Human Umbilical Cord Mesenchymal Stem Cells Efficiently Improve Neurobehavioral Status and Alleviate Brain Injury in Hypoxia/Ischemia-Induced Cerebral Palsy Rat Model Via Down-Regulating the NogoA/NgR/Rho Pathway

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Research

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Abstract

**Background:** Cerebral palsy (CP) is a brain injury disease, which is a global public health issue with an estimated prevalence of 2‰—4‰ and imposes a substantial health burden on many countries. At present, there is no ideal treatment available and most of them will still suffer adverse outcomes. Human umbilical cord mesenchymal stem cells (HUCMSCs) application in many fields of medicine, which can promote nervous system regeneration and inhibit neuroinflammation. The regeneration of central nervous system (CNS) is related to the nervous regeneration inhibitors. NogoA/NgR/Rho pathway is very important to the nerve growth, CP injury is inevitably accompanied by the regeneration and repair of neurons and axons. So we hypothesized that NogoA/NgR/Rho pathway is involved in when using the HUCMSCs to treatment cerebral palsy.

**Purpose:** In this study, we might clarify the NogoA/NgR/Rho pathway functional role in mediating HUCMSCs to improve neurobehavioral status and alleviate brain injury in hypoxia/ischemia-induced CP rat model.

**Methods:** The CP rat model was established by ligating the left common carotid artery and anoxia for 2.5 h, and HUCMSCs were intravenous injected to the modeled rats. The neurobehavioral situation and brain pathological injury in CP rats were determined via a series of assays. The mRNA and protein expression of NogoA\NgR\RhoA\Rac-1\Cdc42 in brain tissue of rats in each group was detected by RT-qPCR and western blot analysis.

**Results:** The CP rats exhibited obvious motor function abnormalities, pathological damage and a lot of brain nerve cell apoptosis. Compared with CP+PBS group and CP group rats, HUCMSCs transplantation can significantly improve the neurobehavioral situation, attenuated brain pathological injury, inhibit apoptosis of brain nerve cells and the activation of astrocytes in CP rats. The expression of NogoA\NgR\RhoA relative mRNA and protein in brain tissues of rats in the CP+PBS group and CP group rats were significantly lower than those of in the sham+PBS and CP+HUCMSCs group. The expression of Rac-1\Cdc42 relative mRNA and protein in brain tissues of rats in the sham and CP+HUCMSCs group was significantly higher than those of in CP+PBS group and CP group rats.

**Conclusion:** This study confirmed that HUCMSCs can efficiently improve neurobehavioral status and alleviate brain injury in hypoxia/ischemia-induced cerebral palsy rat model via down-regulating the NogoA/NgR/Rho pathway.

1 Introduction

Cerebral palsy (CP) is a group of diseases whose common feature are motor disorders, which is the most commonly physical disability in childhood[1]. This disease is a global public health issue with an estimated prevalence of 2‰—4‰ and imposes a substantial health burden on many countries[2]. The children with CP exhibit a complex set of dysfunctions, including behavioral disorders, disturbances in sensation and perception, epilepsy, mental retardation, neurological dysplasia, cerebral anesthesia, poor
learning ability and language deficits accompanied by their whole lifetimes[3]. Brain tissue in the course of the disease is irreversibly damaged, there are many causes of damage lead to CP, such as hypoxia, ischemia, infection, injury or congenital defects[4]. CP as a incurable disease has brought enormous mental and economic burden to the patient's family and society[4].

Nowadays, many treatment strategies have been developed for CP, which include various surgeries on muscles, tendons, bone and nerves, medications, electrical stimulation, patterning, conductive education, orthoses[5]. However, all of the treatment strategies only provide symptomatic relief and none of them are ideal[5]. Therefore, research new efficient strategies to treat CP is in an urgent need[3].

In recent years, HUCMSCs have been proved promising in possible treatment of several diseases and conditions such as diabetes, certain diabetic wounds and brain damage associated with neonatal hypoxia, stroke, autism, acute liver failure, CP and Alzheimer's[6]. HUCMSCs are better in contrast to embryonic and fetal stem cells ethically noncontroversial, inexpensive and readily available source of cells[6]. HUCMSCs are multipotent stem cells, which are easy to obtain materials, rich sources, primitive cells, differentiation ability and secretion of cytokines are strong, immunogenicity is relatively low, and also with no ethical problems and little exogenous pollution and characterized by the ability to differentiate into specific cell types[7]. HUCMSCs are capable of forming many different cell types and it has also become one of the most important cell sources for scientists to study the treatment of CP [8].

