesthesia. Brain tissues were dehydrated in ethanol and embedded in paraffin blocks. Five-micrometer-thick slices were dewaxed in xylene, rehydrated in progressively lower concentrations of ethanol, and then fully stained with haematoxylin and eosin (H&E) (C0105M, Beyotime Institute of Biotechnology) or cresyl violet (G1430, Solarbio).

2.10 Western Blot Analysis

At 60 days after HIE injury, brain tissue homogenates were prepared for Western blot analysis according to the manufacturer's instructions. Briefly, brain tissue was added to a cold lysis buffer (P0013B, Beyotime Institute of Biotechnology), and the supernatants were collected and quantified using a BCA protein assay kit (P0011, Beyotime Institute of Biotechnology). Samples (20 µg) were separated on 10% polyacrylamide gels and transferred to Hybond-P membranes. Incubation of the membranes with a BSA solution (5%; ST025, Beyotime Institute of Biotechnology) at room temperature for 45 minutes was used to block non-specific binding. Primary antibodies against the following proteins were then added and incubated at room temperature for 45 minutes: B-cell lymphoma-2 (BCL-2; 12789-1-AP, Proteintech Group, Inc. Rosemont, IL, USA), BCL-2-associated X protein (BAX; AF0057, Beyotime Institute of Biotechnology), BCL-2-associated agonist of cell death (BAD; AF1009, Beyotime Institute of Biotechnology), neurofilament H/M (NF-H/M; 835403, BioLegend), microtubule-associated protein-2 (MAP-2; 822501, BioLegend), Caspase3 (AB3623, Sigma-Adlrich, St. Louis, MO, USA), and Caspase7 (AB256474, Abcam). GAPDH (AF1186, Beyotime Institute of Biotechnology) served as the loading control. The blots were then incubated with an HRP-conjugated goat anti-rabbit secondary antibody (A0208, Beyotime Institute of Biotechnology) for 45 minutes at room temperature, and subsequently analyzed using an enhanced chemiluminescence (ECL) kit (Beyotime Institute of Biotechnology). ImageJ (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/, v1.8.0) was employed to analyze the greyscale values.

2.11 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA kits were used to determine the concentrations of tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-10 and transforming growth factor (TGF)-β1 in brain tissue, as well as IL-10 and TGF-β1 in conditioned culture medium, according to the manufacturer's instructions (**Supplementary Table 1**, MLBio, Shanghai, China).

2.12 Statistical Analysis

Data are presented as the mean and standard deviation (mean $\pm$ standard deviation (SD)). One-way analysis of variance (ANOVA) was used for comparisons between groups. Statistical analysis was performed using SPSS software version 22 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1 Characteristics of Human Placenta-Derived MSCs

PD-MSC were derived from placental villous tissues procured from healthy women during labor. The cells exhibited a bipolar spindle-like shape and displayed a high capacity to adhere to plastic when maintained in standard

