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Original article

Effects of human umbilical cord blood CD34$^+$ cell transplantation in neonatal hypoxic-ischemia rat model

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Abstract

Perinatal brain injury can cause death in the neonatal period and lifelong neurodevelopmental deficits. Stem cell transplantation had been proved to be effective approach to ameliorate neurological deficits after brain damage. In this study we examine the effect of human umbilical cord blood CD34$^+$ cells on model of neonatal rat hypoxic–ischemic brain damage and compared the neuroprotection of transplantation of CD34$^+$ cells to mononuclear cells from which CD34$^+$ cells isolated on neonatal hypoxic-ischemia rat model. Seven-day-old Sprague-Dawley rats were subjected to hypoxic-ischemic (HI) injury, CD34$^+$ cells (1.5×10^4 cells) or mononuclear cells (1.0×10^6 cells) were transplanted into mice by tail vein on the 7 day after HI. The transplantation of CD34$^+$ cells significantly improved motor function of rat, and reduced cerebral atrophy, inhibited the expression of glial fibrillary acidic protein (GFAP) and apoptosis-related genes: TNF-α, TNFR1, TNFR2, CD40, Fas, and decreased the activation of Nuclear factor kappa B (NF-κB) in damaged brain. CD34$^+$ cells treatment increased the expression of DCX and lectin in ipsilateral brain. Moreover, the transplantation of CD34$^+$ cells and MNCs which were obtained from the same amount of human umbilical cord blood had similar effects on HI. Our data demonstrated that transplantation of human umbilical cord blood CD34+ cells can ameliorate the neural functional defect and reduce apoptosis and promote nerve and vascular regeneration in rat brain after HI injury and the effects of transplantation of CD34+ cells were comparable to that of MNCs in neonatal hypoxic-ischemia rat model.

Keywords: Cell therapy; Hypoxic-ischemic; Umbilical cord blood; CD34$^+$ cells; Neuroprotection

1. Introduction

Perinatal hypoxic-ischemic (HI) brain injury is a major cause of death in the neonatal period and many surviving infants are disabled continually. The treatment for HI is limited to supporting intensive care at present. Therefore, it is necessary to find new approaches to reduce the neurologic injure of HI infants. There are

Abbreviations: MNCs, mononuclearcells; HI, hypoxia-ischemic; E-BST, elevated body swing test; GFAP, glial fibrillary acidic protein; DCX, Doublecortin

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many clues in animal studies showing that stem cell can be used to improve recovery from HI injury [1–3].

Stem cells derived from umbilical cord blood, bone marrow, and embryonic tissues have been used for brain damage repair research [4]. Human umbilical cord blood is available readily and it is a source of hematopoietic stem/progenitor cells, endothelial progenitors, and mesenchymal stem/progenitor cells. Thousands of transplantations have been carried out as a therapy for hematological disorders since the first successful umbilical cord transplantation was reported [5]. Besides being used for the treatment of hematological diseases, umbilical cord blood was proved to have a possible therapeutic effect on different models of CNS damage, including stroke [6], spinal cord injury [7] and cerebral palsy [8–10]. And clinical studies to evaluate the safety and feasibility of umbilical cord blood on cerebral palsy have been conducted [11].

$CD34^+$ cells are considered to be hematopoietic stem cell/endothelial progenitor cells. It is easier to complete the quality control and clinical standardization of homogeneous $CD34^+$ cells than inhomogeneous mononuclear cells. In the last few years, some animal studies which investigated the effects of human umbilical cord blood $CD34^+$ cells on animal models of brain damage are conducted [12,13]. These studies indicate that human umbilical cord blood $CD34^+$ cells can reduce inflammation and enhance angiogenesis and then promote neurogenesis after stroke. However, limited data about transplantation of human umbilical cord blood $CD34^+$ cells in neonatal hypoxic-ischemia rat model is available. In this research we mainly studied the therapeutic effect of human umbilical cord blood $CD34^+$ cells on the HI model and compared the effects of transplantation of $CD34^+$ cells with MNC on this brain damage model.

2. Experimental procedures

2.1. Preparation of cells

Human umbilical cord blood from full-term deliveries were obtained from the Women and Child Health Hospital of Hunan Province. The mothers gave their informed consent. After centrifugation at 400*g* for 30 min, the mononuclear cells were isolated by Ficoll density gradient. We then isolated human $CD34^+$ cells from mononuclear cells using a $CD34^+$ cell isolation kit (Miltenyi Biotec) by using magnetic cell sorting, according to the manufacturers protocol. The purity of $CD34^+$ cells was analyzed by flow cytometry using phycoerythin-conjugated (PE-conjugated) anti-34 antibody. Cells were re-suspended in bovine fetal serum with 10% DMSO, and frozen in liquid nitrogen until it is used.

