Expression of hypoxia-inducible factor 1 alpha and oligodendrocyte lineage gene-1 in cultured brain slices after oxygen-glucose deprivation☆

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Abstract

Oligodendrocyte lineage gene-1 expressed in oligodendrocytes may trigger the repair of neuronal myelin impairment, and play a crucial role in myelin repair. Hypoxia-inducible factor 1α, a transcription factor, is of great significance in premature infants with hypoxic-ischemic brain damage. There is little evidence of direct regulatory effects of hypoxia-inducible factor 1α on oligodendrocyte lineage gene-1. In this study, brain slices of Sprague-Dawley rats were cultured and subjected to oxygen-glucose deprivation. Then, slices were transfected with hypoxia-inducible factor 1α or oligodendrocyte lineage gene-1. The expression levels of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 were significantly up-regulated in rat brains prior to transfection, as detected by immunohistochemical staining. Eight hours after transfection of slices with hypoxia-inducible factor 1α, oligodendrocyte lineage gene-1 expression was upregulated, and reached a peak 24 hours after transfection. Oligodendrocyte lineage gene-1 transfection induced no significant differences in hypoxia-inducible factor 1α levels in rat brain tissues with oxygen-glucose deprivation. These experimental findings indicate that hypoxia-inducible factor 1α can regulate oligodendrocyte lineage gene-1 expression in hypoxic brain tissue, thus repairing the neural impairment.

Key Words

neural regeneration; brain injury; biological factors; hypoxia-inducible factor 1α; oligodendrocyte lineage gene-1; oxygen-glucose deprivation; brain slice culture; immunohistochemistry; oligodendrocyte; myelin repair; premature delivery; rat; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) Brain slices in premature rats were subjected to oxygen-glucose deprivation to generate human brain slice culture models of premature infants with hypoxic-ischemic brain damage.
(2) The expression levels of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 were dynamically observed in a broader attempt to explore their expression during hypoxic-ischemic brain damage and their roles in nerve repair.
(3) Hypoxia-inducible factor 1α can regulate oligodendrocyte lineage gene-1 in hypoxic brain tissue, thus repairing the neural impairment.

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INTRODUCTION

Despite recent developments in the fields of perinatal and neonatal medicine, there is still less improvement in treatments for abnormal cognition and behavior in premature infants[1-3]. Repair mechanisms and treatments for brain injury in prematurity are increasingly attracting the attention of neonatal and perinatal medicine experts[4-5]. However, the molecular biological mechanisms underlying brain injury in premature infants are also poorly understood.

Prenatal, perinatal and postnatal brain injury is regarded as the leading cause of long-term severe neurological defects[6-7]. Early brain injury results from many potential causes, rather than a single factor[8]. The main mechanisms of pathogenesis proposed include hypoxia/ischemia and infection[9-11]. Ischemia causes the activation of microglial cells, triggers an inflammatory reaction, increases local blood flow, and eventually changes the functions of glial cells and neurons[12]. Endothelial cells are also involved in the inflammatory reaction through generating proinflammatory mediators; these mediators alter local blood flow and vascular permeability, and promote leukocyte adhesion[13]. Cytokines are toxic to cerebral white matter by inhibiting differentiation of oligodendrocyte progenitor cells, inducing glial cell apoptosis and triggering myelin degradation[14].

Most studies on functional rehabilitation after brain injury have been confined to the protection of neurons, while few have focused on glial cells such as oligodendrocytes. Oligodendrocytes play important roles in the development and prognosis of periventricular leukomalacia[15]. Therefore, knowledge of the mechanism underlying oligodendrocyte injury could contribute to better treatment of white matter damage, provide a new therapeutic strategy for neurological defects, and serve as a new target for promoting the recovery of neurological functions.

