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Transplantation of umbilical cord blood mononuclear cells attenuates the expression of IL-1β via the TLR4/NF-κB pathway in hypoxic-ischemic neonatal rats

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umbilical cord blood mononuclear cell (UCBMC); hypoxic ischemic brain damage (HIBD); toll-like receptor 4 (TLR4); nuclear factor-kappa B (NF-κB); TLR4/NF-κB pathway; neonatal rats

ABSTRACT
Objective: This study aims to observe the effects of transplantation of umbilical cord blood mononuclear cells (UCBMCs) on the expression of interleukin (IL)-1β and explore the mechanism via the toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB) pathway in hypoxic-ischemic neonatal rats.

Methods: Seven-day-old Sprague–Dawley neonatal rats were randomly divided into Sham, hypoxic-ischemic brain damage (HIBD), and UCBMC groups. The HIBD model was prepared by Rice–Vannucci method, and UCBMC were transplanted 24 h after HIBD in the UCBMC group. At 7 days after transplantation, changes in neurons and the TLR4 protein were examined by neuronal nuclei (NeuN)/TLR4 immunofluorescence staining. The expression of pNF-κB and IL-1β proteins was detected by immunohistochemical staining and enzyme linked immunosorbent assay (ELISA).

Results: The percentage of NeuN/DAPI+ cells in the injured cortex in the UCBMC group was significantly lower than that in the Sham group (P < 0.05). The number of NeuN/TLR4+DAPI+ cells in the UCBMC group was significantly lower than that in the HIBD group (P < 0.05) but higher than that in the Sham group (P < 0.05). More pNF-κB+ cells were observed in the HIBD group than in Sham and UCBMC groups (P < 0.05), and more pNF-κB+ cells were observed in the UCBMC group than in the Sham group (P < 0.05). ELISA results showed that the IL-1β expression in the injured cerebral cortex in the UCBMC group was significantly lower than that in the HIBD group but remained higher than that in the Sham group (P < 0.05).

Conclusions: UCBM transplantation could inhibit the IL-1β protein expression in the injured cortex, thereby alleviating HIBD in neonatal rats. The underlying mechanism might be associated with the down-regulation of TLR4 and pNF-κB proteins.

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1 Introduction

Hypoxic-ischemic encephalopathy (HIE), a common but serious disease among infants and children, results from oxygen deprivation around the time of birth and could cause hypoxic-ischemic brain damage (HIBD). The morbidity of HIE in developing countries has increased in recent years [1]. Hypoxic-ischemic encephalopathy could result in cerebral palsy (CP), epilepsy, mental retardation, and so on, which cause heavy burden for the family and society [2]. However, the pathogenic mechanisms of HIE remain unknown, and no effective therapeutic agents have been developed. Therefore, scholars should explore effective therapeutic strategies for HIE. Inflammation injury plays an important role in the pathogenesis of HIE [3]. Toll-like receptor 4 (TLR4) is a widely studied member of mammalian TLRs and plays a great important role in regulation of inflammation [4, 5]. The TLR4 signaling pathway is very active in the regulation of immune reactions in the central nervous system, and TLR4 is involved in the release of inflammatory mediators after ischemic injury [6]. Nuclear factor-kappa B (NF-kB), a key downstream signal molecule of the TLR4 signaling pathway, could activate neural cells to secrete many pro-inflammatory mediators, including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, and induce brain injury [7]. Umbilical cord blood mono-nuclear cells (UCBMCs) contain a large number of stem and progenitor cells and could modulate immune reactions in many central nervous system diseases [8]. Previous studies showed that UCBMC treatment could alleviate HIBD, and improve CP and other neurological disorders [9]. UCBMC have been proved to produce many cytokines, which could regulate immune inflammation responses after brain injury [8]. In the present study, the effects of UCBMC transplantation on HIBD and pro-inflammatory mediator IL-1β were observed in hypoxic-ischemic neonatal rats. The associated mechanism was explored via the TLR4/NF-κB signaling pathway in order to provide scientific theoretical basis for clinical application of UCBMC transplantation.