2.2. Neonatal hypoxic-ischemic brain damage and cell transplantation

All animal experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines and approved protocols at Central South University. Postnatal 7(P7) Sprague-Dawley rat pups had the right common carotid artery ligated [2] using 6–0 silk suture under isoflurane anesthesia. After 2-h recovery, pups were placed in a hypoxia chamber with a flow of O_2 (8%) and N_2 (92%) for 2.5 h. The hypoxia chamber was kept in a temperature-controlled water bath, which to maintain the temperature inside the chamber at 37 °C [14]. Sham controls underwent anesthesia and incision only. The pups were returned to their dams, and the litter size was adjusted to 6–8 pups per dam. 1×10^6 MNCs or 1.5×10^4 $CD34^+$ cells which was sorted from 1×10^6 MNCs suspended in 10 μl 0.9% saline were transplanted into mice through cervical vein injection on day 7 following hypoxic-ischemic (HI).

2.3. Behavioral testing

The HI-injured rats were tested by elevated body swing test (EBST) at days 14 post-transplantation. The EBST provided a motor asymmetry parameter and involved in handling the animal by its tail and recorded the direction of the biased body swings [15]. The EBST consists of 20 trials with numbers of swings ipsilateral and contralateral to the ischemic hemisphere recorded and expressed in percentage to determine the biased swing activity.

2.4. Histological and immunohistochemical analysis

Animal were anesthetized by isoflurane on 21th day after hypoxic-ischemic (HI) and then fixed by perfusion with a solution of 4% paraformaldehyde in 0.1 M phosphate-buffered saline through the heart. Brains were removed and further fixed in 4% PFA (Sigma) at 4 °C for 24 h before they were sunk through 30% sucrose for cryoprotection.

Brains were sectioned coronally at 10 mm with cryomicrotomy. The primary antibodies were diluted 1:1000 in PBS-T for both: mouse anti-GFAP (Millipore #AB5804) and mouse anti-NFκB, P65 subunit (Clone 12H11, Millipore #MAB3026), and 1:100 for goat anti-Dcx (Clone C-18, Santa cruz biotechnology #SC-8066). After overnight incubation, primary antibody staining was revealed using fluorescence-conjugated secondary antibodies. The secondary antibodies were diluted 1:1000 for both Goat anti-Rabbit IgG (Santa cruz biotechnology #SC-2012) and Goat anti-Mouse IgG (Santa cruz biotechnology #SC-3738).

2.5. Real-time reverse-transcription polymerase chain reaction analysis

All primer sequences were determined by using established rat GenBank sequences [7]. Tissues of brains of ischemic areas were collected on 21th day after HI. RNAs were extracted with Trizol (Invitrogen) and RNA quality was determined by running a sample with RNA loading dye on a 1% agarose gel and checking for distinct 18S and 28S rRNA bands, indicating a lack of degradation. Total RNAs were reverse transcribed into first-strand cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Relative quantification by real-time polymerase chain reaction was performed (ABI Prism 5700 Sequence Detection System; Perkin Elmer). Real-time PCR was carried out under the following conditions: one cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Values were normalized to GAPDH gene transcript levels. The primers we used are shown in Table 1.

2.6. Western blotting analysis

We collected brain tissues nearby ischemic areas on 21th day after HI. Nucleoprotein was extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo scientific#78833) according to the manufacturers' protocol. Nucleoprotein was separated with SDS-denaturing electrophoresis in polyacrilamide gel under non-reducing conditions according to standard procedures. Separated proteins were transferred to a PVDF membrane (Millipore) with subsequent staining by monoclonal rabbit anti-rat antibodies against p65(clone 12H11, Millipore #MAB3026) diluted 1:500 in PBS-T or p50 (clone E-10, Santa cruz biotechnology #sc-8414) diluted 1:100 in PBS-T overnight at 4 °C and with secondary polyclonal HRP-conjugated goat anti rabbit IgG antibodies (Santa cruz biotechnology #sc-2004) for 1 h at room temperature. Visualization was performed by using horseradish peroxidase substrate.

Table 1
The primers used for qRT-PCR analysis of the genes.