Transcription factor oligodendrocyte lineage gene-1 expression in oligodendrocytes may trigger the repair of damaged neuronal myelin[16-18]. Therefore, oligodendrocyte lineage gene-1 plays a crucial role in myelin repair[16-18]. Oligodendrocyte lineage gene-1 is expressed in oligodendrocytes and repairs myelin after brain demyelination injury[16]. Oligodendrocyte lineage gene-1 is essential for the development of myelin, and knockout mice for this gene show shortened survival[17]. Oligodendrocyte lineage gene-1 knockout mice also

show glial cell retardation[18]. In addition, oligodendrocyte lineage gene-1 is of importance in the later stages of oligodendrocyte development[19]. Expression of oligodendrocyte lineage gene-1 also influences myelin repair in the brain tissues of rodents and multiple sclerosis patients[20]. Oligodendrocyte lineage gene-1 is necessary for myelin regeneration after neural progenitor cells transplantation in virus-induced demyelination models[21]. Oligodendrocyte lineage gene-1 is also expressed during the maturation and regeneration of human oligodendrocytes[22].

Similarly, hypoxia-inducible factor 1 plays an extremely key protective role following hypoxic-ischemic brain damage. Hypoxia-inducible factor 1α and tissue adaptability-related protein expressed in oligodendrocytes may protect against neurological impairment[23]. Hypoxia-inducible factor 1α promotes the proliferation and differentiation of endogenous neural stem cells[24]. Hypoxia-inducible factor 1α is also involved in the functions of neural stem cells and post-stroke regeneration[25]. Hypoxia-inducible factor 1α exerts neuroprotective effects and neurotoxic effects based on the cell type and severity of hypoxia/ischemia[26].

In this study, brain slices of immature rats were subjected to oxygen-glucose deprivation to simulate human premature infants with hypoxic-ischemic brain damage. Expression of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 protein expression was detected using immunohistochemistry. The goal of this study was to detect the expression of these two proteins after hypoxic-ischemic brain damage, and to explore the roles of the two in nerve repair.

RESULTS

Brain slice groupings

Brain slices from 3-day-old rats were randomly divided into four groups: a blank control group (brain slices were cultured in complete medium, without hypoxia and glucose deprivation); an oxygen-glucose deprivation group [brain slices were cultured in hypoxia (8% oxygen + 92% nitrogen) and deprived from glucose (glucose-free medium) for 60 minutes, and then oxygen glucose supply was restored]; a hypoxia-inducible factor 1α transfection group (brain slices were deprived from oxygen-glucose and transfected with hypoxia-inducible factor 1α); and an oligodendrocyte lineage gene-1 transfection group (brain slices were deprived from oxygen-glucose and transfected with oligodendrocyte lineage gene-1). Five

brain slices in each group were collected at 4 hours, 8 hours, 1 day and 3 days after transfection.

General observation of cultured brain slices

The cultured brain slices grew well and became thinner as the culture advanced. The slice thickness decreased from 450 μm to 150 μm at 1 week under an inverted microscope, and was maintained at 150 μm for 30 days. The brain slice structure could be clearly seen (Figure 1).
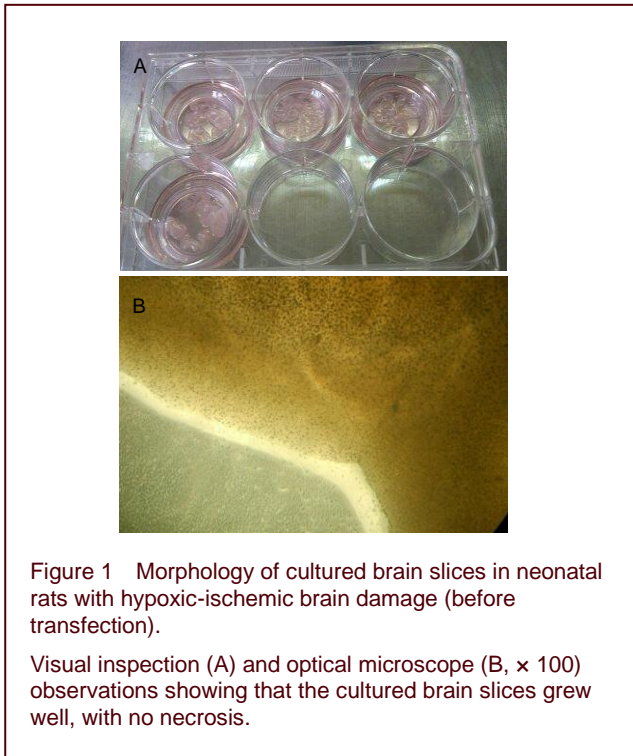

Figure 1   Morphology of cultured brain slices in neonatal rats with hypoxic-ischemic brain damage (before transfection).