The key molecules of Rho/ROCK signaling pathway include NogoA, NgR, RhoA, Rac-1, Cdc42. NogoA protein is an important membrane protein, which is mainly expressed in oligodendrocytes and neurons of the central nervous system[9]. NogoA belongs to the reticular family, a member of the neurite outgrowth inhibitor (Nogo) family, which is the most important and strongest inhibitor of axon growth in the central nervous system[10]. Binding of NogoA to specific receptor NgR can induce cone collapse, axon growth collapse and atrophy, inhibit axon regeneration, and play a negative regulatory role in central nerve injury[11]. NogoA can cause nerve damage by activating Rho. In vivo experiments showed that blocking NogoA expression may result in an increasing in dendrite spine density[12]. Rac1 is involved in axonal formation of nerve cells in vivo[13]. Rac1GTPase is essential for axon formation during hippocampal neuron development and can stimulate axon growth expansion, axon guidance, dendrite growth and neuronal polarity of nerve cells[14]. Many studies have shown that Rac1 silencing can reduce and improve neuronal oxidative stress injury, and abnormal Rac1 activity may lead to axonal contraction and neuronal death after ischemia-reperfusion in models[15]. CDC42 can regulate microfilaments, mediate the formation of filamentous pseudopods, participate in vesicle transport, regulate axon and dendrite formation and synaptic plasticity[16, 17].

Many studies demonstrated that HUCMSCs could migrate to the sites of brain injury in CP rats, reduce functional deficits, protect against white matter injury [18]. The main purpose of using stem cells treatment of cerebral palsy is to use their regenerative capacity to rebuild damaged brain tissue, which can differentiate into nervous system regeneration and inhibit neuroinflammation[19]. However, the data on the effectiveness of HUCMSCs therapy in cerebral palsy are still insufficient. So we need continue to
research more about the HUCMSCs therapy mechanism for CP. CP injury is inevitably accompanied by
the regeneration and repair of neurons and axons. Nerve regeneration is noticed to play an important role
in the CP treatment. The regeneration of central nervous system (CNS) is related to the existence of
factors against regeneration in the microenvironment of CNS. However, the effect of NogoA/NgR/Rho
pathway on the regulation of HUCMSCs activity in CP is rarely reported and the specific mechanism is
still unclear [20].

We hypothesized that NogoA/NgR/Rho pathway is involved in when use the HUCMSCs to treat CP. The
aim of our study is to explore the role of NogoA/NgR/Rho pathway in the nerve regeneration mechanisms
of the HUCMSCs and evaluate its potency as a new target for treating CP or the injury and diseases of
central nervous system.

To further validate the therapeutic mechanisms of the NogoA/NgR/Rho pathway, HUCMSCs were injected
into rats with hypoxia/ischemia-induced CP. In this article, we explain the effect in rat with CP and
NogoA/NgR/Rho pathway potential molecular mechanisms by analyzing these changes, including
NogoA, NgR, RhoA, Rac-1, Cdc42 expression levels of related nerve regeneration genes. This in vivo study
in the rat CP model may provide new treatment method for CP patient in future.

2 Materials And Methods

2.1 Ethics approval

All experiments in this study were performed with human ethics approval from Gannan Medical
University of Jiangxi province in China (approval number: LLSC-20190720). Written informed consent was
obtained from all participants prior to collection of HUCMSCs. All experiments were performed following
the National Health and Medical Research Council guidelines [20].

2.2 Animal ethics, selection and welfare

The adult Sprague-Dawley (SD) rats were purchased from Hunan SJA Laboratory Animal Co., Ltd in China
for reproduction rat pups. The rats were fed and watered freely in the SPF animal laboratory for 12 h
day/night cycle, temperature (23-25)°C and humidity (45-60%). A total of 80 rat pups were borned by
adult SD rats and used for CP model. Rat pups were randomly assigned to experimental groups, based on
CP model surgery times. This study was approved by the First Affiliated Animal Ethics Committee of
Gannan Medical University (Approval No. 20200057). All animal experiments were conducted to relieve
their pain in accordance with the international guidelines for the Care and Use of Laboratory Animals of
the National Institute of Health.