TNF-α	Sense	5′CCGAGATGTGGAACTGGCAGA3′
	Antisense	5′CCCATTTGGGAACTTCTCCT3′
TNFR1	Sense	5′GCTACCTGAGTGAGACGCATTT3′
	Antisense	5′CTGGAGGTAGGCACAGCTT3′
TNFR2	Sense	5′CACACATCCCTGTGTCCT3′
	Antisense	5′GGGCTTCTTTTTCCTCTG3′
CD40	Sense	5′CGAACTCAATCAAGGGCTTC3′
	Antisense	5′GCAGGGTTGGCAGACAGTAT3′
Fas	Sense	5′CTGGAATCCCAAGTCCTGAA3′
	Antisense	5′CATAGGTGGCAGGCTCTCT3′

qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; TNF-α, tumor necrosis factor-α, TNFR1, tumor necrosis factor receptor 1; TNFR2, tumor necrosis factor receptor 2.

2.7. Measurement of cerebral blood flow

Experimental rats were positioned in a stereotaxic frame and cerebral cortical blood flow was measured with a laser doppler perfusion imaging (LDPI; Moore Instruments, Devon, UK) in an anesthetized state (chloral hydrate). The animal's skull was exposed by a midline scalp incision and the skull surface was illuminated continuously by a 780 nm laser light. The penetration depth of the laser is approximately 500 μm. The light was filtered and detected by a charge coupled device (CCD) camera positioned above the animal's head. Color-coded blood perfusion images were obtained.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software). Numeric continuous data were analyzed using one-way analysis of variance (ANOVA) or unpaired *t*-test. Kruskal-Wallis analysis of variance were used for nonparametric distribution data. $P < 0.05$ was considered statistically significant, and all probabilities were 2-tailed.

3. Results

3.1. $CD34^+$ cells transplantation after HI reduces nerve damage

$CD34^+$ cells were isolated from mononuclear cells using magnetic cell sorting. The purity of $CD34^+$ cells was >90%. Seven days post HI, the rats were tested by brain tissue' anatomical structure analysis. In the ipsilateral hemisphere, there existed extensive cortical and striatal atrophy before cell transplantation (Fig. 1A). And the rats that underwent HI had significant brain injury in the ipsilateral hemisphere at 21-day post HI. $CD34^+$ cells transplantation could slightly decreased ipsilateral atrophy ($p > 0.05$) (Fig. 1B). In order to evaluate the functional outcomes of $CD34^+$ cells treatment on HI injury pups, we monitored the body weight which is an indicator of general health of HI rats. Pups body weight was reduced after HI, and $CD34^+$ cells treatment increase the body weight significantly ($P < 0.05$) (Fig. 1C). To detect neurological function, the elevated body swing test (EBST) was conduct. Fourteen days after cells injection, the EBST outcome of $CD34^+$ cells-treated animals have better performance than control rats significantly ($p < 0.05$) (Fig. 1D).

3.2. Cells transplantation after HI reduced the activation of astrocyte

Astrocytes broadly distributed throughout the human central nervous system (CNS), playing a key role