Visual inspection (A) and optical microscope (B, × 100) observations showing that the cultured brain slices grew well, with no necrosis.

Identification of oxygen-glucose deprived models

At 4 hours after modeling, the blank control group showed clear cell structure, intact nuclear membrane, clear nucleoli, and evenly distributed chromatin (Figures 2A–D). In the oxygen-glucose deprivation group, cytoplasm rupture, nuclear pyknosis, karyorrhexis, and karyolysis were observed (Figures 2E–H).

Compared with the blank control group, the neuronal nuclear pyknosis and karyorrhexis were seen in rat cerebral cortex, hippocampus and lateral ventricle were significantly attenuated after oxygen-glucose deprivation (Figure 3).

Expression of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 in hypoxic-ischemic brain damage

After oxygen-glucose deprivation, hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 expression levels were both up-regulated in the brain tissue of

oxygen-glucose deprived rats, with a more obvious increase in the expression level of hypoxia-inducible factor 1α (Figures 4, 5).
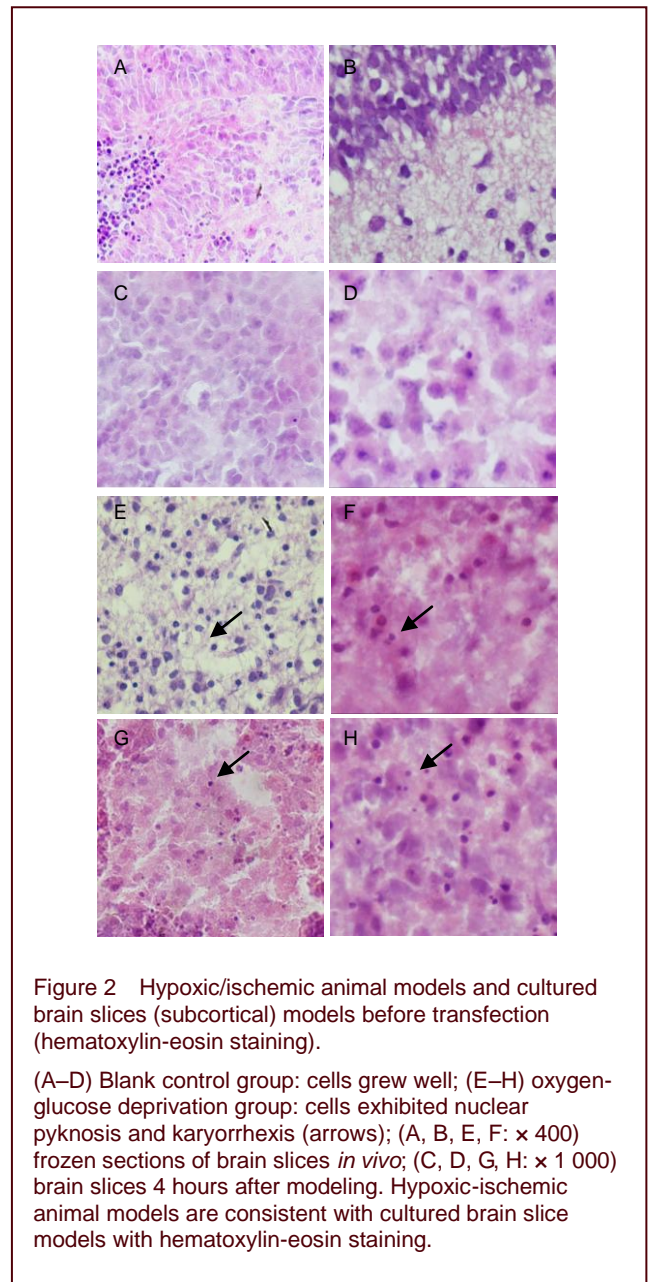

Figure 2   Hypoxic/ischemic animal models and cultured brain slices (subcortical) models before transfection (hematoxylin-eosin staining).