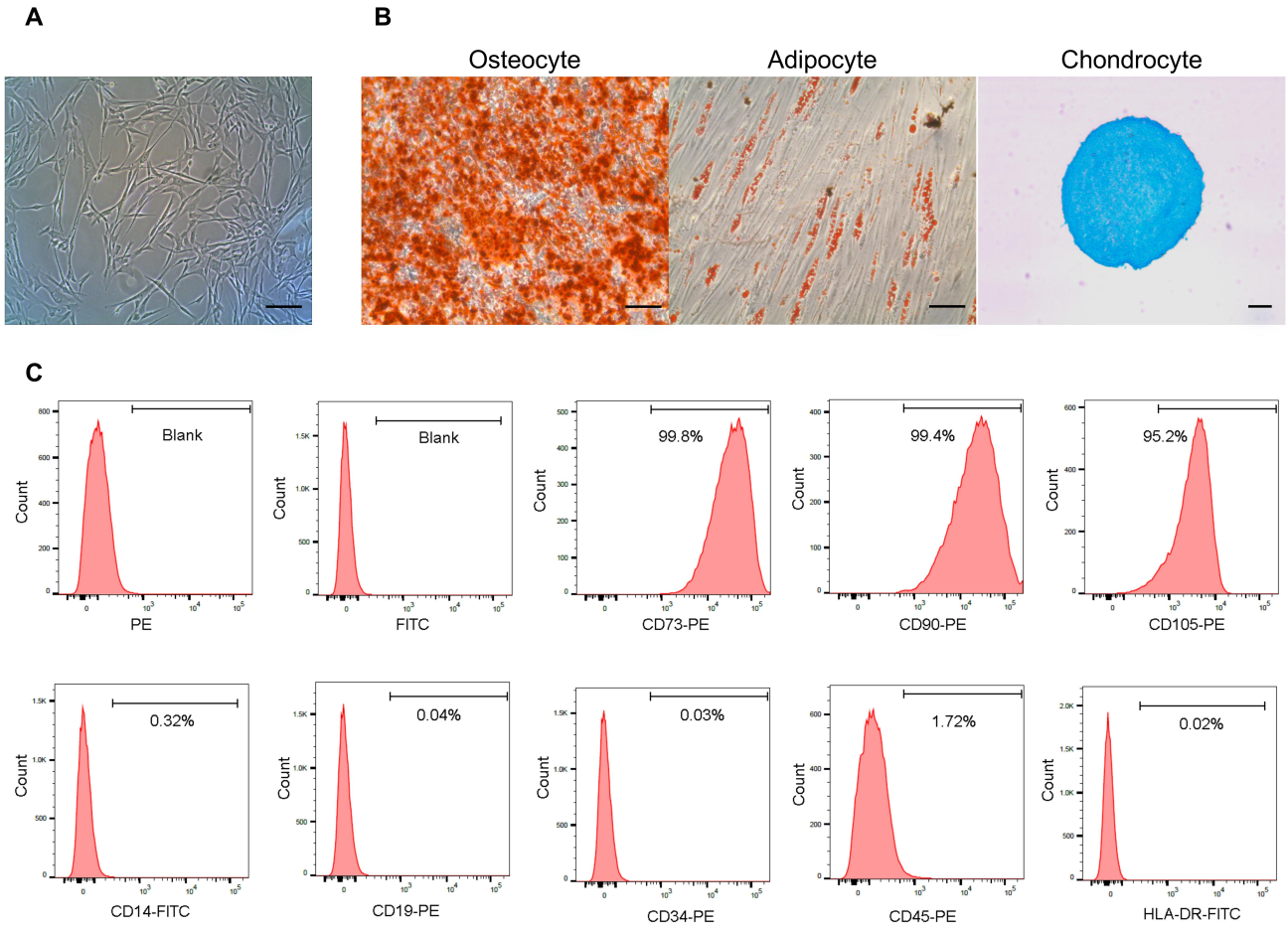

Fig. 1. Characteristics of human placenta-derived mesenchymal stem cells. (A) Morphology of MSCs. (B) Differentiation of MSCs was observed after induction (original magnification × 200). Alizarin Red S staining of extracellular calcium deposits confirmed osteogenic differentiation; Oil-red-O-positive lipid droplets in the cytoplasm indicated adipocytic differentiation; Alcian Blue staining of aggrecan confirmed chondrogenic differentiation. (C) Flow cytometry results showing the immunophenotype of PD-MSCs. Scale bars = 50 µm. MSCs, mesenchymal stem cells; PD-MSCs, placenta-derived mesenchymal stem cells.

culture conditions using tissue culture flasks (Fig. 1A). PD-MSCs showed the capacity to differentiate into adipocytes, chondrocytes, and osteoblasts after being cultured in the appropriate induction conditions (Fig. 1B). Flow cytometry showed that PD-MSCs had positive expression of CD73 (99.8%), CD90 (99.4%) and CD105 (95.2%), and negative expression of the hematopoietic lineage markers CD14 (0.32%), CD19 (0.04%), CD34 (0.03%), CD45 (1.72%) and HLA-DR (0.02%). These differentiation abilities and the surface marker expression profile were in accordance with typical MSC standards (Fig. 1C).

3.2 PD-MSC Injection Improved Neurological Development and Function in an HIE Animal Model

We developed a new type of intrauterine HI rat model in order to study the therapeutic efficacy of PD-MSCs and the underlying mechanism of action. Rats in the HIE group had lower body weight than those in the control group. After treatment with PD-MSCs, the mean weight was higher in the P7 (266.62 ± 10.91 g), P14 (263.13 ± 10.94 g) and P28 (244.00 ± 10.61 g) groups compared to the HIE (241.89 ± 12.68 g) and HIE + normal saline (NS) groups (243.91 ± 12.06 g, Fig. 2A).

The neurological behaviors of motor coordination and balance in the HIE model were assessed using the rotarod and traction tests. The rotarod test revealed a prolonged mean latency time in the P7 (27.19 ± 12.50 s) and P14 (15.56 ± 8.06 s) groups after PD-MSC transplantation compared with the HIE (8.29 ± 5.27 s) and HIE + NS (9.24 ± 5.17 s) groups (Fig. 2B). A significant difference was observed between the P7 and the HIE groups ($p < 0.01$). The mean scores for the traction tests were higher in the three PD-MSC treatment groups than in the HIE and HIE + NS groups (Fig. 2C). The P7 group (2.81 ± 0.18) had a higher score than the P14 (2.56 ± 0.41) and P28 (2.11 ± 0.41) groups.

Morris water maze tests were performed to evaluate the capacity to recover learning memory function in the HIE model. No significant differences in escape latencies between each group were observed on the first day of the water

