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hUC-MSCs Exert a Neuroprotective Effect via Anti-apoptotic Mechanisms in a Neonatal HIE Rat Model

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Abstract

In this study, we investigated how human umbilical cord mesenchymal stem cells exerted a neuroprotective effect via anti-apoptotic mechanisms in a neonatal hypoxic-ischemic encephalopathy rat model. A total of 78 10-day old (P10) rats were used. After human umbilical cord mesenchymal stem cells were collected from human umbilical cords and amplified in culture, they were administered to rat subjects 1 h after induced hypoxic-ischemic encephalopathy treatment. The short-term (48 h) and long-term (28 day) outcomes were evaluated after human umbilical cord mesenchymal stem cells treatment using neurobehavioral function assessment. Triphenyltetrazolium chloride monohydrate staining was performed at 48 h. Beclin-2 and caspase-3 levels were evaluated with Western blot and real time polymerase chain reaction at 48 h. Human umbilical cord mesenchymal stem cells were collected and administrated to hypoxic-ischemic encephalopathy pups by intracerebroventricular injection. Hypoxic-ischemic encephalopathy typically induced significant delay in development and caused impairment in both cognitive and motor functions in rat subjects. Human umbilical cord mesenchymal stem cells were shown to ameliorate hypoxic-ischemic encephalopathy-induced damage and improve both cognitive and motor functions. Although hypoxic-ischemic encephalopathy induced significant expression of caspase-3 and Beclin-2, human umbilical cord mesenchymal stem cells decreased the expression of both of them. Human umbilical cord mesenchymal stem cells may serve as a potential treatment to ameliorate brain injury in hypoxic-ischemic encephalopathy patients.

Keywords

hUC-MSCs, hypoxic-ischemic encephalopathy (HIE), apoptosis, caspase-3, Beclin-2

Introduction

Hypoxic-ischemic encephalopathy (HIE) is a major cause of morbidity and mortality in infants[1]. It causes improper development of the immature brain and subsequently leads to long-term disabilities such as epilepsy, mental retardation, cerebral palsy, and behavioral difficulties[1]. Several studies demonstrated that HIE causes delayed cell death via oxidative stress, inflammation, apoptosis, and excitotoxicity[2–5].

Previous studies have shown the newborn brain is primed to respond to various traumas with the activation of apoptotic cascades, because cell death is a normal part of development in the central nervous system. Thus, there is a high expression of pro-apoptotic proteins in the developing brain[6]. As a result, mitochondrial dysfunction occurs and ultimately signals pathways of apoptosis[7,8]. Specifically, the release of cytochrome c by the mitochondria leads to activation of caspase-3[9]. Therefore, controlling apoptosis can ameliorate brain damage.

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Human umbilical cord mesenchymal stem cells (hUC-MSCs) can secrete a variety of cytokines and neurotrophic factors and promote nerve regeneration. However, the neuroprotective properties and mechanisms of hUC-MSCs have not yet been explored following HIE. Therefore, we sought to explore the effects of hUC-MSCs in a neonatal HIE rat model.

Materials and Methods

The study was approved by the Ethics Committee of Guizhou Medical University and Soochow University. Animals were cared for according to the guidelines provided by the committee. Sprague-Dawley rat mothers with litters of 10–12 pups produced a total of 78 10-day old unsexed Sprague-Dawley rat pups that were then utilized in this study. All animals were randomly assigned to groups using Microsoft Excel. All researchers performing animal testing were blinded to the treatment conditions.

HIE Model

HIE was performed as previously described[10,11]. An aseptic technique was used throughout the study. After induction of anesthesia, the neck of the rats was prepared and draped using standard sterile techniques. Next, a small midline incision on the anterior neck was made. Using gentle blunt dissection, the right carotid artery was isolated and gently separated from surrounding structures including the vagus nerve, trachea, and esophagus. The left carotid artery was then ligated (tied). After the surgical procedure was completed the rats were allowed to wake and recover for 1 h. After the 1 h recovery period, rats were exposed to the hypoxia (8% oxygen concentration and 92% nitrogen) using the standard published protocols of the Rice-Vannucci model for 2 h at 37. The flow rate was monitored continuously in the chamber. After exposure to hypoxia, all animals were monitored closely for any signs of distress, failure to thrive, infection, or serious disability. After hypoxia, animals were assessed and returned to their mothers.