(A–D) Blank control group: cells grew well; (E–H) oxygen-glucose deprivation group: cells exhibited nuclear pyknosis and karyorrhexis (arrows); (A, B, E, F: × 400) frozen sections of brain slices *in vivo*; (C, D, G, H: × 1 000) brain slices 4 hours after modeling. Hypoxic-ischemic animal models are consistent with cultured brain slice models with hematoxylin-eosin staining.

As shown in Figures 4 and 5, the expression levels of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 were the same after oxygen-glucose deprivation. Their expression levels began to increase at 8 hours after oxygen-glucose deprivation, reaching a peak at 1 day, and gradually decreased at 3 days. The expression of hypoxia-inducible factor 1α in the oxygen-glucose deprivation group was significantly higher than that in the control group at 8 hours, 1 day and 3 days ($P < 0.05$), while oligodendrocyte lineage gene-1 expression in the oxygen-glucose deprivation group was significantly higher than that in the control group at 1 day ($P < 0.01$).
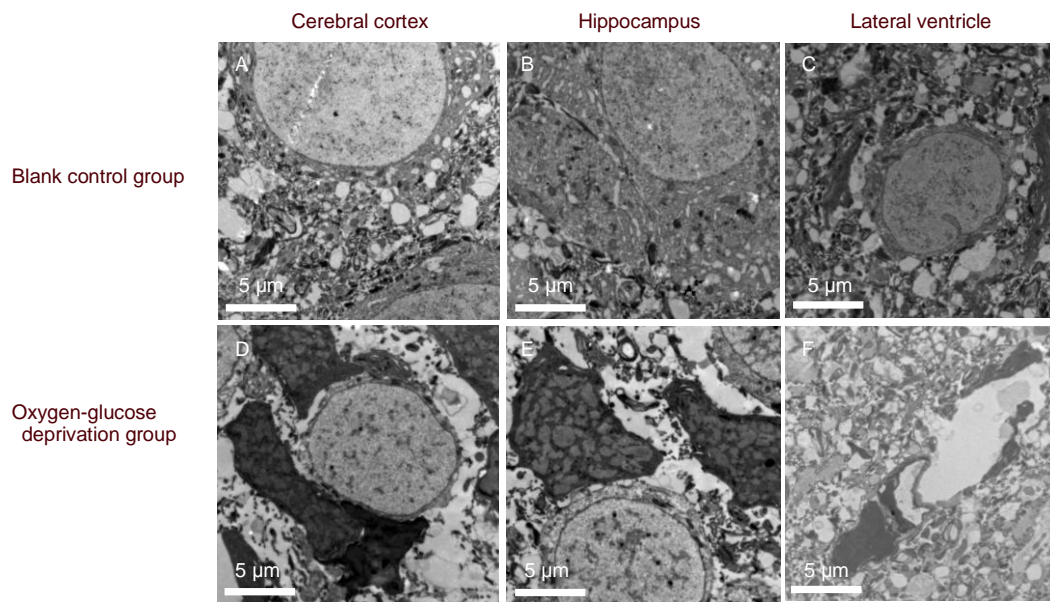
Figure 3   Ultrastructure of rat brain slices in oxygen-glucose deprived rats before transfection (uranyl acetate-lead citrate staining, transmission electron microscopy, × 3 000).