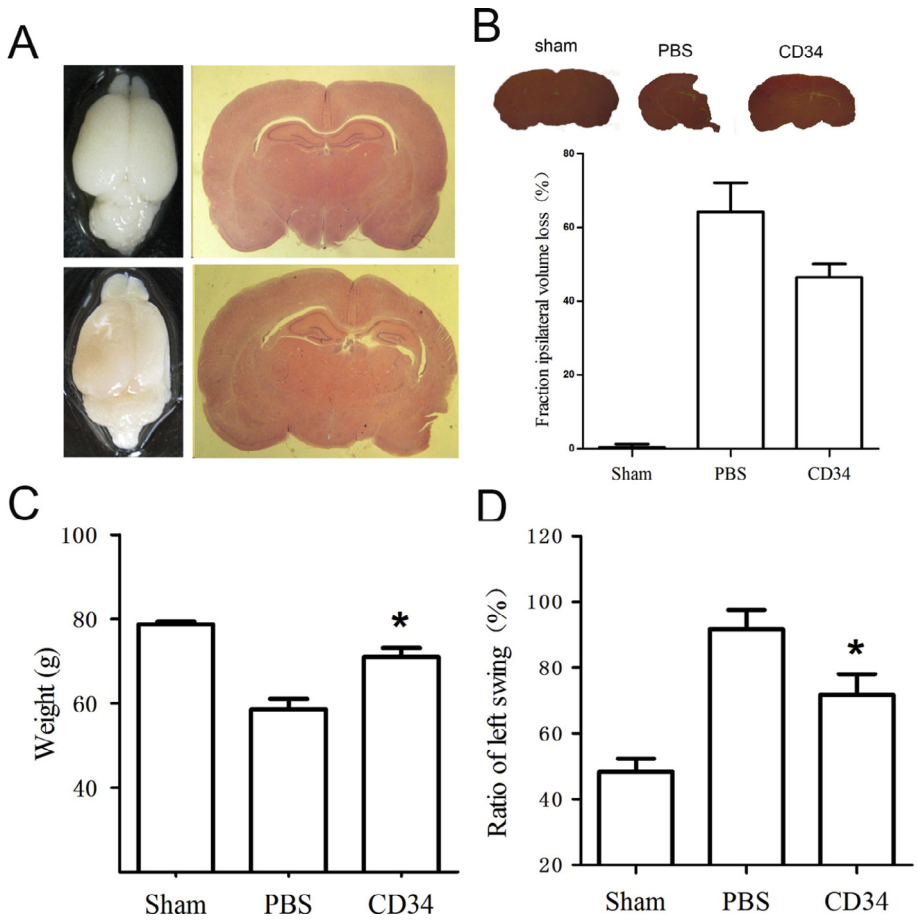

Fig. 1. HI caused newborn rat brain damage which was inhibited after cell therapy. (A) 7 days after HI injury, ipsilateral hemisphere emerged necrosis atrophy and contralateral hemisphere was intact. (B) Representative brain tissues and sections show effect of mononuclear cells (MNCs) and $CD34^+$ cells transplantation on lesion size. Quantification of damage using hematoxylin and eosin (HE) staining ($^*P < 0.05$ versus PBS control, $n = 3$). $CD34^+$ cells significantly inhibit the loss of weight after HI, and there is no obvious difference between the body weight of the two cell-treatment groups (C). $CD34^+$ cells transplantation can reduce the ratio of left swing which was abnormally elevated after HI damage (D). (*: $P < 0.05$ versus PBS, $n = 3$–5).

in keeping neuronal homeostasis and regulating synaptic plasticity. Reactive astrocytes existing close to the injury site are responsible for the glial scar formation. HI damage increased the expression of GFAP which is thought to be a marker of astrocyte activation in ipsilateral hemisphere. Fourteen days after cell transplantation, the expression of GFAP in $CD34^+$ cell treated rat's brain was lower than control (Fig. 2).

3.3. Cells treatment down-regulated NF-kB after HI

NF-κB is a transcription factor and can regulate expression of genes involved in inflammation, cell survival, and apoptosis. To measure the activity of NF-κB, we detected nuclear translocation of p65 subunit. P65 immunofluorence staining showed that there was more NF-κB protein in the nuclear of brain tissues after HI. $CD34^+$ cells treatment prevented the entry of NF-κB to nuclear (Fig. 3).

TNF-α is a target of NF-κB transcription factor and participate in regulating apoptosis. HI induced the transcription of TNF-α. 14 days after cell transplantation, the delivery of $CD34^+$ cells significantly decreased the expression of apoptotic relative genes TNF-α, TNFR1, TNFR2, Fas, and CD40 ($P < 0.05$) (Fig. 4).

3.4. $CD34^+$ cells had the similar effect to MNCs on the HI model

In order to understand whether the effect of $CD34^+$ cells was similar to that of MSC from which the $CD34^+$ cells were obtained, we compared the therapeutic effect of transplantation of $CD34^+$ cells with MNC treatment. Transplantation of MNCs significantly decreased ipsilateral atrophy and there was no statistically significant difference between the $CD34^+$ cells and MNCs-treated rats (Fig. 5A). The expression of TNF-αin the brain tissue of group CD34 was similar