2 Materials and methods

2.1 Experimental animals and study design

Forty-two 7-day-old Sprague-Dawley (SD) neonatal rats (11.2–15.0 g) were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Jinan, China) and randomly divided into three groups: (1) Sham group (n = 14), where the left common carotid artery was separated without ligation 7 days after birth, and 2 μL of normal saline was injected into the left lateral ventricle 8 days after birth; (2) HIBD group (n = 14), where the HIBD model was made and 2 μL of normal saline was injected into the left lateral ventricle 24 h after HIBD; (3) UCBMC group (n = 14), where the HIBD model was made and 3 × 10⁶/2 μL of UCBMC were injected into the left lateral ventricle 24 h after model preparation.

2.2 HIBD model

The HIBD model was prepared by Rice–Vannucci method [10]. Seven-day-old rats were anaesthetized by inhaling ether. The left common carotid artery was separated and permanently sutured between double ligatures with 4-0 sterile surgical silk. The rats were placed into the hypoxic chamber with oxygen concentration of 8.00% ± 0.01%, controlled temperature of (36 ± 1) °C, and humidity of 70% ± 5% for 2 h. Finally, the rats were returned to the mother rat cage for feeding.
2.3 UCBMC sources, preparation, and transplantation
Consent form was obtained from the participants. In brief, 50 mL of umbilical cord blood was collected from healthy full-term neonates and diluted with an equal volume of 0.01 mol/L sterilized phosphate buffer. The mononuclear cell separation solution was placed into the sterilized centrifuge tubes and added slowly with the diluted cord blood. After centrifugation at 2000 rpm for 20 min, the mononuclear cells were aspirated and washed with normal saline. Finally, the cell concentration was adjusted to 1.5 × 10^6/μL with normal saline. The solution was placed on ice prior to transplantation. At 24 h after HIBD, animals in the UCBMC group were transferred to a stereotaxic apparatus for lateral ventricle injection of the cell suspension with microsyringe (Hamilton, Reno, NV, USA) at coordinate: AP: −0.5 mm, ML: −2.5 mm, DV: −2 mm. Rats in Sham and HIBD groups were injected with 2 μL of normal saline at the same coordinate.

2.4 Tissue preparation for microscopy and ELISA examination
Seven days after transplantation, rats in each group were sacrificed by deep anesthesia and divided into two parts: (1) the first part (n = 8) was prepared for immunofluorescence double staining and immunohistochemical staining. After heart perfusion, the brain tissue was fixed in 4% paraformaldehyde overnight, regularly paraffin embedded, and coronally cut into 4 μm slices from 1.0 mm before bregma to 0.8 mm after bregma. For the other part (n = 6), the left cerebral cortex in each group was collected from 1.0 mm before bregma to 0.8 mm after bregma. The tissue was placed into liquid nitrogen to freeze and stored in a refrigerator at −86°C for ELISA examination. All animals were included in the analysis of the experimental results.

2.5 Immunofluorescence double-labeling staining
The slices were dewaxed and hydrated to water, and antigen was retrieved in citrate buffer at 98°C for 10 min. The slices were blocked with normal goat serum and placed in an incubator at 37°C for 1 h. The primary antibodies were added and incubated in a refrigerator at 4°C overnight. The primary antibodies were mouse anti-neuronal nuclei (NeuN; 1:100, Chemicon, USA) and rabbit anti-TLR4 (1:100, Millipore, USA). On the next day, after thorough washing, the slices were incubated with Alexa 488-conjugated goat anti-rabbit and Alexa Fluor 594 goat anti-mouse (1:100, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at 37°C for 1 h in the dark. The slices were washed and covered with fluorescence DAPI seal (F6057, Sigma, USA). Positive cells were counted and analyzed by fluorescence microscope (BX-51, Olympus, Japan) and analysis software (Cellens 1.6 software).

2.6 Immunohistochemical staining
The slices were dewaxed with xylene and rehydrated by gradient ethanol. Antigen retrieval was performed at 98°C for 10 min in citrate buffer. The sections were immersed in 3% H2O2 for 30 min to block endogenous peroxidases. The slices were incubated with normal goat serum for 1 h at 37°C. The sections were incubated with rabbit anti pNF-αB (1:100, CST, USA) overnight at 4°C. After thorough washing, the slices were incubated with two-step conjugated antibody (PV9001, Beijing Zhongshan Golden Bridge Biological Reagent Co., Ltd., Beijing, China). Finally, the slices were stained by diaminobenzidine (DAB) and covered with neutral gum.