2.3 Animal surgery to induce CP model and assessment of
brain Injury

The rat pups were anesthetized with 1% pentobarbital sodium via intraperitoneally injection on 7 days
age. The left carotid artery was ligation and bupivacaine was used for pain relief [20]. Then the rat pups
were placed with their mother for an hour to recover. Then the rat pups were immediately placed in an anoxic chamber at 37°C, and filled with a mixture of 8% oxygen and 92% nitrogen with the flow rate of 1 L/min for 2.5h [20]. The whole process of anoxic was continuously monitored through an oxygen meter [20]. The left carotid artery of the sham group rat pups was only isolated, but with neither ligation performed nor hypoxia treatment. After surgery, the rat pups were returned to their mother with a 37°C heating pad for 1-hour recovery. Finally, all the rat pups returned to the cage with their mother. The CP model assessment of rat pups was conducted through behavior test and TTC staining of brain tissue.

2.4 Brain tissue TTC staining testing to assessment of brain injury

The 36-day-old rats were sacrificed for TTC (Sigma, USA) staining. The brain tissue sections were cut into 5 coronal sections with 2 mm thickness and immediately incubated with 2% TTC in 37°C phosphate buffered saline (PBS) for 30 minutes. The viable cells stained by TTC are dark red and infarcted cells are white.

2.5 Behavioral testing

Behavioral testing was conducted to assessment of CP rat pups after 3 weeks of the surgery and the HUCMSCs therapeutic effect 7 days after the last time administration of HUCMSCs. The scoring rules were as follows:

Suspension test[21]: Put the rat pups forelegs grabbed the 0.5 cm glass rod and recorded how long it takes to fall. The time of falling: < 10 s was 1 point, 10–30s was 2 points, 31–119 s was 3 points, 2–5 min was 4 points, > 5 min was 5 points.

Slope test[21]: The rat pups were placed on a 45° slope to observe the turning time.

Open field test[21]: A box with length and width of 36 cm was divided into 9 equal size lattices at the bottom of the box. When more than 1/2 of the body part of the rat entered the adjacent grid was 1 point, and the hind limb of the rat stood was 1 point. The sum of the two points was the total score.

2.6 Isolation and characterization of HUCMSCs

The HUCMSCs used in this study were isolated from human umbilical cord tissue from voluntarily donated, which was obtained from normal healthy birth after a rigorous screening process from the First Affiliated Hospital of Gannan Medical Universit of Jiangxi province in China. HUCMSCs were obtained through the enzymatic digestion of UC Wharton's jelly using collagenase 1.67% (Sigma, USA) at 37°C. Then HUCMSCs were cultured in cell culture plates using α-MEM(Gibco, USA) supplemented with 4 mM GlutaMax (Gibco, USA) and 10% inactivated fetal bovine serum (Cyagen, USA) and 1% streptomycin/penicillin in 37°C incubator with 5% CO2[22].
The HUCMSCs from passage numbers 0-5 were characterized with flow cytometry (Becton, Dickinson and Company, USA). The cells were added with rabbit polyclonal antibodies conclude CD73, CD44, CD90, CD105, CD34, CD45, HLA-DR (Proteintech Inc, USA) (1:100 dilution) to detect the surface antigens of HUCMSCs[23].

The Multi-potency of HUCMSCs was tested by StemPro® Osteogenesis (Gibco, USA) and Adipogenesis (Gibco, USA) differentiation Kits (37°C, 5% CO2) according to the instructions[24].

2.7 Preparation and transplantation of HUCMSCs

The rat pups were divided into 4 groups and 15 rat pups in each group. The CP+HUCMSCs group rat pups received multiple doses of HUCMSCs at 7 days (PND14), 14 days (PND21), 21 days (PND28), 28 days (PND35) after surgery. HUCMSCs were counted and viability determined using trypan blue. The cells were resuspended in PBS at a concentration of 1×10^6 cells/ml.

The migration assessment of HUCMSCs was performed through HUCMSCs labele with Did and injured brain stain with DAPI. HUCMSCs were labeled with Did 30 minutes before intravenous injection according to the instructions. The Did (Beyotime Biotechnology Co., Ltd., China) can stain the HUCMSCs to red fluorescence.