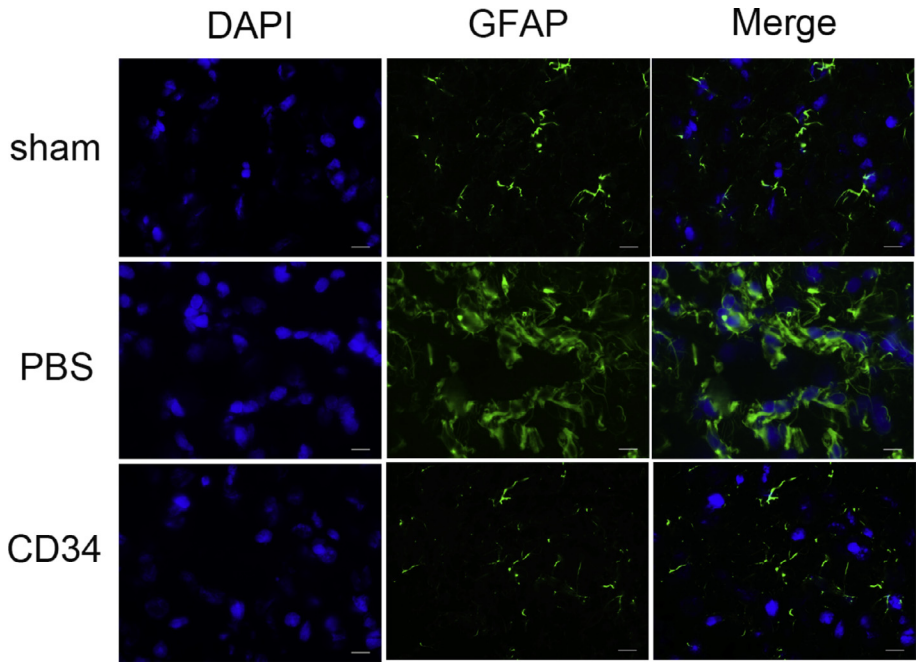

Fig. 2. Representative GFAP staining show CD34$^+$ cells transplantation reduced the expression of ipsilateral GFAP which was elevated after HI damage and inhibit the excessive activation of reactive astrocytes. Scale bar = 20 μm.

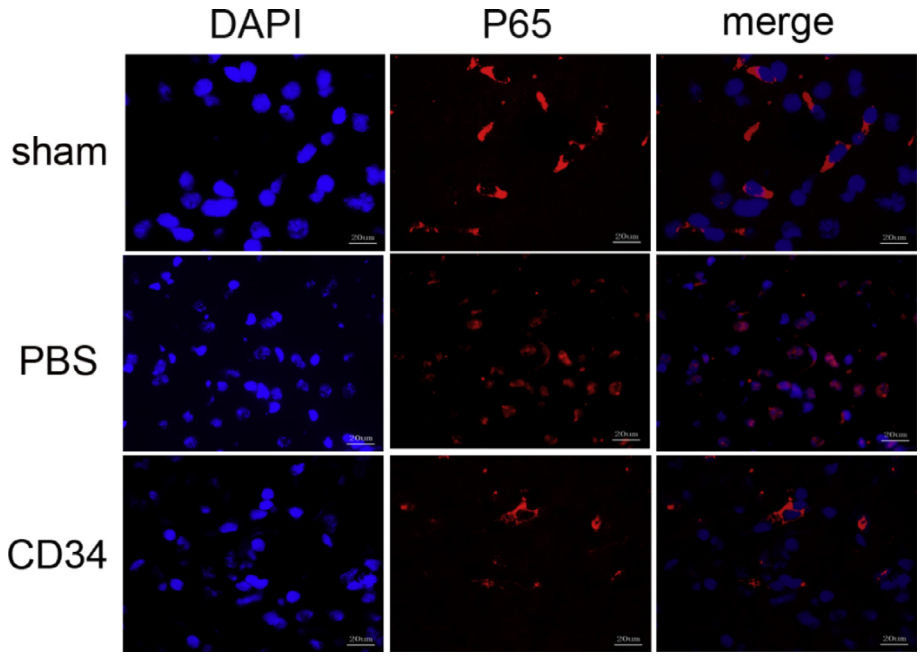

Fig. 3. CD34$^+$ cells treatment inhibited the HI-induced nuclear translocation of NF-κB. Immunofluorescence staining show that CD34$^+$ cells treatment reduce the expression of NF-κB (p65 subunit) in ipsilateral brain cell nucleus after HI. Scale bar = 20 μm.

to that of group MNC (Fig. 5B). Furthermore, we examined the levels of NF-κB protein (p50 and p65) in nucleus by western blot. High concentration of p50 and p65 were detected in the nucleus of brain post-HI. CD34$^+$ cells transplantation sharply reduced the concentration of p50 and p65 in nucleus and the ability of CD34$^+$ cells transplantation to inhibit the activation of NF-κB was comparable to that of MNCs. (Fig. 5C). To examine transplantation of CD34$^+$ cells could promote the regeneration of nerve cell in damaged region, we analyzed the expression of Doublecortin (DCX), a marker of immature or early neurons, in rats' brain. The results showed that both CD34$^+$ cells and MNCs treatment increased the expression of DCX (Fig. 5D).