Compared with the blank control group, nuclear pyknosis, karyorrhexis, and myelinated axons in rat cerebral cortex, hippocampus and lateral ventricle were significantly attenuated after oxygen-glucose deprivation. Neuropil edema and mitochondrial swelling were visible.
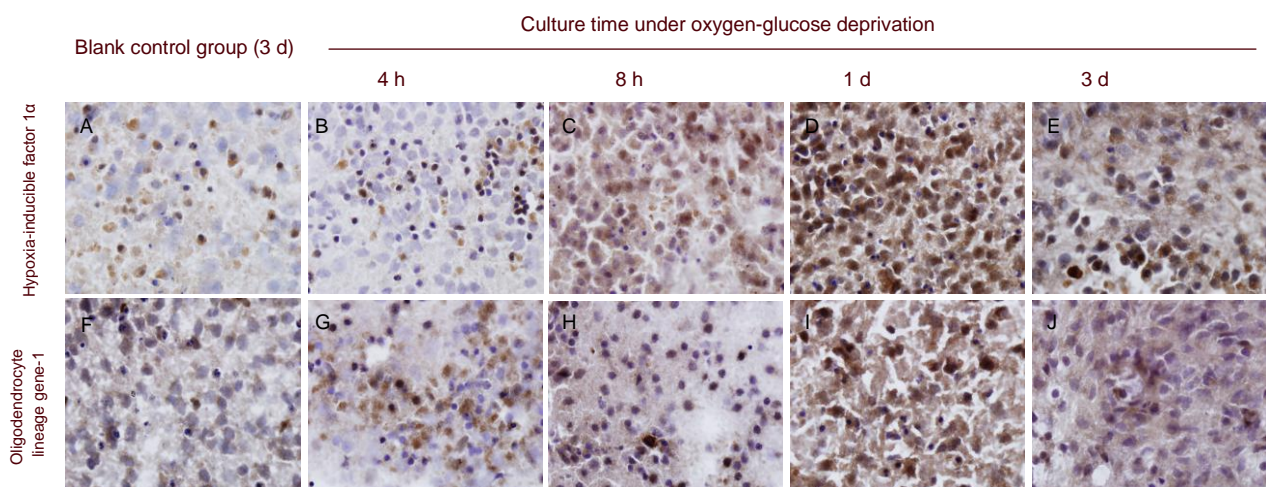

Figure 4   Expression of hypoxia-inducible factor 1α (A–E) and oligodendrocyte lineage gene-1 (F–J) in brain tissue of oxygen-glucose deprived rats prior to transfection (immunohistochemical staining, × 1 000).