Culture of hUC-MSCs and transplantation

The human hUC-MSCs used in this experiment came from the obstetrics department of the Affiliated Hospital of Guizhou Medical University. hUC samples were collected after deliveries with informed maternal consent. The isolation and expansion of hUC-MSCs was conducted as previously reported[12]. In this experiment, the number of cells was small, and only hUC-MSCs were amplified from umbilical cord culture of a parturient.

The newborn umbilical cords of about 10 cm in length were collected. The ages of the pregnant women ranged from 20 to 35 years old. All the subjects were healthy and free from acute and chronic diseases. Newborns were full term and healthy, each with body weight greater than 2500 g. To extract the hUC-MSCs, the umbilical cord outer layer and arteriovenous structures were removed, place in Wharton glue then mechanically digested. The tissue was shredded into 1 mm × 1 mm × 1 mm pieces and incubated in L-Dulbecco's modified eagle's medium (DMEM) + fetal bovine serum (FBS) solution in a 75 cm^2 tissue culture flask at 5% CO^2 at 37. They were monitored daily using an inverted phase-contrast microscope. After 5 days, the medium was replaced with L-DMEM containing 10% FBS. The cells were fused to 50% and the tissue mass was removed and cultured. The cells were passaged after reaching 90% confluency. After four passages, the levels of CD90, CD44, CD105, and CD73 were detected by flow cytometry and negative control was set up.

The timing of the hUC-MSC transplantation is an hour following the hypoxic-ischemia. Intracerebroventricular drug administration was performed as previously described[12]. Briefly, rats were placed in a stereotaxic apparatus under 2.5% isoflurane anesthesia. The scalp area was sterilized and Bregma was exposed. Using Bregma as a reference point, the following stereotactic coordinates were measured: 1.0 (rostral) and 1.0 mm (lateral). A burr hole (1 mm) was drilled. A needle was inserted at a rate of 1 mm/min at the depth of 1.8 mm from the dura, where the lateral ventricle is located. In the test subjects, 5 μl (1×10^6 cells/ml) of hUC-MSC suspension were administered by intracerebroventricular (ICV) injection within several minutes of HIE treatment.

Proposal

Experiment I: To culture hUC-MSCs.

Experiment II: To assess the effect of ICV hUC-MSC injection after HIE, neurobehavioral function testing was performed 48 h after surgery. A total of 30 rats were divided into groups as follows: sham group (n=10), HIE + vehicle group (n=10), and HIE + hUC-MSCs group (5 μl ICV) (n=10). Body righting and negative geotaxis tests were performed. Sham: We exposed the right carotid artery only and did not ligate the carotid artery. They were not exposed to hypoxia (low oxygen). Vehicle: We gave the HIE model by intracerebroventricular phosphate buffered saline (PBS) administration as the vehicle.

Experiment III: After the neurobehavioral function assessment, to determine the infarct volume triphenyltetrazolium chloride monohydrate (TTC) staining was performed 48 h post-surgery. The samples for sham and HIE groups from experiment II were used. A total of 18 rats were divided into groups as follows: sham group (n=6), HIE + vehicle group (n=6), and HIE + hUC-MSCs group (5 μl ICV) (n=6).

Experiment IV: To investigate the long-term effect of ICV injection of hUC-MSCs after HIE, neurobehavioral function assessment was performed 28 days after surgery. A total of 30 rats were divided into groups as follows: sham group (n=10), HIE + vehicle group (n=10), and HIE + hUC-MSCs group (5 μl ICV) (n=10).

Experiment V: To further clarify the essential role of caspase-3/Beclin-2 signaling pathway, Western blot and real time polymerase chain reaction (RT-PCR) were performed to detect the expression of caspase-3 and Beclin-2. A total of 18 rats were divided into groups as follows: sham group (n=6), HIE + vehicle group (n=6), and HIE+ hUC-MSCs group (n=6).

Infarct Volume

At 48 h post-HIE treatment animals were deeply anesthetized before being perfused transcardially with PBS. After the subjects' brains were removed, they were sectioned into 2 mm slices and immersed in a 2% 2,3,5-TTC solution (Fisher Scientific, Waltham, MA, USA) at 37°C for 5 min, followed by a 10% formaldehyde solution. Image J software was used to trace and analyze infarct volume[13].