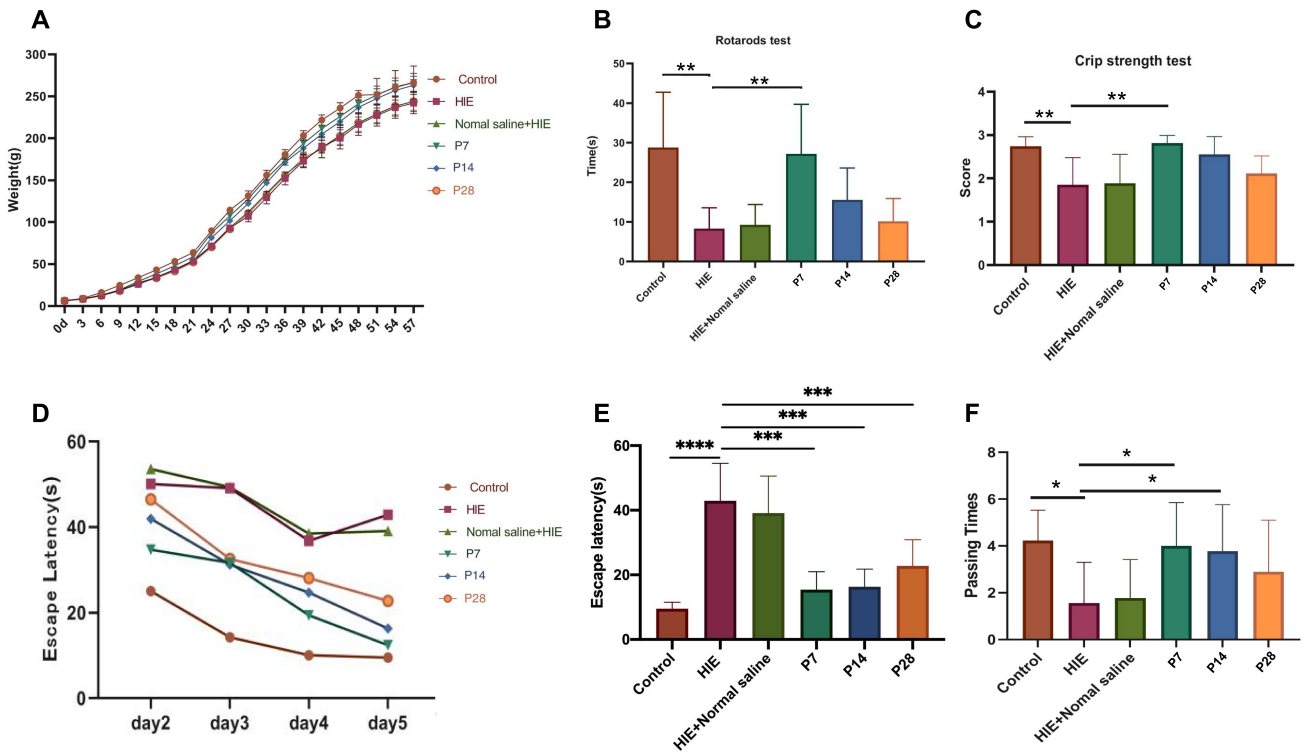

Fig. 2. Body weight and neurological evaluation in the HIE model. (A) Weight gain by the rats. At 56 days after PD-MSC injection, the mean body weight increased until it was almost equal to the control group. (B) Rotarod test. The significantly longer time spent on the rotarod indicated that PD-MSC treatment improved coordination after HIE injury. (C) Traction test. Significant improvement in muscle strength was observed after PD-MSC injection. (D) Escape latencies for each group from the second to the fifth day of the test. The escape latencies to locate the hidden platform gradually decreased in all three PD-MSC treatment groups. (E) The escape latencies for each group on the fifth day. The P7 group showed the best results compared to the P14 and P28 PD-MSC injection times. (F) Passing times of probe trains. The significant reduction in time spent locating the platform and the increased number of passages confirmed that PD-MSC injection improved learning memory function after HIE injury. Data are mean ± SD, n = 9. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. HIE, Hypoxic-ischaemic encephalopathy. SD, standard deviation.

maze test, suggesting that neither HIE injury nor PD-MSC injection impaired rat motility and vision. The test was then performed with the hidden platform from the second to fifth days, with the escape time becoming progressively shorter with increasing training time (Fig. 2D). Compared to the HIE group (42.90 ± 34.42 s), rats in the P7, P14 and P28 groups took significantly less time to find the platform on the fifth day (12.40 ± 16.64, 16.31 ± 16.07, 22.79 ± 23.86, respectively; $p < 0.01$, Fig. 2E). Probe trains performed on the sixth day revealed that the frequency of crossing the platform was significantly greater in the P7 (4.00 ± 1.85) and P14 (3.78 ± 1.99) groups (Fig. 2F), suggesting that PD-MSC injection improved HIE pup motor function and learning ability. Of the three PD-MSC treatment groups, the P7 group displayed the best recovery from HIE injury.

3.3 PD-MSC Transplantation Reduced Brain Damage in the HIE Animal Model

Histological examination and Nissl staining were carried out to evaluate the degree of brain tissue damage in the HIE model. The H&E results revealed that PD-MSC transplantation reduced histopathological damage to the brain tissue caused by HIE (Fig. 3A). Furthermore, Nissl staining showed frequent neuronal degeneration and loss in the cerebral cortex after HIE injury (Fig. 3B). PD-MSC transplantation alleviated brain injury, and cell counts showed an increased number of neurons in the PD-MSC treatment groups (Fig. 3C). Western blot analysis showed a low level of MAP-2 expression in the HIE group. However, PD-MSC injection on the 7th day after HIE injury (P7 group) increased the level to close to the normal value (Fig. 3D). The P7 group had more neurons and greater MAP-2 expression than both the P14 and P28 groups, suggesting the optimal treatment time was approximately the 7th day after HIE injury.

3.4 PD-MSC Transplantation Regulates the Expression of Apoptosis-Related Proteins and Inflammatory Factors in an HIE Animal Model

Since the number of normal neurons was notably higher in the three PD-MSC transplantation groups, we further evaluated the expression levels of apoptosis-related