HUCMSCs were labeled with Did 30 minutes before intravenous injection according to the instructions. The Did (Beyotime Biotechnology Co., Ltd., China) can stain the HUCMSCs to red fluorescence.

The DAPI (Beijing Solarbio Science & Technology Co., Ltd., China) can stain the brain tissue cell nucleus to blue fluorescence. The frozen slices of rat brain tissues were stained with DAPI (Beijing Solarbio Science & Technology Co., Ltd., China) to see whether HUCMSCs migrated to the damaged brain after intravenous injection the next day according to the instructions.

The CP+HUCMSCs group rats received 1×10^6 HUCMSCs on each cell administration day via tail intravenous injection in a volume of 100 µl/100g body weight. The sham group rat pups and CP+PBS group rat pups only received the same volume of PBS alone through tail intravenous injection. The CP group rat pups received neither PBS nor HUCMSCs.

2.8 Anatomy and detection of rat brain tissue

After 24 h of the first and last times CP rats behavioural evaluation test finished, 5 rats from each group were euthanized by intraperitoneal injection of 1% pentobarbital sodium[25]. The heart was exposed by opening the chest, and the left ventricle was inserted into the ascending aorta with injection syringe and fixed with hemostatic forceps[25]. Then about 200 mL saline containing 12.5 U/L heparin sodium was quickly injected. After all the blood was washed out, 200 mL 10% formalin was perfused. All rats right and left brains were dissected. Then the rat brain tissues in each group were fixed in 4% paraformaldehyde for 24 h. Then the rat brain tissues were sliced with the thickness of 4 µm and examined using paraffin embedded sections. The rat brain tissue slices were staind use HE staining. Each
group of the rat brain tissues were randomly selected with five slices for observe. The stained rat brain tissues were observed under an optical microscope (Olympus, Tokyo, Japan).

At the same time, the other 10 rats brain tissue taken from each group were removed and rapidly put into liquid nitrogen for the detection of RT-qPCR and western blot analysis.

### 2.9 Real-time quantitative PCR

The rat brain tissue total RNA samples were extracted using Trizol (Invitrogen, USA) according to the manufacturer’s instructions. Real-time quantitative PCR analysis was performed using Applied Biosystems 7500 Real-Time PCR Systems (Applied Biosystems). The housekeeping gene GAPDH was used as an internal control for relative quantification. The mRNA expression levels of NogoA, NgR, RhoA, Rac-1 and Cdc42 were measured by qPCR. The primers used in this study were synthesized and purchased from Invitrogen Co., Ltd., China. The PCR products were examined by electrophoresis with 2% agarose gel. The sequence information for all the primers in this study is listed in Table 1.

| Gene   | Sequence       |
|--------|----------------|
| NogoA  | F: TGCAGTGTATGATGATGATGATGATGATG | R: CTATCTGCACCTGATGATGCCGT |
| NgR    | F: CGCATCTCTCTCTGATGGC | R: GTGCAAGAGGAGACGGGTCA |
| RhoA   | F: AGAGTGTATGATGATGATG | R: TGTCTGGGGTAGGAGAGG |
| Rac-1  | F: GAGACGGCGACGGCTGGTAAA | R: TGTCAGACGGTTGGGATG |
| Cdc42  | F: GCAGGAGAGGCTGGTCAAA | R: ACCAACAGCACCATCACC |

### 2.10 Western blot assay

The protein expression of NogoA, RhoA, Rac-1, Cdc42 and Rnd3 was measured by western blot[26]. The total protein of each group rat brain tissues were extracted by a RIPA buffer (Solarbio, China) with 1% phenylmethylsulfonyl fluoride (PMSF). The rat brain tissue protein concentration was determined referring to the instructions of the bicinchoninic acid assay (Boster Biological Technology, Ltd., China). The internal
control is β-Actin. The proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and electro transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat dry milk (prepared by 1 × Tris-buffer saline and 0.05% Tween 20%) for 2 h and then incubated with the antibody (NogoA, RhoA, Rac-1, Cdc42 and Rnd3 antibody, dilution ratio of 1:2000, Proteintech Inc, USA) for 12 h at 4°C. Afterwards, the membrane was incubated with goat anti-rabbit IgG (dilution ratio of 1:5000; Proteintech Inc, USA) labeled with horseradish peroxidase (dilution ratio of 1:5000, Proteintech Inc, USA). The membrane was rinsed and incubated at room temperature for 2 h with Horizontal shaker (Proteintech Inc, USA). Finally, the membrane was rinsed again and incubated with the chemically luminescent solution for exposure. Protein intensity was measured using Image Lab software.