Neurological Examination

The effects of HIE and treatment with hUC-MSCs on the development of rats were evaluated using body righting and negative geotaxis tests. Tests were performed daily in a blinded fashion starting 48 h after treatment. We also tested the effects of both HIE and hUC-MSC treatment on cognitive function using a water maze and on motor function using a foot-fault test at 28 days[12].

Western Blots

Beclin-2 and caspase-3 levels were evaluated 48 h post-treatment by Western blot according to the manufacturer's recommendations. Primary antibodies used were Beclin-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands were revealed by an Enhanced Chemiluminescence Kit (Pierce, New Brighton, MN, USA) and analyzed using Quantity One software version 4.6.2 (Bio-Rad Laboratories, Hercules, CA, USA). Protein expression level was normalized against β-actin[12].

RNA Extraction and Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed to detect the expression levels of caspase-3 and Beclin-2 mRNA in the brain tissues[13]. Total RNA was isolated from the dissected animals using RNAiso Plus (Takara, Kusatsu, Shiga, Japan) according to the manufacturer's protocol. Total RNA was stored at -80 until use. The concentration and purity of RNA were measured at a wavelength setting of 260/280 nm. The RNA samples were considered of sufficient quality for reverse transcription reaction when A260/A280 ratios ranged between 1.8 and 2.2. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using PrimeScript® RT Reagent kit with gDNA eraser (Takara, Kusatsu, Shiga, Japan). The reverse transcription reaction conditions consisted of a 37 incubation for 15 min and 85 incubation for 5 s, and an addition of 80 μl of diethyl pyrocarbonate (DEPC) to the reaction mixture for a total volume of 100 μl. Primers were designed and synthesized by Takara (Takara, Kusatsu, Shiga, Japan). The primers used for PCR amplification were as follows: rat caspase-3 forward (CTCGCTCTGGTACGGATGTG) and reverse (TCCCATAAATGACCCCTTCATCA), rat Beclin-2 forward (CTGAGGAGCAACAGGACGGT) and reverse (GCTGCCTTCTGGATGCTACT), and rat GAPDH forward (GGAGCGAGATCCCTCCAAAAT) and reverse (GGCTG TTGTCATACTTCTCATGG). cDNA was amplified using Bio-Rad CFX96 Real Time System (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR® Premix Ex TaqTM II (Takara, Kusatsu, Shiga, Japan). β-actin and GAPDH were used for normalization. The qRT-PCR cycling conditions consisted of a 95 incubation for 30 s, followed by a total of 40 cycles of 95 incubations for 5 s and 60 incubations for 30 s, and a melting curve analysis from 65 to 95°C (5 s). After checking the amplification and melting curves, data were analyzed by Bio-Rad CFX96 Manager (Bio-Rad Laboratories, Hercules, CA, USA)[14].

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad software). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Values were expressed as mean ± SD, where p values <0.05 were considered statistically significant.

Results

Culture, Expansion, and Identification of hUC-MSCs

Within 1 week of inoculation, cell migration from the umbilical cord tissue and adherence to the tissue culture flask surface were evident. The cells became 50% confluent 14 days after inoculation. Once the remainder of tissue segments was removed, the cells grew rapidly. After 20 days of culture, cell confluency reached 90%; this can be passed on to the next generation. hUC-MSCs exhibiting adherent behavior and healthy growth can be obtained from umbilical cords by employing a mechanical isolation method and utilizing Wharton glue. After culture and subculture, a large number of hUC-MSCs satisfying clinical needs can be obtained by purification and expansion (Figure 1(a)). The results of flow cytometry assays showed the cells were positive for CD90, CD44, CD105, and CD73 after three passages (Figure 1(b)–(d)), which is in accordance with the biological characteristics of MSCs.