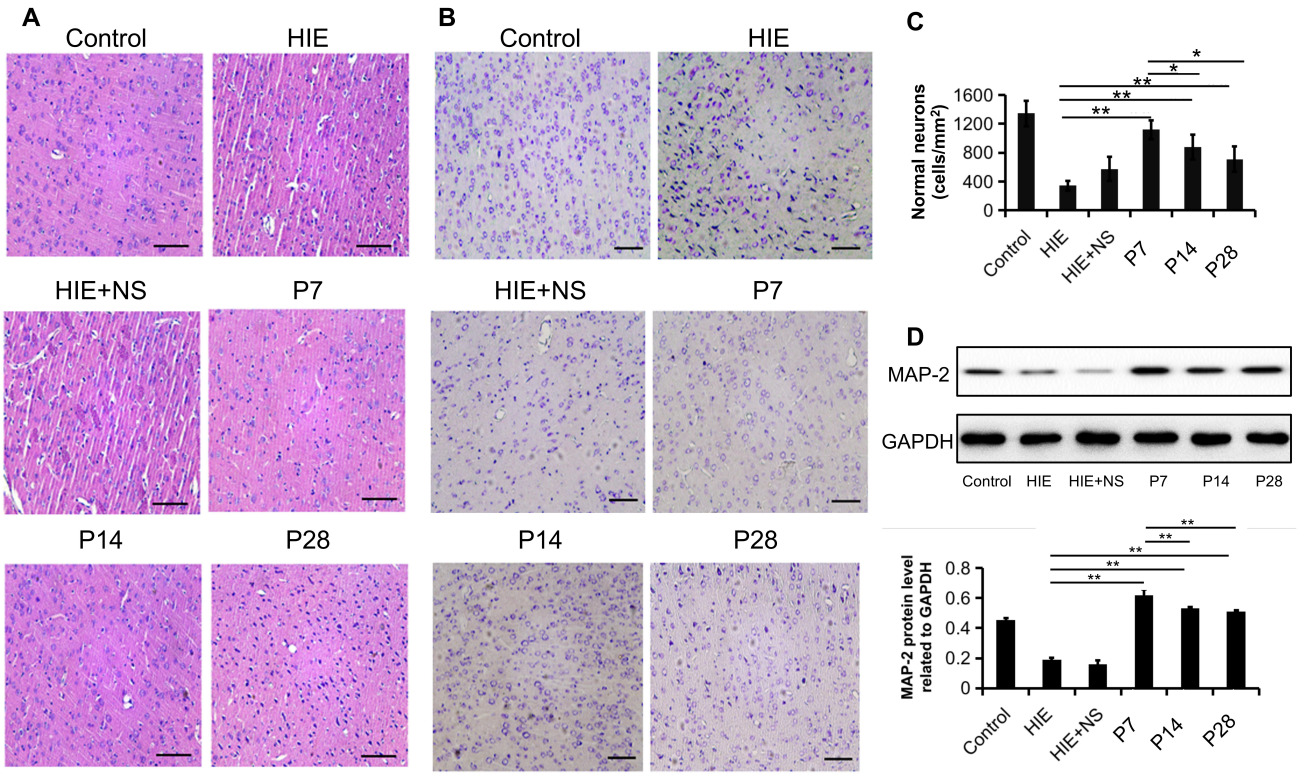

Fig. 3. Histopathological results and neuron counts. (A) H&E examination of histopathological damage in brain tissues. (B) Nissl staining was used to assess neurons 60 days after HIE damage. The number of Nissl bodies was reduced in the HIE group, with this trend reversed in the PD-MSC transplantation groups. Scale bars = 100 µm. (C) A significant increase in the number of normal neurons was found after PD-MSC transplantation, with the P7 group showing the best treatment effect. Data are mean ± SD, n = 5. (D) The MAP-2 expression level indicated that PD-MSC transplantation after HIE injury reduced brain damage compared with the HIE group. The P7 group had the highest expression of MAP-2. Results shown are mean ± SD, n = 5. * $p < 0.05$; ** $p < 0.01$. H&E, haematoxylin and eosin; MAP-2, microtubule-associated protein-2.

proteins in brain tissue, including BAX, BAD and BCL-2. Western blot analysis showed that BCL-2 expression was upregulated after PD-MSC transplantation, whereas BAD and BAX expression levels were downregulated (Fig. 4A). The BCL-2 expression level was the highest in the P7 group, suggesting that PD-MSCs showed the best anti-apoptosis effects on the 7th day after HIE injury.

To study the inflammation processes in the HIE model, the expression levels of anti-inflammatory cytokines were also examined. PD-MSC transplantation reduced production of the proinflammatory cytokines IL-1β and TNF-α compared to the HIE group (Fig. 4B,C and **Supplementary Document 1**). The anti-inflammatory factors IL-10 and TGF-β1 were expressed at low levels in the HIE group, but were upregulated after PD-MSC injection (Fig. 4D,E and **Supplementary Document 1**). The best moderating effects were observed when the PD-MSC were transplanted 7 days after HIE injury, which was determined to be the optimal time for treatment.

3.5 PD-MSCs Secrete Anti-Inflammatory and Anti-Apoptotic Cytokines in an in Vitro H/R Model

We next conducted flow cytometry with annexin V/propidium iodide (PI) to investigate the effects of PD-MSCs on the apoptosis of injured neurons. As shown in Fig. 5A, approximately 8% of neurons were apoptotic ($p < 0.05$) after co-culture with PD-MSCs, whereas the percentage apoptosis in the H/R model was approximately 20%. The reparative effect of PD-MSCs on the injured neurons was manifested after 24 hours of co-culture, and increased with prolonged culture time. Immunofluorescent staining showed shrinkage of the cell bodies and axons in the H/R model, while the co-culture group showed a dense neural fiber network and typical morphological characteristics (Fig. 5B).

Protein extracted from the brain tissue of HIE model animals was used to stimulate PD-MSCs. The results showed that protein stimulation significantly upregulated the expression levels of IL-10 and TGF-β1 in PD-MSCs ($p < 0.05$), which indirectly reflects the feedback regulatory effect of the HIE microenvironment on PD-MSCs (Fig. 6A,B and **Supplementary Document 1**). The ex-

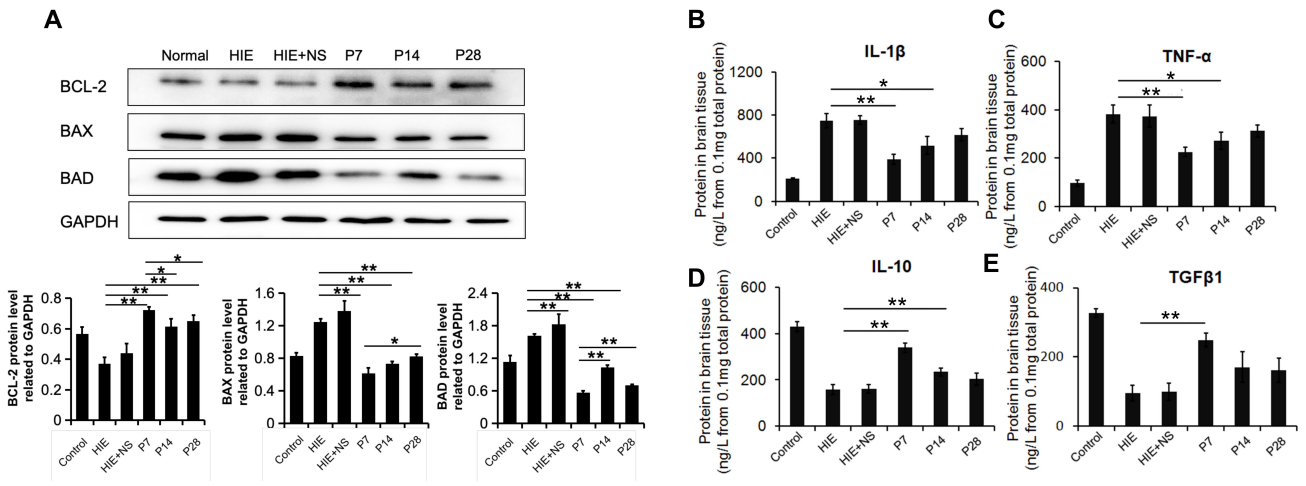

Fig. 4. Expression of apoptosis-related proteins and inflammatory factors. (A) The expression of apoptosis-related proteins as determined by Western blot assay. PD-MSC transplantation increased the level of BCL-2, which inhibits cell apoptosis. In contrast, PD-MSC transplantation decreased the levels of BAX and BAD, which promote apoptosis. GAPDH was used as a loading control. (B–E) Protein expression levels of IL-1β, TNF-α, IL-10 and TGF-β1 in brain tissue, as determined by ELISA. Results shown are mean ± SD, n = 5. * $p < 0.05$; ** $p < 0.01$. BAX, BCL-2-associated X protein; BAD, BCL-2-associated agonist of cell death; ELISA, Enzyme-linked Immunosorbent Assay.