**Statistical analysis**

Statistical analysis of the data was conducted using SPSS software. Data were presented in the form of mean ± SD (standard deviation). For the measurement data that conformed to the normal distribution, t-tests were employed for comparison between the two groups. p < 0.05 was considered statistically significant.

3 Results

3.1 Characterization of CP model in rats

The results of the suspension test showed that CP group rats spent shorter time on the glass rod than sham group (p < 0.001) (Fig. 1). The results of the slope experiment results showed that CP group rats spent more time on the slope than the sham group (p < 0.001) (Fig. 1). The results of open field test showed that the open field score in the CP group was lower than that in the sham group (p < 0.001) (Fig. 1).

TTC staining results showed that the CP group brain infarction and necrosis (Figure 2). The total area of the infarct was higher in the CP group (Figure 2) than the sham group. That the volume of left brain in CP rats was atrophied and lower than the sham group rats (Fig. 2), while there is no obvious differences in the right brain (Fig. 2). In all, the results revealed that rat CP models were successfully established. The successfully-modeled rats were divided into 4 groups, 15 rats in each group.

3.2 HUCMSCs can improve the neurobehavioral situation in CP rats

After transplantation of HUCMSCs in CP rats, the results of suspension test suggested that the suspension test score in rats of the CP and CP+PBS groups were lower than that in the CP+HUCMSCs group (all P < 0.05) (Fig. 3A). The results of slope test indicated that the slope test time of CP and CP+PBS groups rats was obviously longer than that in the CP+HUCMSCs group (P < 0.01) (Fig. 3B). The results of
open field test showed that the open field score in the CP and CP+PBS groups were lower than that in the CP+HUCMSCs group (P < 0.01) (Fig. 3C).

3.3 Morphological observation and identification of HUCMSCs

Most of the HUCMSCs showed irregular shape, the morphology and size of the cells showing a fiber strip (Fig. 4). Specific surface markers of isolated cells were identified by flow cytometry. The results showed that HUCMSCs surface markers of CD73(97.2%), CD44(98.2%), CD90(97.9%), CD105(98.8%) were highly expressed, while surface markers of hematopoietic stem cells CD34(0.1%), CD45 (0.1%), HLA-DR(0.1%) were lowly expressed in cultured HUCMSCs, which were identified as the immunophenotypic characteristics of HUCMSCs (Fig. 5).

After differentiation of cultured osteoblasts, a large number of calcium nodules could be seen under an alizarin red staining microscope (Fig. 6), showing that the cells have good osteogenic differentiation function. After the intervention of the fat-inducing agent, the red-stained particles could be seen in the cytoplasm of the cells through the oil-red O staining, and a large amount of lipid droplets were visible (Fig. 6), suggesting the existence of adipogenesis differentiation in cells. These results suggest that rat HUCMSCs were successfully cultured.

3.4 HUCMSCs can alleviated brain pathological injury in CP rats

The pathological morphology of brain tissues in rats was observed by HE staining. In the sham group, the neuronal cells of the brain were intact, with abundant rough endoplasmic reticulum, free ribosome and mitochondria, clear mitochondrial ridge, regular arrangement, clear nuclear membrane, dominant chromatin in nucleus and obvious nucleolus (Fig. 7A). The CP group rats (Fig. 7B) and CP+PBS group rats (Fig. 7C) brain tissue structure was destroyed and white matter was disordered, which was presented with multiple irregular cystic cavities, cell swelling, degeneration, disordered arrangement, inflammatory cell infiltration and local round softening foci. After transplantation of HUCMSCs in CP rats, the CP+HUCMSCs group rats, the degree of cell swelling was reduced, inflammatory infiltrating cells were decreased, and cystic degeneration was decreased, the pathological degree of brain tissue was further alleviated and the cells were arranged neatly (Fig. 7D).