Expression levels of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 were up-regulated at each time point, especially hypoxia-inducible factor 1α.
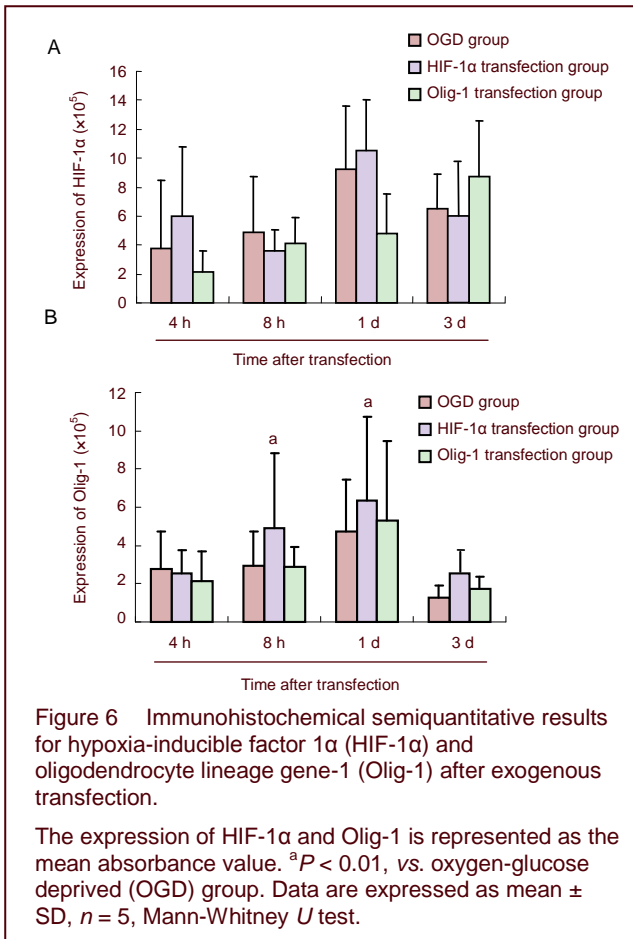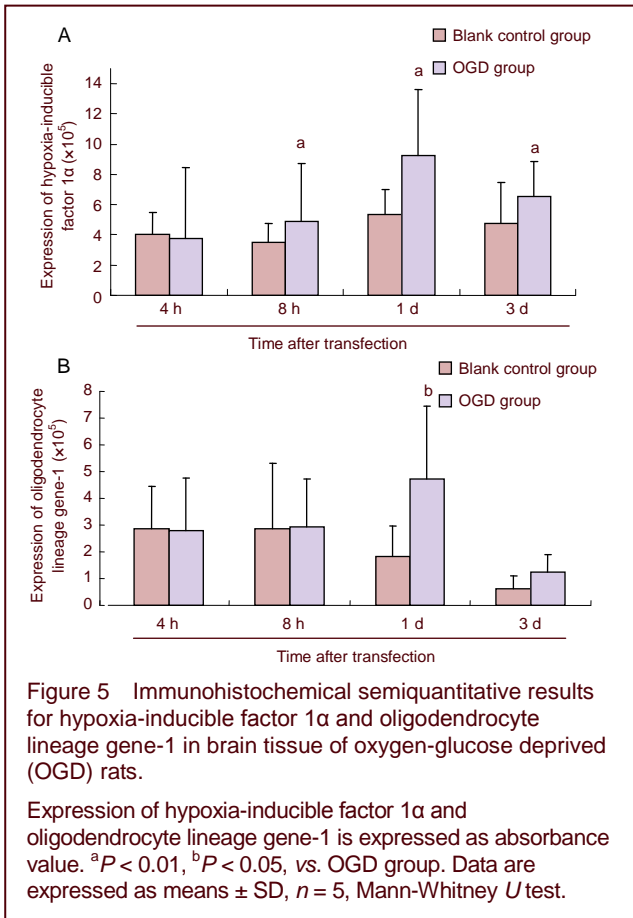
Expression of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 in rat brains after exogenous transfection

At 8 hours after exogenous transfection of slice cultures, hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 expression in brain slices began to rise, reaching a peak at 1 day. The expression of oligodendrocyte lineage gene-1 at each time point was significantly increased after transfection, compared with the oxygen-glucose deprivation group ($P = 0.018$, 0.001; Figure 6).

DISCUSSION

The survival rates of premature infants with very low birth weight increase. However, neurodevelopmental sequelae are evident in the growth and development process, mainly due to brain injury in infants.

Figure 5   Immunohistochemical semiquantitative results for hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 in brain tissue of oxygen-glucose deprived (OGD) rats.

Expression of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 is expressed as absorbance value. $^aP < 0.01$, $^bP < 0.05$, *vs*. OGD group. Data are expressed as means ± SD, $n = 5$, Mann-Whitney U test.

Figure 6   Immunohistochemical semiquantitative results for hypoxia-inducible factor 1α (HIF-1α) and oligodendrocyte lineage gene-1 (Olig-1) after exogenous transfection.

The expression of HIF-1α and Olig-1 is represented as the mean absorbance value. $^aP < 0.01$, *vs*. oxygen-glucose deprived (OGD) group. Data are expressed as mean ± SD, $n = 5$, Mann-Whitney U test.