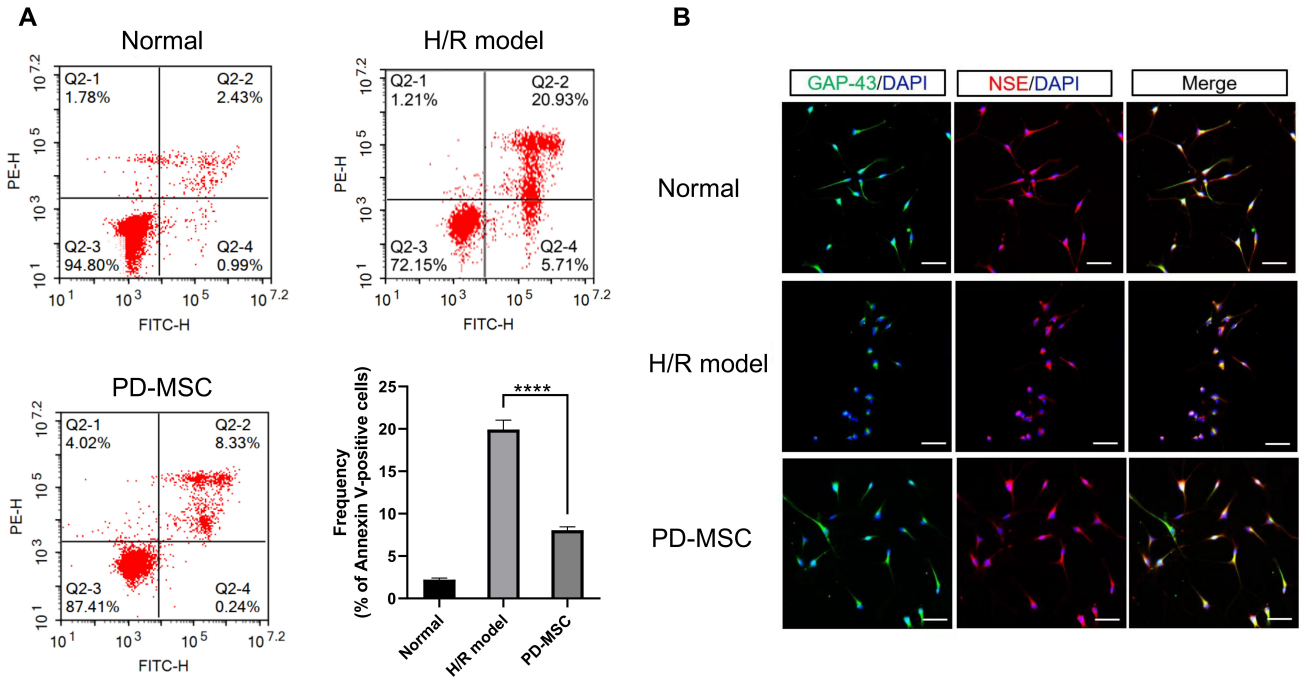

Fig. 5. Apoptosis was inhibited by PD-MSCs *in vitro*. (A) Annexin V-FITC/propidium iodide (PI) flow cytometry was performed to investigate neuronal apoptosis. Results are mean ± SD, n = 3. (B) Morphology of neurons. Immunofluorescence showing the cell bodies labelled with NSE (red), neurites labelled with GAP-43 (green), and nuclei stained with DAPI (blue). **** $p < 0.01$, Scale bars = 50 µm. NSE, neuron-specific enolase; GAP, growth-associated protein; DAPI, 4′,6-diamidino-2-phenylindole; H/R, hypoxic-reoxygenated.

pression levels of IL-10, TGF-β1, FGF2, Caspase3 and Caspase7 were also evaluated after co-culture of PD-MSCs with H/R neurons. ELISAs showed that the expression levels of IL-10, TGF-β1 and FGF2 were upregulated (Fig. 6C–E and **Supplementary Document 1**), while Western blotting showed decreased expression of Caspase3 and Caspase7 (Fig. 6F–H). These results suggest that the neuroprotective effects of PD-MSCs may be partly due to the secretion of anti-inflammatory and anti-apoptotic cytokines.