Effects of hUC-MSCs on Short-Term Neurological Function at 48 h post-HIE

hUC-MSC treatment was shown to reduce the infarct volume when compared to vehicle 48 h post-HIE (Figure 2(a) and (b)). Furthermore, hUC-MSCs significantly improved

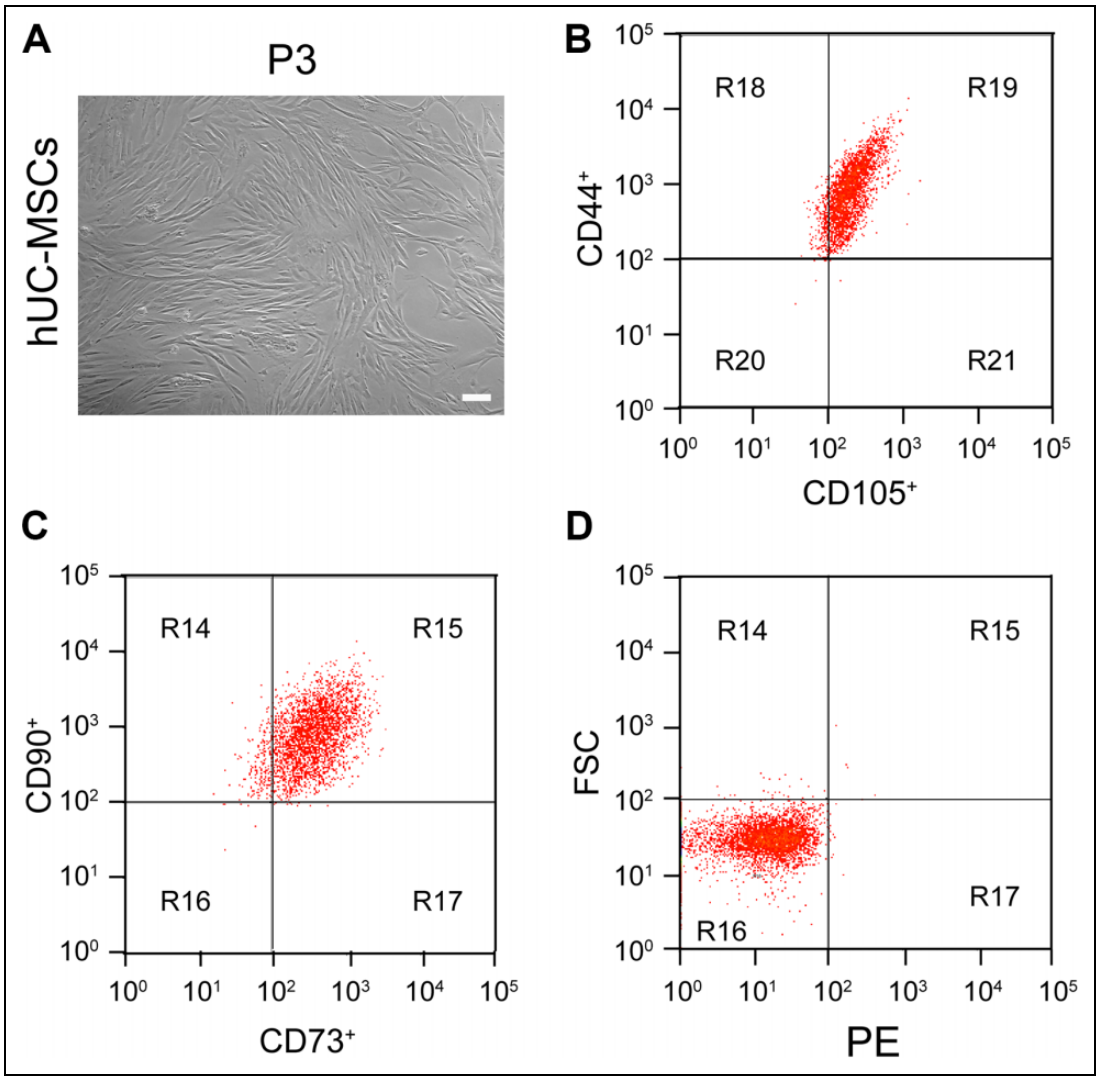

Fig 1. Human umbilical cord mesenchymal stem cell (hUC-MSCs) morphology and MSC surface markers ($CD44^+$, $CD105^+$, $CD90^+$, and $CD73^+$). These indicate the biological characteristics of mesenchymal stem cells. (a) Long spindle-shaped phenotype of hUC-MSCs. (b) Expression of hUC-MSCs surface markers $CD44^+$, $CD105^+$. (c) Expression of hUC-MSCs surface markers $CD90^+$,$CD73^+$. (d) Expression levels of NOT-hUC-MSCs surface markers were very low. Scale bar = 50 μm. FSC (forward scatter), PE (dyestuff).

neurological deficits 48 h post-HIE (evaluated by both negative geotaxis and righting reflex tests) (Figure 2(c) and (d)).