3.5 HUCMSCs can migrate to the injured rat brain hippocampal tissue

The DAPI successfully stained the brain hippocampal tissue blue (Fig. 8). HUCMSCs labeled with Did and stained with DAPI showing that the HUCMSCs can successfully reached the site of PC+HUCMSCs group rats damaged brain tissue (Fig. 8), the PC+PBS showed nothing (Fig. 8).

3.5 RT-qPCR and Western blot in brain tissues
The expression of NogoA\textsuperscript{\textsuperscript{\textregistered}}NgR relative mRNA and protein in brain tissues of rats in the CP group was significantly higher than those of sham group (P < 0.05), while RhoA relative mRNA and protein in brain tissues of rats in the CP group was significantly higher than those of sham group (P < 0.001) (Fig. 9A\textsuperscript{\textregistered}B\textsuperscript{\textregistered}C). The expression of NogoA\textsuperscript{\textsuperscript{\textregistered}}NgR relative mRNA and protein in brain tissues of rats in the CP and CP+PBS groups were significantly higher than those of in CP+HUCMSCs group (P < 0.01), while the expression of NogoA\textsuperscript{\textsuperscript{\textregistered}}NgR relative mRNA and protein in brain tissues of rats in the CP and CP+PBS groups were significantly higher than those of in CP+HUCMSCs group (P < 0.001) (Fig. 9A\textsuperscript{\textsuperscript{\textregistered}}B\textsuperscript{\textsuperscript{\textregistered}}C).

The expression of Rac-1 relative mRNA and protein in brain tissues of rats in the sham group was significantly higher than those of CP group (P < 0.01) (Fig. 9E). The expression of Cdc42 relative mRNA and protein in brain tissues of rats in the sham group was significantly higher than those of CP group (P < 0.05) (Fig. 9A\textsuperscript{\textsuperscript{\textregistered}}F). The expression of Rac-1 relative mRNA and protein in brain tissues of rats in the CP+HUCMSCs group was significantly higher than those of in CP and CP+PBS groups (all P< 0.05) (Fig. 9E). The expression of Cdc42 relative mRNA and protein in brain tissues of rats in the CP+HUCMSCs group was significantly higher than those of in CP and CP+PBS groups (all P< 0.01) (Fig. 9F). These results suggesting the HUCMSCs can regulate the NogoA/NgR/Rho pathway in the CP rats.

4 Discussion

CP is a most readily identifiable consequence of perinatal brain injury. The incidence of premature CP has increased over time\textsuperscript{27}. This condition may occur due to chronic or acute injury sustained following birth in the new born period or even before \textsuperscript{27}. In recent years, numerous methods have been developed for CP therapy, including occupational and physical therapies\textsuperscript{28}. However, current therapies are far from ideal. Therefore novel therapeutic strategies for CP are urgently needed.

Previously, more and more studies have showed HUCMSCs can promote central nerve regeneration and treatment of cerebral palsy efficiently\textsuperscript{29}. However, the detailed mechanism by which HUCMSCs regulate central nerve regeneration and CP therapeutic have not defined \textsuperscript{30}.

After central nervous system injury, the regeneration ability of central nervous system is very limited. One of the most important reasons is that inhibitory factors can affect the regeneration of the nervous system. NogoA is the most important axon growth inhibitory factor in the central nervous system, which can bind to specific receptor NgR to inhibit axon regeneration and play a negative regulatory role in central nervous injury\textsuperscript{31}. CP is often accompanied by a large number of neuronal apoptosis, which might closely relate to the NogoA/NgR/Rho pathway\textsuperscript{32}. Therefore, we hypothesized that the NogoA/NgR/Rho pathway was involved in when use HUCMSCs to treatment. Our study of western blot and RT-PCR results showed that hUCMSCs significantly inhibited the expression level of NogoA, NgR and RhoA, and increased the expression level of Rac1 and CDC42. In addition, our study also showed that HUCMSCs can improve neurobehavioral status, alleviate brain pathological damage, and promote nerve regeneration. McDonald\textsuperscript{31} confirmed that UCB can regulate the neuropathology after perinatal brain injury in rat pups, alleviate brain tissue damage, improve encephalopathy and recover behavioral defects.
Wang[32] confirmed that NgR and PirB nucleic acid vaccines can be used as therapeutic strategies for central nervous system diseases and injuries such as SCI.