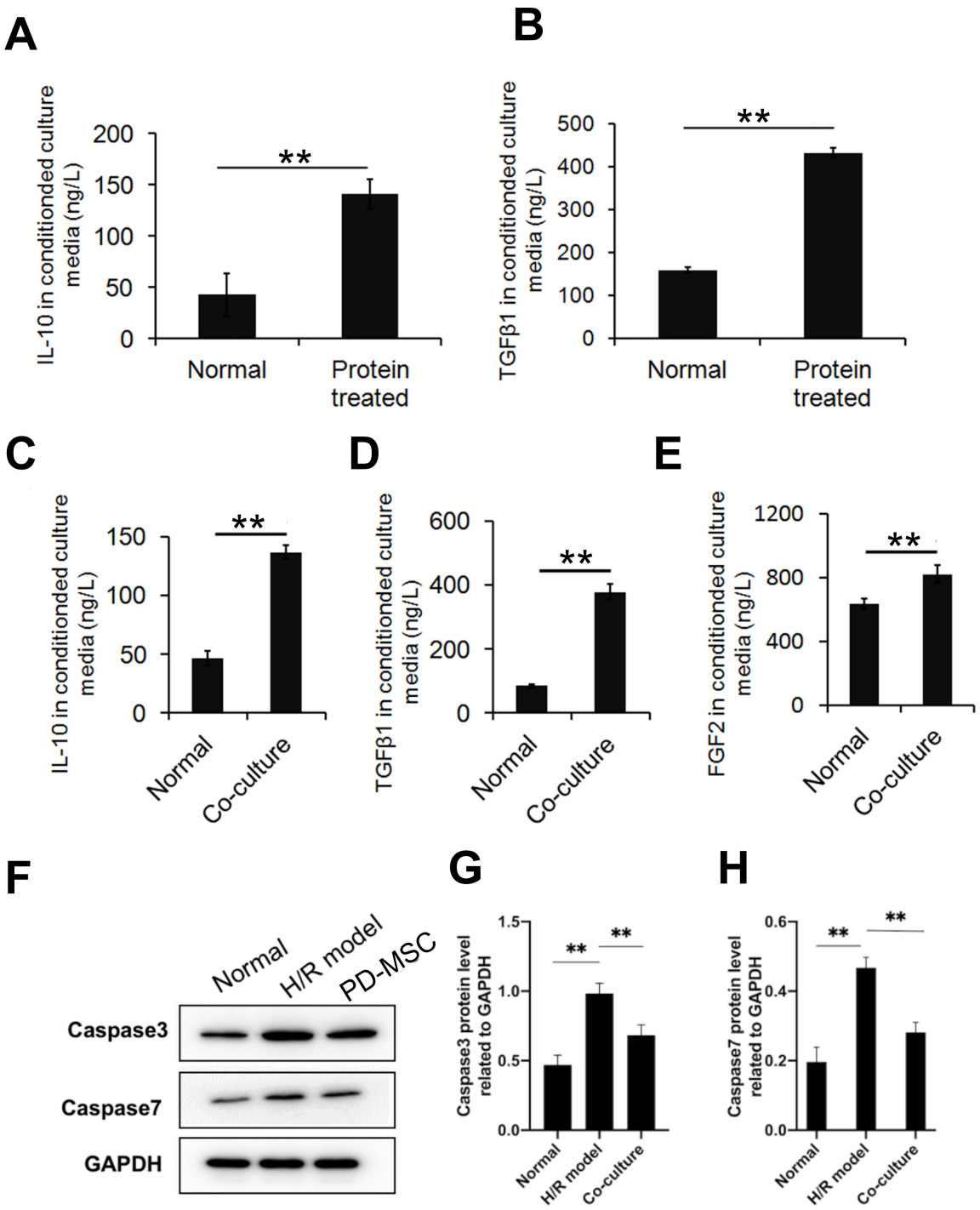

Fig. 6. Expression levels of apoptosis-related proteins and inflammatory factors *in vitro*. (A,B) Anti-inflammatory cytokines in PD-MSCs were upregulated after stimulation with proteins from the HIE model. Results shown are mean ± SD, n = 5. (C–E) The expression levels of IL-10, TGF-β1 and FGF2 were upregulated in the co-culture model. Results shown are mean ± SD, n = 5. (F–H) The expression levels of Caspase3 and Caspase7 were downregulated in the co-culture model. Results shown are mean ± SD, n = 3. ** $p < 0.01$.

4. Discussion

HIE is a type of neonatal encephalopathy caused by systemic hypoxaemia and/or reduced cerebral blood flow resulting from an acute peripartum or intrapartum event. Researchers usually use the common carotid artery block method to develop animal models of neonatal HIE brain injury. However, this model does not accurately reflect intrauterine HI injury caused by decreased placental perfusion or disruption in the delivery of oxygen and glucose [20,21]. Besides, the focal distribution of cerebral injuries

caused by carotid artery block is more similar to a neonatal stroke rather than the deeper and widespread damage in neonatal HIE. Therefore, we developed a new type of intrauterine HI rat model by clipping the bilateral uterine arteries, which better reflects the physiological process of HIE caused by intrauterine ischaemia and hypoxia. We then conducted human PD-MSC transplantation experiments using this model, which we believe more closely reflects intrauterine HI injury. The model allowed investigation of the neuroprotective efficacy and optimal treatment timing of PD-MSCs in newborn pups with HIE.

Our results showed that PD-MSC treatment for HIE could improve growth, ameliorate behavioral function, and repair brain damage. Rats in the HIE group were insufficiently groomed and had low motor activity. This could be ameliorated by PD-MSC transplantation, which significantly reduced neuron death and improved neurological functions including motor coordination, muscle strength, and learning and memory abilities. The optimal effects of PD-MSC transplantation were observed in the P7 group, in which the rats were almost identical to normal controls in terms of weight gain, recovery of brain damage and neurological function. Ding *et al*. [22] reported similar results in rats suffering from HI brain injury caused by common carotid artery block. Previous studies have suggested that MSC transplantation immediately after damage, and especially before the peak of the inflammatory response, could result in better therapeutic efficacy [23,24]. However, it is almost impossible to complete the transplantation of MSC within hours, unless these cells have been collected and prepared in advance. We found that PD-MSC transplantation 7 days after HIE insult had good therapeutic effect, and that early treatment gave better results than later transplantation. These results provide strong support for the clinical application of PD-MSC autologous reinfusion for HIE treatment.

The research focus for MSC therapeutic mechanisms has shifted from engraftment and differentiation into neurons, to multiple functions such as reduction of inflammation, inhibition of apoptosis, and decreased fibrosis [7,23,25]. Shortly after HIE damage, the primary phase of injury is caused by activation of excitotoxicity, oxidative stress, multiple programmed cell death pathways, and inflammatory reactions. Apoptosis has been described as an important mechanism in both the early and chronic phases of HIE [4]. To investigate the cellular mechanisms and potential therapeutic effects of PD-MSC transplantation for repairing damage caused by hypoxic-ischaemic injury to the neuron *in vitro*, we established the H/R cells model for further research on anti-inflammatory and anti-apoptotic effects of PD-MSC through paracrine pathway. In the present study, PD-MSCs were found to suppress the inflammatory response by upregulating anti-inflammatory proteins such as IL-1β and TNF-α, and downregulating pro-inflammatory proteins such as IL-10 and TGF-β1. This had the effect of preventing secondary neurological damage both *in vivo* (Fig. 4D,E and **Supplementary Document 1**) and *in vitro* (Fig. 6C–E and **Supplementary Document 1**). We also observed that PD-MSCs inhibited apoptosis in HIE rat and cell models. PD-MSCs exerted their anti-apoptosis effects by upregulating BCL-2 expression and downregulating BAX, BAD, Caspase3 and Caspase7 expression. MSCs can produce and release microvesicles and exosomes containing a variety of cytokines, growth factors, miRNAs, circRNAs, and mtDNA. These biologically active substances play important roles in the neuroprotective functions of MSCs [12,26–28]. Our results showed that intravenous injection was an effective way to transplant PD-MSCs, suggesting it may be a noninvasive and acceptable route for clinical use. *In vitro*, PD-MSCs could reduce neuron apoptosis and inflammation only through the exchange of culture medium. PD-MSCs administered via intravenous injection can barely pass through the blood-brain barrier, but still provide a neuroprotective effect. This indicates that the mechanism involving PD-MSCs is more likely to be the paracrine pathway and not the replacement of damaged neurons.