Effects of hUC-MSCs on Short-Term Neurological Function at 28 d post-HIE

To test the effects of hUC-MSC treatment on the long-term neurological impairments induced by neonatal HIE, neurological function was assessed by water maze and rotarod tests 4 weeks post-HIE. hUC-MSC treatment animals showed significantly improved memory and learning abilities as it took them less time to get to the platform, they travelled a shorter distance and spent more time in the platform quadrant in the probe trail ($p > 0.05$ vs. sham, Figure 3(a) and (b)). Furthermore, vehicle group animals had significantly worse foot-fault performances compared to the sham group, whereas hUC-MSC-treated animals showed significantly reduced foot-fault performances compared with vehicle group rats ($p < 0.05$, Figure 3(c)).

Expression of Beclin-2 and Caspase-3 post-HIE

Endogenous Beclin-2 mRNA and caspase-3 mRNA were significantly upregulated 48 h post-HIE. hUC-MSC treatment showed that Beclin-2 mRNA and caspase-3 mRNA decreased 48 h post-HIE, as shown in Figure 4(a). Expression of endogenous Beclin-2 and caspase-3 were significantly upregulated 48 h post-HIE. hUC-MSC treatment showed that expression of Beclin-2 and caspase-3 decreased 48 h post-HIE, as shown in Figure 4(b) and (c).