Periventricular leukomalacia is one of the most common brain injuries in premature infants, and the leading cause of premature death and neurological sequelae. Brain injury in premature infants is often induced by hypoxia/ischemia and septicemia[27-30]. The selective vulnerability of oligodendrocyte precursor cells to hypoxic/ischemic injury provides a pathological explanation for the white matter damage in preterm infants. Free radicals and glutamate-mediated toxicity and infection may cause oligodendrocyte injury. Oligodendrocyte precursor cells injury hinders myelin formation in white matter, and results in cerebral palsy and mental retardation. Therefore, understanding of the repair mechanisms after oligodendrocyte injury could provide a new strategy for the prevention of the neurological defects associated with periventricular leukomalacia. In addition, it can be regarded as a new target for promoting the recovery of neurological function. Hypoxia-inducible factor 1 is a transcription factor that regulates the expression of more than 70 target genes. Many genes such as vascular endothelial growth factor have been confirmed as target genes of hypoxia-inducible factor 1[31-34]. When hypoxia/ischemia occurs, hypoxia-inducible factor 1 can strongly modulate target gene expression[35-38], improve energy metabolism disorder, promote cerebral hemodynamic recovery, inhibit excitatory amino acid toxicity, and reduce apoptosis[39]. The hypoxia-inducible factor 1α expression level decreases as hypoxia is attenuated[40-43]. Hypoxia-inducible factor 1α gene knockout (hypoxia-inducible factor 1α$^{+/-}$) aggravated cerebral ischemia and reduced survival rates in mice[36]. When brain white matter injury occurs, accompanying myelin-associated glycoprotein loss and oligodendrocyte apoptosis, hypoxia-inducible factor 1 is expressed in oligodendrocyte cell nuclei[44]. Hypoxia-inducible factor 1α and tissue adaptability-related protein expression in oligodendrocytes play a neuroprotective role, and antagonize the injury[23]. In addition, hypoxia-inducible factor 1α promotes the proliferation and differentiation of endogenous neural stem cells[24]. Therefore, hypoxia-inducible factor 1α is of great significance in the prevention of hypoxic-ischemic brain damage. Hypoxia and ischemia mediate nerve demyelination, and expression of oligodendrocyte lineage gene-1 is closely correlated with myelin repair. In the myelin repair process, oligodendrocyte lineage gene-1 expression undergoes a dynamic change. Under normal circumstances, oligodendrocyte lineage gene-1 is located in the cytoplasm of stationary oligodendrocyte precursor cells. After myelin is damaged, oligodendrocyte lineage gene-1 expressed in the nuclei of oligodendrocyte precursor

cells. When cells begin to differentiate, oligodendrocyte lineage gene-1 returns to the cytoplasm[20].

No work to date has tried to explore the correlation between hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 in premature infants with hypoxic-ischemic brain damage. Studies on hypoxia-inducible factor 1α expression in astrocytes[37, 45] have shown a rapid rise in hypoxia-inducible factor 1α expression at 30–60 minutes after the onset of hypoxia. By contrast, in this study we observed increased expression at 8 hours after the onset of hypoxia. The variation depends on the study subject: the former study focused on astrocytes, while our study focused on brain slices. Generally, organs will show changes later than cells. The rapidly expressed hypoxia-inducible factor 1α can quickly regulate target gene expression, and allows adaptive and protective changes in cells and tissues.

Brain slice culture is a new method for nervous system research. The methods for establishment of cell culture and animal models have developed considerably, and are widely used for physiological and pathological studies *in vivo*. However, the shortcomings are inevitable. Cell culture is characterized by easy operation, clear sample features, and economic efficiency. But *in vitro* culture environment may lose links between tissues and cells, as well as related biochemical properties. Although animal models can reflect pathological and physiological reactions, experimental operations are limited and affected by many factors *in vivo*. Therefore, brain slice culture represents a platform between cell culture and animal models, being suitable for *in vitro* experiments owing to easy operation and easy observation, but providing a tissue structure close to the *in vivo* tissue structure.

We used 3-day-old Sprague-Dawley rats to establish brain slice culture models. On one hand, 3-day-old rats are equivalent to human infants at metaphase pregnancy, namely premature infants at 23–32 weeks[46]; on the other hand, 3-day-old rat brains are highly plastic and highly tolerant to mechanical damage, thus increasing culture success rates. In this study, growing concerns were paid to the following. (1) Brain slices were cultured using a microporous membrane, because of the simplicity of operation, long neuronal survival, and good cell reactivity. (2) Brain slices were placed at the gas-liquid interface, immersing them in medium so they could absorb nutrition and exposing them to the air to obtain a sufficient oxygen supply. (3) Because of mechanical damage in the process of preparing brain

slices, too thin brain slices are not conducive to cell survival. Too thick brain slices may hinder oxygen and nutrient supply, and decrease cell survival rates. Therefore, the thickness of brain slices in this study was 450 μm. (4) To ensure the survival of brain slices, slices should be harvested as quickly as possible and maintained at low temperatures in aseptic conditions.