The satisfactory therapeutic effect achieved by PD-MSCs in this intrauterine HI model hints at their potential for treating neonatal HIE. To date, several clinical trials have reported that umbilical cord blood cells and umbilical cord tissue-derived MSCs are feasible and safe for HIE treatment [29–31]. Compared to MSCs obtained from adult fat or bone marrow, MSCs derived from birth-associated gestational tissues have better reparative effects and a faster rate of cell proliferation [23]. However, since umbilical cord blood coagulates within a few minutes of delivery, it is difficult to collect sufficient amounts for MSC preparation after completing neonatal rescue. Moreover, the small number of MSCs present in umbilical cord blood and umbilical cord tissue limits their clinical application. PD-MSCs have significant advantages over other MSCs in terms of cell number, quantity and ease of access. The placenta is stable and clinicians have sufficient time to preserve the tissue and prepare it for MSC extraction. MSCs extracted from the placenta for autologous reinfusion may also prevent rejection reactions, viral infections, and ethical concerns. Although the preparation of PD-MSCs for autologous reinfusion took several days after birth, our study found that therapy started 7 days after delivery was still effective. Considering the high safety and acceptance of PD-MSC autologous transplantation, it is worthwhile collecting and preparing PD-MSCs for the supportive treatment of HIE when HI injury is suspected during delivery. Autologous reinfusion therapy with PD-MSCs may thus be a new method to treat HIE.

5. Conclusions

In summary, human PD-MSC transplantation improves neurological function in rats with intrauterine hypoxic-ischaemic encephalopathy by reducing apoptosis and inflammatory reactions. PD-MSCs showed anti-

inflammatory and anti-apoptotic effects of PD-MSCs through paracrine pathway *in vitro*. Intravenous injection of PD-MSCs at 7, 14 and 28 days after HI damage in an intrauterine HI rat model was found to improve learning, memory and motor function by inhibiting apoptosis and inflammatory damage. PD-MSC transplantation at 7 days after HI damage showed the best effect of neuroprotection, which may be the optimal time of PD-MSC transplantation in the tertiary phase of HIE. These findings indicate the potential application of autologous reinfusion therapy with PD-MSCs for the treatment of HIE.

Abbreviations

BAD, BCL-2-associated agonist of cell death; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma-2; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; GAP-43, growth-associated protein-43; H&E, haematoxylin and eosin; HI, hypoxia-ischaemia; HIE, hypoxic-ischaemic encephalopathy; IL, interleukin; MAP-2, microtubule-associated protein-2; MSC, mesenchymal stem cells; NSE, neuron-specific enolase; PD-MSC, placenta-derived mesenchymal stem cells; ROS, reactive oxygen species; TGF, transforming growth factor; TNF, tumour necrosis factor.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

LWY, YQZ, YMZ, YZ and RLP designed the research study. LWY, YQZ, YYX, YMZ, FH, RLP and YMH performed the research. YMZ, LWY, RLP, JRC and YMH provided help and advice on the material or patients. LWY, YQZ, YYX, RLP, JRC, FH and YMH analyzed the data. LWY, YQZ, YYX, JRC and RLP wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The use of rats and the studies performed were carried out in accordance with the Public Health Service policy on the humane care and use of laboratory animals, and the protocol was approved by the Institutional Animal Care and Use Committee of Zhejiang Provincial People's Hospital (ZJPPHEC 2021020). The use of human placenta was approved ty the Ethics Committee of Zhejiang Provincial People's Hospital (ZJPPHEC 2020O055) and written informed consent was obtained from all subjects.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2904139.

References

[1] Millar LJ, Shi L, Hoerder-Suabedissen A, Molnár Z. Neonatal Hypoxia Ischaemia: Mechanisms, Models, and Therapeutic Challenges. Frontiers in Cellular Neuroscience. 2017; 11: 78.

[2] Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG. Cooling for newborns with hypoxic ischaemic encephalopathy. The Cochrane Database of Systematic Reviews. 2013; 2013: CD003311.

[3] Executive summary: Neonatal encephalopathy and neurologic outcome, second edition. Report of the American College of Obstetricians and Gynecologists' Task Force on Neonatal Encephalopathy. Obstetrics and Gynecology. 2014; 123: 896–901.

[4] Douglas-Escobar M, Weiss MD. Hypoxic-ischemic encephalopathy: a review for the clinician. JAMA Pediatrics. 2015; 169: 397–403.

[5] Tagin MA, Woolcott CG, Vincer MJ, Whyte RK, Stinson DA. Hypothermia for neonatal hypoxic ischemic encephalopathy: an updated systematic review and meta-analysis. Archives of Pediatrics & Adolescent Medicine. 2012; 166: 558–566.

[6] Queensland Clinical Guidelines. Supplement: Hypoxic-ischaemic encephalopathy (HIE). 2022. Available at: https://www.health.qld.gov.au/qcg (Accessed: 22 November 2022).

[7] Nair S, Rocha-Ferreira E, Fleiss B, Nijboer CH, Gressens P, Mallard C, *et al*. Neuroprotection offered by mesenchymal stem cells in perinatal brain injury: Role of mitochondria, inflammation, and reactive oxygen species. Journal of Neurochemistry. 2021; 158: 59–73.

[8] Choi JH, Seok J, Lim SM, Kim TH, Kim GJ. Microenvironmental changes induced by placenta-derived mesenchymal stem cells restore ovarian function in ovariectomized rats via activation of the PI3K-FOXO3 pathway. Stem Cell Research & Therapy. 2020; 11: 486.

[9] Ceccarelli S, Pontecorvi P, Anastasiadou E, Napoli C, Marchese C. Immunomodulatory Effect of Adipose-Derived Stem Cells: The Cutting Edge of Clinical Application. Frontiers in Cell and Developmental Biology. 2020; 8: 236.