Our study showed that the expression level of NogoA, NgR and RhoA in CP rats brain tissue decreased after hUCMSCs treatment, while the expression level of Rac1 and CDC42 increased. These results obtained in this study are basically consistent with those of previous studies, so we speculate that NogoA/NgR/Rho pathway plays an important role in the repair of brain tissue injury after hUCMSCs treatment.

This study suggests that regulate the NogoA/NgR/Rho pathway may provide a new promising therapeutic strategy for using the HUCMSCs to treat CP.

5 Conclusion

To sum up, we confirmed that HUCMSCs transplantation can efficiently improve neurobehavioral status and alleviate brain injury in hypoxia/ischemia-induced cerebral palsy rat model via NogoA/NgR/Rho pathway. Thus, this work establishes a new therapeutic strategy when use the HUCMSCs transplantation can to treat CP via regulate the NogoA/NgR/Rho pathway.

Abbreviations

1. CP:Cerebral palsy
2. HUCMSCs:Human umbilical cord mesenchymal stem cells
3. CNS:The regeneration of central nervous system

Declarations

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Competing interests

All the authors declare that there are no conflicts of interests.

Availability of data and material declaration

All of the data and materials in this article where the data supporting their findings can be found are availability.

Author information

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Author contributions

Yaoling Luo conceived, designed, performed the experiments and analyzed the data, Zhengyi He performed the experiments, analyzed the data and wrote the paper. Minhong Zhang, Lincai Li, Zhengwei Zou, Jiayang Qu, Qianqian Xu performed the experiments. Junsong Ye gave some advices for this study. Yaoling Luo and ZhengYi He are the Co-first author, Junsong Ye is the Corresponding Author, and all the authors report no declarations of interest conflict. All authors reviewed and approved to be accountable for all aspects of the final manuscript.

Ethics declarations

This study was approved by the local ethics committee for animal and human use at the First Affiliated Hospital of Gannan Medical College. All experiments in this study were performed with human ethics approval from Gannan Medical University of Jiangxi province in China (approval number: LLSC-20190720). Written informed consent was obtained from all participants prior to collection of HUCMSCs. All experiments were performed following the National Health and Medical Research Council guidelines. This study was approved by the First Affiliated Animal Ethics Committee of Gannan Medical University (Approval No. 2020057). All animal experiments were conducted to relieve their pain in accordance with the international guidelines for the Care and Use of Laboratory Animals of the National Institute of Health.

Consent for publication

All the authors consent for publication this manuscript.
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**Figures**

![Figure 1](image-url)
Behavioural testing results of suspension test, slope experiment, open field test. Compared to sham group rats, behavioural testing results demonstrating that the neurobehavioral status of CP group rats are successfully-modeled . * P< 0.05, ** P< 0.01, *** P< 0.001.

Figure 2

Rat brain tissue stained with TTC. TTC staining reveals infarction and necrosis around the hippocampus of CP rat group. The sham rat brain showed normal appearance; The CP rat brain showed Cerebral infarction in the ligated side of the brain as a significant atrophic and destructive area.
Figure 3

Behavioural testing results of suspension test (Fig.1A), slope experiment (Fig.1B), open field test (Fig.1C). Compared to CP and CP+PBS group rats, behavioural testing results demonstrating that the neurobehavioral status of CP+HUCMSCs group rats are significantly alleviated. * P< 0.05, ** P< 0.01, *** P< 0.001.

Figure 4

The morphological observation of P0-4, P1-2, P2-2 HUCMSCs.
Figure 5

The flow cytometry analysis results of HUCMSCs specific surface markers.

Figure 6

The osteogenesis and adipogenesis test results of HUCMSCs.
Figure 7

The rat brain tissues slices stained with HE. Fig. 7A: The sham group rats; Fig. 7B: The CP group rats, Fig. 7C: The CP+PBS group rats; Fig. 7D: The CP+HUCMSCs group rats.

Figure 8
The DAPI successfully stained the brain tissue blue. The CP+HUCMSCs group rats damaged brain tissue showing many red points, and the CP+PBS group rats damaged brain tissue showing nothing.

**Figure 9**

The expression of NogoA, NgR, RhoA, Rac-1, and Cdc42 mRNA and protein in brain tissues of rats. * P< 0.05, ** P< 0.01, *** P< 0.001.