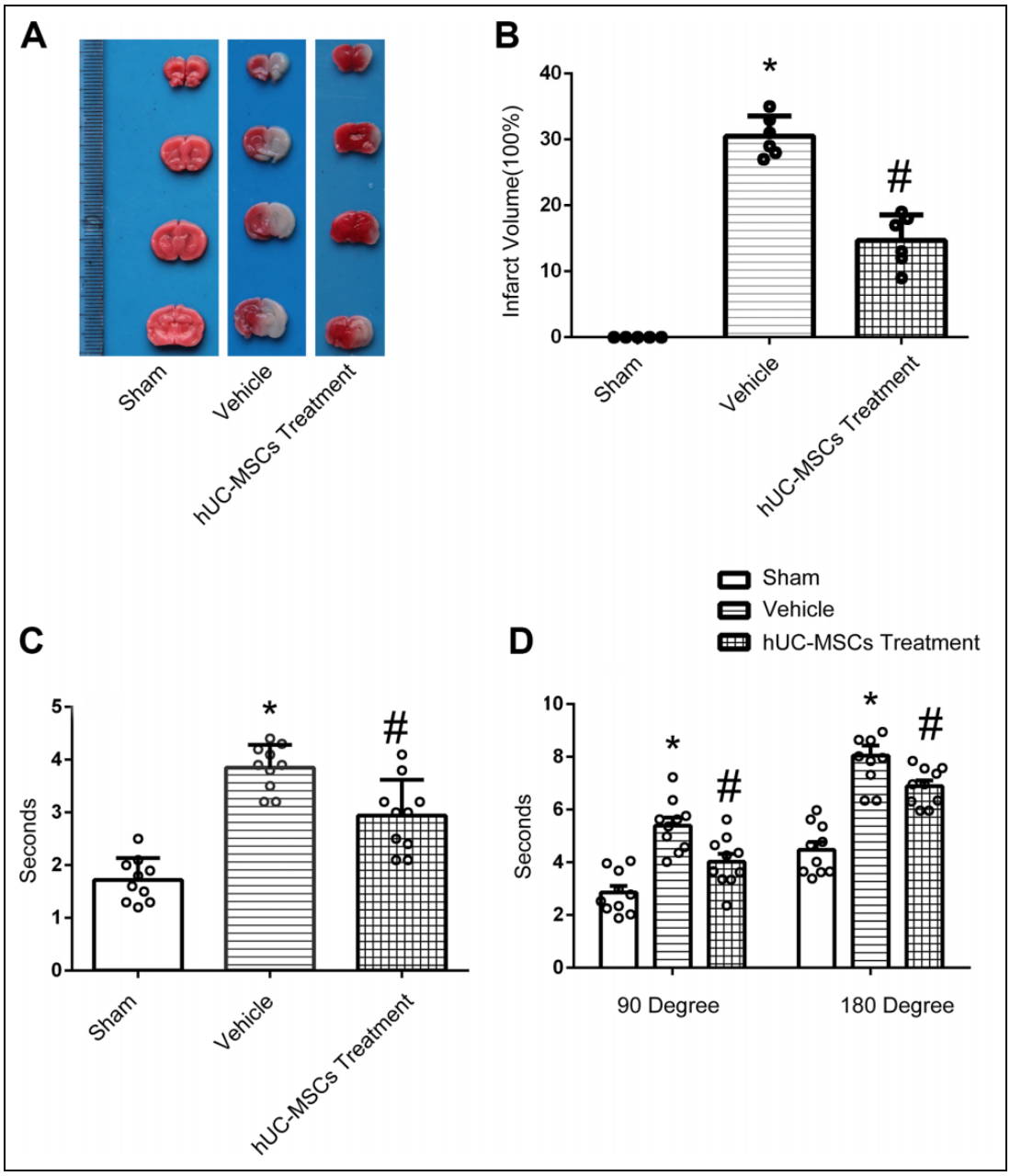

Fig 2. Representative TCC stained brain sections (a) and triphenyltetrazolium chloride monohydrate (TTC) analysis (b) of 48 h post-hypoxic-ischemic encephalopathy (HIE) with human umbilical cord mesenchymal stem cell (hUC-MSC) treatment. Tilt table test (c) and righting reflex (d) of 48 h post-HIE with hUC-MSC treatment. Data are presented as mean ± SD. *p<0.05 vs sham, #p<0.05, p<0.05 vs vehicle, n=10 per group, difference between groups were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's test.

Discussion

hUC-MSCs are abundant and easy to collect. They can be prepared by mechanical isolation, culture, and expansion, and identified by flow cytometry[15]. Several studies showed that hUC-MSCs do not cause any adverse reactions in mothers and their newborns[3,15,16]. These cells are more primitive than other MSCs and have a strong proliferative ability. They are not easily differentiated over a long period of time. The infection and transmission probability of a virus or other pathogenic microorganism is relatively low, and it does not involve social, ethical, and legal disputes. hUC-MSC is considered the ideal seed cell for tissue engineering[17]. The unique biological characteristics of hUC-MSCs, especially their multidirectional differentiation potential, have shown attractive application prospects and have become a hot topic in the field of tissue engineering.

The results of this study showed that hUC-MSCs can migrate into the periventricular tissue space after