[10] Anastasiadou E, Ceccarelli S, Messina E, Gerini G, Megiorni F, Pontecorvi P, *et al*. MiR-200c-3p maintains stemness and proliferative potential in adipose-derived stem cells by counteracting senescence mechanisms. PLoS ONE. 2021; 16: e0257070.

[11] Ding H, Zhang H, Ding H, Li D, Yi X, Ma X, *et al*. Trans-

plantation of placenta-derived mesenchymal stem cells reduces hypoxic-ischemic brain damage in rats by ameliorating the inflammatory response. Cellular & Molecular Immunology. 2017; 14: 693–701.

[12] Otani T, Ochiai D, Masuda H, Abe Y, Fukutake M, Matsumoto T, *et al*. The neurorestorative effect of human amniotic fluid stem cells on the chronic phase of neonatal hypoxic-ischemic encephalopathy in mice. Pediatric Research. 2019; 85: 97–104.

[13] Gonzales-Portillo GS, Reyes S, Aguirre D, Pabon MM, Borlongan CV. Stem cell therapy for neonatal hypoxic-ischemic encephalopathy. Frontiers in Neurology. 2014; 5: 147.

[14] Serrenho I, Rosado M, Dinis A, M Cardoso C, Grãos M, Manadas B, *et al*. Stem Cell Therapy for Neonatal Hypoxic-Ischemic Encephalopathy: A Systematic Review of Preclinical Studies. International Journal of Molecular Sciences. 2021; 22: 3142.

[15] Derrick M, Luo NL, Bregman JC, Jilling T, Ji X, Fisher K, *et al*. Preterm fetal hypoxia-ischemia causes hypertonia and motor deficits in the neonatal rabbit: a model for human cerebral palsy? The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2004; 24: 24–34.

[16] Luo Z, Zhang M, Niu X, Wu D, Tang J. Inhibition of the PI3K/Akt signaling pathway impedes the restoration of neurological function following hypoxic-ischemic brain damage in a neonatal rabbit model. Journal of Cellular Biochemistry. 2019; 120: 10175–10185.

[17] Semenov OV, Koestenbauer S, Riegel M, Zech N, Zimmermann R, Zisch AH, *et al*. Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of stemness after isolation. American Journal of Obstetrics and Gynecology. 2010; 202: 193.e1–193.e13.

[18] Peng Y, Xiong WC, Mei L. Culture of dissociated hippocampal neurons. Methods in Molecular Biology (Clifton, N.J.). 2013; 1018: 39–47.

[19] Diao M, Qu Y, Liu H, Ma Y, Lin X. Effect of carbamylated erythropoietin on neuronal apoptosis in fetal rats during intrauterine hypoxic-ischemic encephalopathy. Biological Research. 2019; 52: 28.

[20] Lyu H, Sun DM, Ng CP, Chen JF, He YZ, Lam SY, *et al*. A new Hypoxic Ischemic Encephalopathy model in neonatal rats. Heliyon. 2021; 7: e08646.

[21] Gotchac J, Cardoit L, Thoby-Brisson M, Brissaud O. A Rodent Model of Mild Neonatal Hypoxic Ischemic Encephalopathy. Frontiers in Neurology. 2021; 12: 637947.

[22] Ding HF, Zhang H, Ding HF, Li D, Yi XH, Gao XY, *et al*. Therapeutic effect of placenta-derived mesenchymal stem cells on hypoxic-ischemic brain damage in rats. World Journal of Pediatrics: WJP. 2015; 11: 74–82.

[23] Ahn SY, Park WS, Sung SI, Chang YS. Mesenchymal stem cell therapy for intractable neonatal disorders. Pediatrics and Neonatology. 2021; 62 Suppl 1: S16–S21.

[24] Chang YS, Choi SJ, Ahn SY, Sung DK, Sung SI, Yoo HS, *et al*. Timing of umbilical cord blood derived mesenchymal stem cells transplantation determines therapeutic efficacy in the neonatal hyperoxic lung injury. PLoS ONE. 2013; 8: e52419.

[25] Abe Y, Ochiai D, Sato Y, Otani T, Fukutake M, Ikenoue S, *et al*. Amniotic fluid stem cells as a novel strategy for the treatment of fetal and neonatal neurological diseases. Placenta. 2021; 104: 247–252.

[26] Yu Y, Yan Y, Luo Z, Luo P, Xiao N, Sun X, *et al*. Effects of human umbilical cord blood $CD34^+$ cell transplantation in neonatal hypoxic-ischemia rat model. Brain & Development. 2019; 41: 173–181.

[27] Zhou X, Gu J, Gu Y, He M, Bi Y, Chen J, *et al*. Human Umbilical Cord-Derived Mesenchymal Stem Cells Improve Learning and Memory Function in Hypoxic-Ischemic Brain-Damaged Rats via an IL-8-Mediated Secretion Mechanism Rather than Differentiation Pattern Induction. Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology. 2015; 35: 2383–2401.

[28] Joerger-Messerli MS, Oppliger B, Spinelli M, Thomi G, di Salvo I, Schneider P, *et al*. Extracellular Vesicles Derived from Wharton's Jelly Mesenchymal Stem Cells Prevent and Resolve Programmed Cell Death Mediated by Perinatal Hypoxia-Ischemia in Neuronal Cells. Cell Transplantation. 2018; 27: 168–180.

[29] Tsuji M, Sawada M, Watabe S, Sano H, Kanai M, Tanaka E, *et al*. Autologous cord blood cell therapy for neonatal hypoxic-ischaemic encephalopathy: a pilot study for feasibility and safety. Scientific Reports. 2020; 10: 4603.

[30] Cotten CM, Murtha AP, Goldberg RN, Grotegut CA, Smith PB, Goldstein RF, *et al*. Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. The Journal of Pediatrics. 2014; 164: 973–979.e1.

[31] Cotten CM, Fisher K, Kurtzberg J, Simmons R. Phase I Trial of Allogeneic Umbilical Cord Tissue-Derived Mesenchymal Stromal Cells in Neonates with Hypoxic-Ischemic Encephalopathy. Cytotherapy. 2020; 22: S192.