2.7 ELISA
The damaged cerebral cortex tissue in each group was collected. Normal saline was added
to the brain tissue according to quality of the tissue. The brain tissue was homogenized in ice water and centrifuged at 3000 rpm. The supernatant was collected and examined according to instructions of IL-1β ELISA kits (R&D Systems, Minneapolis, MN, USA).

2.8 Statistical analysis

SPSS 18.0 statistical software was used for statistical analysis. All data were presented as mean ± standard deviation (x ± s). Homogeneous variance data were tested by one-way ANOVA. Pairwise comparison between each two groups was conducted by SNK-q. P < 0.05 was considered significant.

3 Results

3.1 UCBMC could inhibit the expression of TLR4 and protect neurons

Neuronal nuclei is a mature neuron marker. Seven days after transplantation, NeuN+ cells were detected by immunofluorescence staining. The nuclei of NeuN+ cells were stained red. The percentage of NeuN+DAPI+ cells in the injured cortex to those in the contralateral cortex in the HIBD group was significantly lower than those in Sham and UCBMC groups. The percentage of NeuN+DAPI+ cells in the UCBMC group was significantly lower than that in the Sham group, and the difference was significant (all P < 0.05).

Fig. 1 UCBMC inhibited the expression of TLR4 and protected the neuron. (A) NeuN/TLR4/DAPI immunofluorescence staining results (400 ×) of the damaged lateral cortex in each group 7 days after transplantation. Nuclei were stained blue with DAPI. Mature neurons were marked by anti-NeuN with red in the nuclei. TLR4 was stained green on the cell membrane. NeuN+DAPI+ cells could be observed in all three groups, and the percentage of NeuN+DAPI+ cells was relatively high in the Sham group. NeuN+TLR4+DAPI+ cells were also observed in all three groups. Unlike NeuN+DAPI+ cells, the number of NeuN+TLR4+DAPI+ cells was obviously lower in Sham group than in the other two groups. White arrows indicate the NeuN+TLR4+DAPI+ cells. Scale bar = 20 μm. (B) The percentage of NeuN+DAPI+ cells in the injured cortex to those in the contralateral cortex in each group. Error bars indicate SD. *, P < 0.05 vs. the HIBD group; †, P < 0.05 vs. the UCBMC group. (C) The number of NeuN+TLR4+DAPI+ cells in the cortex in each group. Error bars indicate SD. *, P < 0.05 vs. the HIBD group; †, P < 0.05 vs. the UCBMC group.
TLR4, which plays an important role in the TLR4/NF-κB signaling pathway, is stained green on the cell membrane by immunofluorescence staining. The number of NeuN+TLR4+DAPI+ cells in the HIBD group increased significantly compared with those in Sham and UCBMC groups \((P < 0.05)\). The number of NeuN+TLR4+DAPI+ cells in the UCBMC group was significantly higher than that in the Sham group \((P < 0.05)\), and the difference was significant (Fig. 1).

### 3.2 UCBMC could reduce the expression of the pNF-κB protein in the damaged cerebral cortex of neonatal HIBD rats

NF-κB is the main downstream signal molecule of the TLR4 signaling pathway, and pNF-κB is a form of NF-κB that is activated by phosphorylation. The pNF-κB+ cells containing activated NF-κB was examined by immunohistochemical staining, with nuclei stained brown-yellow. This finding indicated that the phosphorylated NF-κB being transferred to the nucleus. The number of pNF-κB+ cells in the HIBD group was significantly higher than in the other two groups, whereas that in the UCBMC group was higher than in the Sham group; the difference was significant (all \(P < 0.05\)) [Figs. 2(A) and (B)]. Therefore, UCBMC transplantation could reduce the expression of pNF-κB, that is, UCBMC could inhibit the activation of NF-κB.