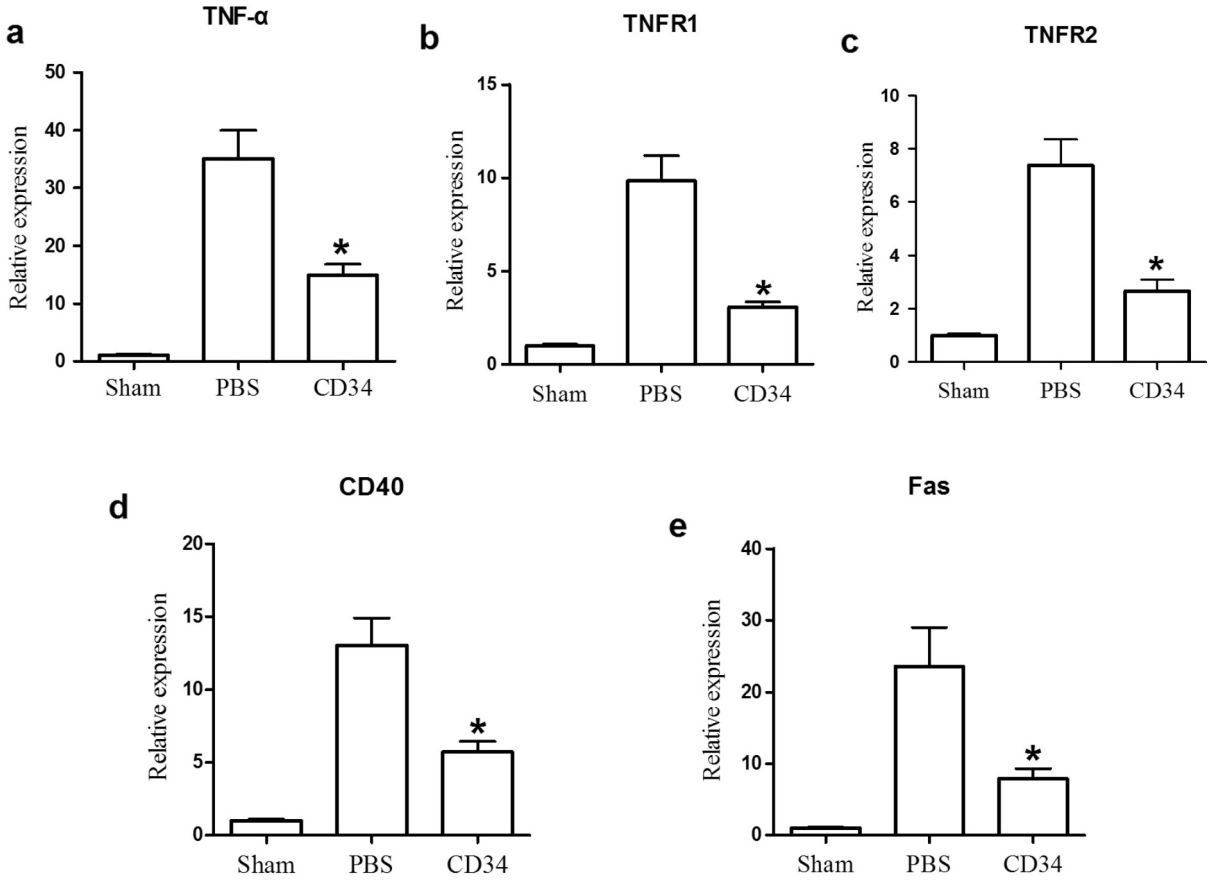

Fig. 4. Real-time polymerase chain reaction analysis. $CD34^+$ cells treatment significantly decreases the expression of relative apoptosis genes in brain after HI. Plots represent the relative expression of TNF-α (a), TNFR1 (b), TNFR2 (c), CD40 (d), Fas (e).

We also analyzed microvascular density in damaged brain tissues by lectin staining. As shown in Fig. 5 D, preservation of microvascular structures had been detected in brain of $CD34^+$ cells and MNCs treatment rats. The blood perfusion detection of brain by LDPI showed that $CD34^+$ cells and MNCs treatment have a little effect on recovery of blood perfusion, but there is no significant difference between the three groups of PBS, MNC and CD34 (Fig. 5E). These fingings suggested that the effect of transplantation of $CD34^+$ cells was comparable to that of MNCs on HI.

3.5. Absence of detectable human derived-cells in HI brain

In order to further study whether the transplanted cells migrated into the damaged brain tissue, we detect the human cells grafts in the rat brain tissues by human nuclei monoclonal antibody. But none human derived-cells are detected in both MNC and CD34 group animal brains in our study (data not shown).

4. Discussion

$CD34^+$ cells represent the majority of umbilical cord blood stem cells. The purified stem/progenitor cells are homogeneous, and it is easier to complete the quality control and clinical standardization of $CD34^+$ cells than inhomogeneous mononuclear cells. In addition, protocols using extracellar matrix components and 3D cultures can promote a 50-fold expansion of $CD34^+$ cells [16]. But it is not clear whether the transplantation of $CD34^+$ cells isolated from MNCs have the similar therapeutic effect to MNCs treatment. In this study, we compared the efficacy of $CD34^+$ cells and MNCs which were acquired from the same amount of human umbilical cord blood on the model of neonatal HI injury. Our results demonstrated that the effects of $CD34^+$ cells on HI injury brain comparable with those of MNCs, which increased the possibility that homogeneous $CD34^+$ cells administration may be a alternative to clinical therapy for cerebral palsy patients.