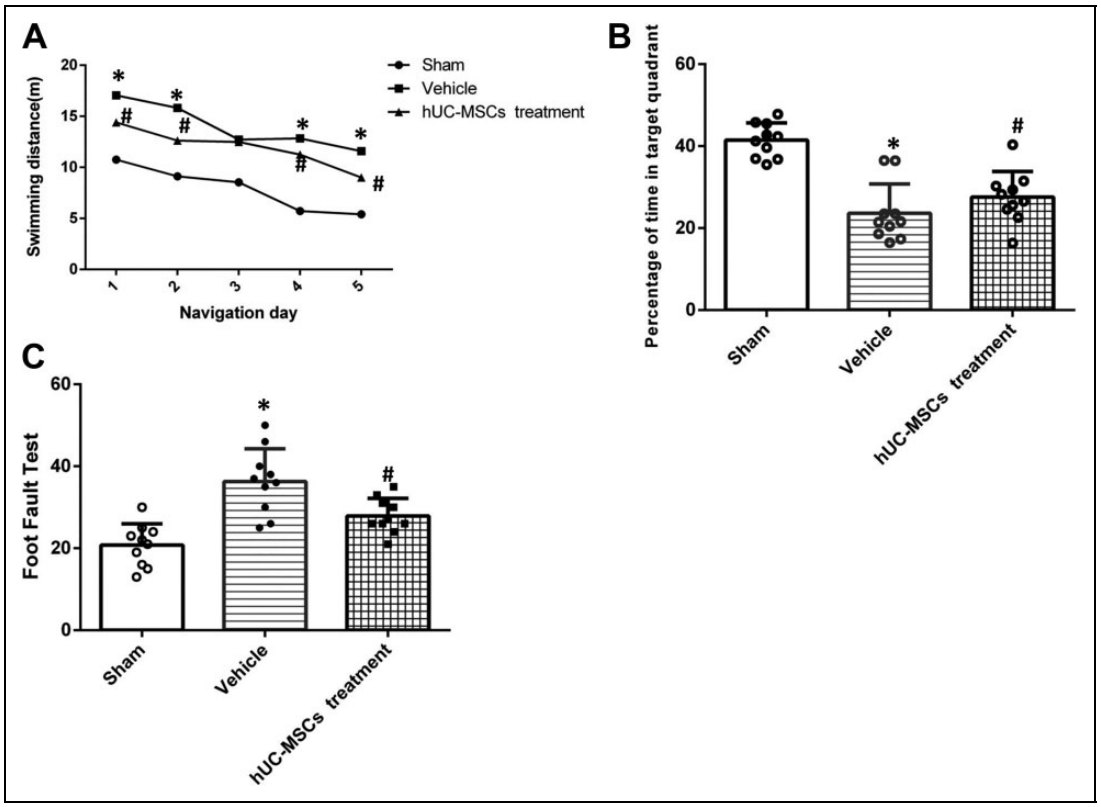

Fig 3. Human umbilical cord mesenchymal stem cell (hUC-MSC) administration ameliorated long-term neurological deficits. (a) Swimming distance; (b) percentage of time in target quadrant; (c) foot fault. Data are presented as mean ± SD, *$p<0.05$, vs sham, # vs vehicle, $n=10$ per group, difference between groups were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's test.

hUC-MSC transplantation. The motor function of neonatal rats after treatment with hUC-MSCs was improved. hUC-MSCs can effectively reduce the hypoxic ischemic injury and decrease the infarct volume of the rat brain. They can also significantly reduce apoptosis of the rat brain and decrease the expression levels of Beclin-2 and caspase-3. The mechanism by which cell transplantation affects the treatment of HIE is very complex. Apart from differentiation and substitution, paracrine action is involved in nerve repair, as well as inhibition of neuronal apoptosis in the ischemic peripheral area.

In recent years, studies have reported that Beclin-2 and caspase-3 are closely related to apoptosis[18–20]. Beclin-2 regulates apoptosis and autophagy by interacting with class III PI3 K complex and Bcl-2. While serving as a regulator of confluence, Beclin-2 also plays a regulatory role between autophagy and G-protein-coupled receptor. It can enhance the lysosomal transport and metabolic diversity, Beclin-family function in cell autophagy and apoptosis, and expression in damaged tissues[21]. As the most critical apoptosis executor downstream of the caspase cascade, caspase-3 plays an important role in the development of disease[22]. Caspase-3 participates in the process of apoptosis of various nerve cells, which can be activated by a series of factors. Caspase-9 is activated after the release of cytochrome c in mitochondria. Activated caspase-9 cuts and activates caspase-3, whereas the activated caspase-3 cleaves its protein substrate and eventually leads to apoptosis[23]. In this experiment, both Beclin-2 and caspase-3 were highly expressed within 48 h of establishing the HIE model. After the hUC-MSC treatment, the expression of both Beclin-2 and caspase-3 decreased. This indicates that hUC-MSCs play a regulatory role in the repair of damaged neonatal rat brains, regulate the apoptosis-related proteins Beclin-2 and caspase-3, and inhibit the apoptosis of brain cells in HIE rats. hUC-MSC transplantation can promote neurological function recovery, reduce infarct volume and ischemic injury, and inhibit neuronal apoptosis in HIE rats. We also have to point out that hUC-MSC transplantation can inhibit inflammation and the apoptosis of host nerve cells and promote axonal and vascular regeneration by secreting various factors[21,24,25]. Furthermore, MSCs have immunomodulatory functions. Therefore, we think hUC-MSC transplantation is promising method to treat HIE.