### 3.3 UCBMC could attenuate the expression of the IL-1β protein in the damaged cerebral cortex in neonatal HIBD rats

IL-1β is one of the most widely used representative pro-inflammatory mediators. The expression of IL-1β was examined by ELISA. According to our examination, the expression of IL-1β in the HIBD group was higher than that in Sham and UCBMC groups, and the difference was significant \((P < 0.05)\). The concentration of IL-1β in the UCBMC group was still higher than

![Fig. 2](image-url)

**Fig. 2** UCBMC attenuated the expression of pNF-κB and IL-1β protein. (A) pNF-κB immunohistochemical staining results (400 × 7 days after transplantation. In the Sham group, only a few pNF-κB+ cells were observed in the nuclei, there existed a large number of pNF-κB+ cells in the HIBD group; and there were some pNF-κB+ cells in the UCBMC group. Scale bar = 20 μm. Black arrows indicate the NeuN-TRLR4/DAPI+ cells. (B) The number of pNF-κB+ cells in the cortex in each group. Error bars indicate SD, * \(P < 0.05\) vs. the HIBD group. \(\dagger\) \(P < 0.05\) vs. the UCBMC group. (C) The concentration of IL-1β protein in the cortex in each group was examined by IL-1β ELISA kit. Error bars indicate SD, * \(P < 0.05\) vs. the HIBD group; \(\dagger\) \(P < 0.05\) vs. the UCBMC group.
that in the Sham group, and the difference was significant \( (P < 0.05) \) [Fig. 2 (C)]. Thus, UCBMC transplantation could inhibit the expression of IL-1β.

4 Discussion and conclusion

HIE is a common disease during the neonatal period and could result in several severe diseases. Inflammation plays an important role in the development of HIBD; therefore, inhibition of inflammation after HIBD plays a major role in the therapy of HIBD [11]. In the present study, HIBD model was prepared to simulate HIE disease and UCBMC were transplanted into the lateral ventricle of the HIBD model to observe the therapeutic effects of UCBMC transplantation and explore its underlying mechanism. Based on the results, neurons in the cerebral cortex were damaged severely in the HIBD model, but UCBMC transplantation alleviated the loss of neurons. This finding indicated that the HIBD model was successfully made and that UCBMC transplantation attenuated HIBD, consistent with previous reports [9]. However, the mechanism of UCBMC transplantation on HIBD should be further elucidated. In this regard, the present work explored the underlying mechanism of UCBMC transplantation.

TLR4 plays a key role in activation and modulation of neuroinflammatory responses and is closely associated with brain injury [12]. In the present study, the expression of the TLR4 protein in neurons was examined by NeuN/TLR4/DAPI immunofluorescence staining 7 days after HIBD. The results showed that, comparing with the Sham group, more NeuN+/TLR4+/DAPI- cells were observed in the HIBD model but less in the UCBMC group. As such, HIBD increased the expression of the TLR4 protein in the membrane of neurons, while UCBMC transplantation inhibited the expression in hypoxic-ischemic neonatal rats. However, whether the increase in TLR4 protein expression could alleviate the following inflammation injury remains uncertain. Studies showed that NF-κB could be activated by the upstream molecule TLR4, phosphorylated, and transferred to nuclei, resulting in inflammation injury [13]. In the present work, the expression of pNF-κB in the nuclei increased significantly in the HIBD model 7 days after HIBD, indicating that hypoxia–ischemia activated NF-κB. After UCBMC transplantation, the expression of pNF-κB in the nuclei decreased 7 days after HIBD. As TLR4 is the upstream molecule of NF-κB in the TLR4/NF-κB pathway and could regulate the activation of NF-κB, UCBMC could regulate the activation of NF-κB by inhibiting the activation of TLR4. Previous studies showed that the activation of NF-κB could increase the expression of inflammatory factors in the damaged brain. As such, we examined the expression of IL-1β protein by ELISA. The expression of IL-1β in the cortex in the HIBD group was higher than that in the Sham group, indicating that HIBD could increase the expression of IL-1β. Meanwhile, the expression of the IL-1β protein in the cortex in the UCBMC group was lower than in the HIBD group but still higher than that in the Sham group. This finding indicated that UCBMC transplantation could inhibit the expression of the IL-1β protein. However, the therapeutic effect of single transplantation of UCBMC is limited and could only attenuate the inflammation injury to some extent. Further studies should determine whether multiple UCBMC transplantation could improve the therapeutic effect of UCBMC transplantation and attenuate the brain damage in the long run.
In conclusion, UCBMC transplantation could alleviate the expression of the IL-1β protein and attenuate brain damage and could play a neuroprotective role in hypoxic-ischemic neonatal rats. The underlying mechanism maybe associated with the TLR4/NF-κB signaling pathway.

Conflict of interests

All contributing authors declare no conflict of interests in this work.

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