Postnatal 7(P7) Sprague-Dawley rat pups were chosen to undergo HI injury, because P7 rat is the age of peak brain growth, which occurs at term in humans [17]. The production of model is based on Levine and Rice-Vannucci model, which was unilateral permanent ligation of left commom carotid artry and subsequent exposure to hypoxic condition. There are many other models have been applied including hypoxia-only models and inflammatory models and so on [18]. These

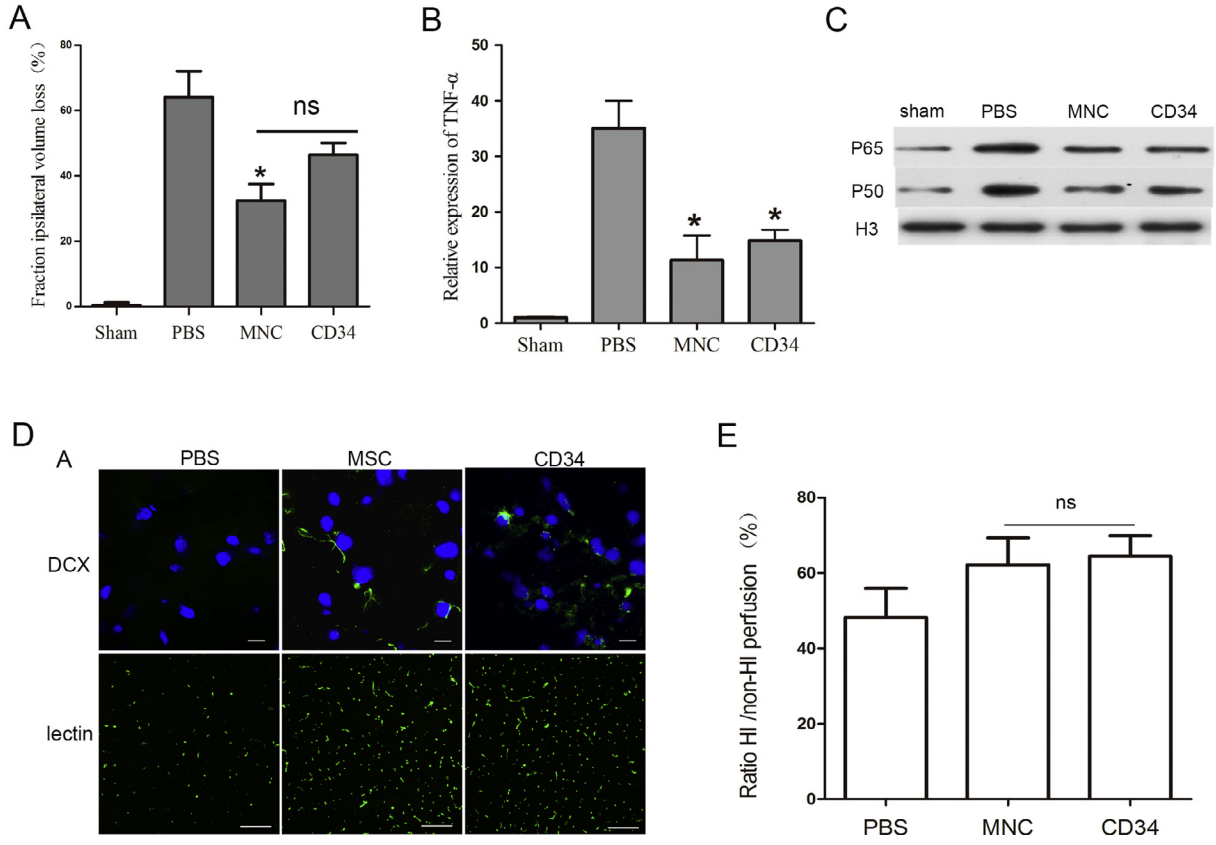

Fig. 5. Comparison of the neuroprotection between $CD34^+$ cells and MNCs transplantation in HI. $CD34^+$ cells and MNCs significantly decreased ipsilateral atrophy (A) and expression of apoptotic relative genes (B), and there were no statistical difference between two cell-treatment groups. (C) Western blot analysis. Nuclear protein from ipsilateral brain tissue was isolated and the concentration of p50 and p65 were detected. $CD34^+$ cells and MNCs treatment reduce NF-κB transferring into the nucleus. Immunofluorescence analysis. (D, up) MNCs and $CD34^+$ cells treatment enhance expression of DCX which is the mark of neurogenesis in brain. Scale bar = 20 μm. (D, down) There are more dense blood vessels in the brain of $CD34^+$ cells and MNCs treatment groups suggest that both of MNCs and $CD34^+$ cells transplantation promoted angiogenesis. Scale bar = 200 μm. Result of LDPI (E) shows that $CD34^+$ cells and MNCs treatment have a similar effect on cerebral blood flow of ipsilateral brain recovery. And there is no significant difference of ipsilateral/nonipsilateral perfusion between three groups.