In summary, the hUC-MSC transplantation mechanism may be related to regulation of Beclin-2 and caspase-3. It can be a part of a safe and effective treatment for HIE, providing an experimental basis for further investigation in clinical application.

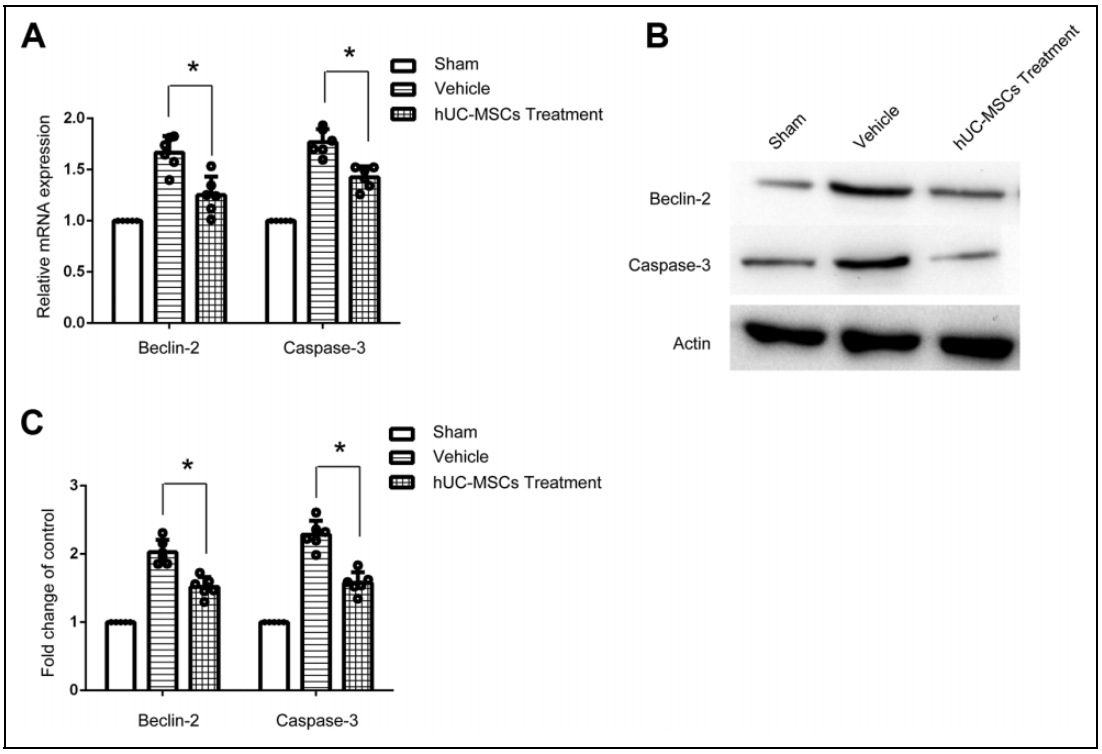

Fig 4. Expression characteristic of Beclin-2 and caspase-3. (a) Endogenous Beclin-2 mRNA and caspase-3 mRNA were significantly upregulated 48 h post-hypoxic-ischemic encephalopathy (HIE). (*p< 0.05), (n=6 per group). Data are expressed as mean ± SD. (b) Representative bands of NHE1 protein. (c) Endogenous Beclin-2 protein and caspase-3 protein increased significantly 48 h post-HIE. (*p< 0.05). Data are expressed as mean ± SD, (n=6 per group).

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Author Contributions

H.Z. and J.X. conceived, designed the experiments, carried out behavioral tests, Western blot, and wrote the manuscript. L.Y. and Z.F. carried out TTC staining, behavioral tests, and immunohistochemistry. X.W. and Y.X. conceived and participated in acquiring and analyzing the presented data. L.W. coordinated the study. All authors read and approved the final manuscript.

Ethical Approval

This study was approved by the Ethics Committee of Soochow University and Guizhou Medical University, Jiangsu Province and Guizhou Province, China.

Statement of Human and Animal Rights

All experimental procedures involving animals were conducted in accordance with the institutional Animal Care guidelines of Soochow University and Guizhou Medical University (No 1800661).

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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