animal models try to replicate the clinical symptom of cerebral palsy, but the etiologies of cerebral palsy are complex and often multifactorial, involving placental abnormalities, intrauterine growth restriction, preeclampsia, maternal infections, circulation disorders and perinatal asphyxia [4]. Preclinical study on pathophysiological mechanisms of neonate HI models uncovered the potential mechanisms of brain injury and searched therapeutic approaches which are safe and effective in neonatal humans. Once cerebral ischemia occur, oxygen and glucose are depleted which eventually causing neuron necrosis and gliosis. Previous studies suggested that the effect of stem cell transplantation was achieved by replacing necrotic cells and improve functional recovery after stroke [19]. But now there is a growing awareness that exogenous stem cells protect nerve function through releasing soluble factors and enhancing neurocyte survival [20–22]. Previous study indicated that systemically delivered human umbilical cord blood cells cannot cross the blood brain barrier (BBB) and entry into the central nervous system after acute stroke [23]. After intravenously delivered of HUCB with Mannitol, the neural growth factor, glial cell line-derived neurotrophic factor, and brain-derived neurotrophic factor were increased through a paracrine effect and only few cells detected in the damaged area suggesting neurotropic factor rather than cell replacement contributed to neural restoration [24]. Our result reveal that neuroprotection after systemically delivery of stem cells was independent on their entry into the central nervous system. We detected neither $CD34^+$ cells nor MNCs in the brain of rat 14 days after cell injection, indicating that the stem cells act without replacing the damaged nervous system. The results are analogous to recently reported observations that HUCB do not exert their effect by engraftment in the brain [6,23].

After stem cell transplantation, we analyzed the expression profiles of apoptotic relative genes at 21 days post-HI, finding efficient down-regulation of these genes after $CD34^+$ cells injection, which were similar to MNCs

transplantation. NF-κB is a ubiquitously expressed transcription factor that regulates expression of genes involved in inflammation, cell survival, and apoptosis. NF-κB activation induces production of proinflammatory cytokines, a pathway proposed to promote neuronal death in vivo. And inhibition of NF-κB has strong neuroprotective effects after neonatal Hypoxia-Ischemia [25,26]. In our study, we observed that the activation of NF-κB in the damaged brain, entering the nucleus largely, was reduced by $CD34^+$ cells transplantation. GFAP is a regular marker of astrocyte, and the increased expression of GFAP demonstrate the proliferation of astrocyte [27]. The decline of GFAP in the cell-treat groups means that $CD34^+$ cells administration can effectively relieve gliosis. In addition, $CD34^+$ cells promoted neovascularization in the brain. And the angiogenesis is conducive to neuronal regeneration [13].

However, there are still some problems need to be solved before the optimized strategy using for clinic treatment. First, although brain atrophy and neurological function deficit were still preserve in the $CD34^+$ cell-treated rat as show in Figs. 1 and 5, suggesting that further research about injection does of stem cells is needed to enrich the neuroprotective function of $CD34^+$ cells. Previous report revealed that intravenous administration about 10 times more stem cells are required to have comparable therapeutic effects with intra- arterial injection [28], which indicated that optimal strategies are associated with route of cell transplantation in addition to cell does. Furthermore, the difference between animal models and clinical situation suggest that a lot of clinical trials need to do to verify previous results before clinical application.

In summary, this study demonstrated that $CD34^+$ cells derived from human umbilical cord blood could ameliorate the neural functional defect and reduce apoptosis and promote nerve and vascular regeneration in brain after HI injury and the effect of $CD34^+$ cell treatment was comparable to that of MNCs. $D34^+$ cell has better uniformity than MNC, and can easier to control the quality and clinical standardization. Furthermore, $CD34^+$ cells can be amplified in vitro culture, and mass production of these cells for clinical use is prospective. So that the pure $CD34^+$ cells from umbilical cord blood may be a viable therapeutic agent for the treatment of cerebral palsy.

Acknowledgments

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Conflict of interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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