In this study, we determined the viability of brain slices by gross and microscopic observations, and then compared frozen sections between conventional brain tissue and brain tissue cultured for 3 days using hematoxylin-eosin staining. As shown in Figure 2, the cultured brain tissue exhibited good viability and clear structure. Brain injury in premature infants usually involves damage to hippocampal neurons and around the lateral ventricle, so here we choose to use whole brain slices for culture. Our experimental findings showed that hypoxia-inducible factor 1α expression was increased at 8 hours after oxygen-glucose deprivation and virus transfection, reaching a peak at 1 day, and then gradually decreasing. The expression level at day 3 was still significantly higher than that in the control group. Compared with the peak times in previous animal models (4–8 hours), the peak time of hypoxia-inducible factor 1α expression in this study was slightly delayed. This difference possibly resulted from the development and metabolism disorders following brain slice culture. After oxygen-glucose deprivation and transfection, oligodendrocyte lineage gene-1 expression remained unchanged at 8 hours, reached a peak at 1 day and began to decrease at 3 days. There was no significant difference in the expression levels of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 following hypoxia/ischemia. Oligodendrocyte lineage gene-1 expression began to rise at 8 hours and reached a peak at 1 day after exogenous transfection of slices with hypoxia-inducible factor 1α. In addition, oligodendrocyte lineage gene-1 expression in the hypoxia-inducible factor 1α transfection group was significantly higher than that in the oxygen-glucose deprivation group at each time point. Conversely, exogenous transfection of oligodendrocyte lineage gene-1 had no impact on the expression level of hypoxia-inducible factor 1α relative to that in the oxygen-glucose deprivation group. This evidence suggested that hypoxia-inducible factor 1α may up-regulate oligodendrocyte lineage gene-1 expression. Our research group previously analyzed the gene sequence of oligodendrocyte lineage gene-1 and found several possible hypoxia-inducible factor 1α binding sites in the promoter region, within the gene and downstream of the gene. Therefore, we speculate that hypoxia-inducible

factor 1α regulates oligodendrocyte lineage gene-1 expression, and accelerates the repair process after hypoxic-ischemic brain damage. In summary, hypoxia-inducible factor 1α, a transcription factor involved in the rapid response to tissue hypoxia, shows increased expression following hypoxic-ischemic brain damage and in turn up-regulates oligodendrocyte lineage gene-1 expression. Oligodendrocyte lineage gene-1 plays an important role in oligodendrocyte development and differentiation, and is also necessary for remyelination, eventually triggering neuroprotective mechanisms and providing a favorable environment for tissue repair.

MATERIALS AND METHODS

Design
A randomized, controlled, animal experiment.

Time and setting
Experiments were performed in the Department of Pediatrics, Beijing Friendship Hospital Affiliated to Capital Medical University from May 2009 to May 2010.

Materials
Twelve 3-day-old Sprague-Dawley specific pathogen-free rats, irrespective of gender, weighing 8–10 g, were provided by Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., China. The license number is SCXK (Beijing) 2007-0001. Experimental disposals were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[47].

Methods
Preparation of brain slices
According to the methods of Adamchik *et al* [48-49] with some modifications, brain slices were prepared. In brief, Sprague-Dawley rats were disinfected with 75% alcohol and killed. The brains were quickly placed in precooled anatomical buffer for 15 minutes. The buffer contained 64% Dulbecco's modified Eagle's medium, 32% Hank's balanced salt solution (Gibco, New York, NY, USA), 6.5 g/L D-glucose, 2.98 mg/L hydroxyethyl piperazine ethanesulfonic acid (Sigma, St. Louis, MO, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin (Gibco). The meninges were removed under an anatomical microscope (Leica, Wetzlar, Germany). Brain tissues were cut into slices using a microtome (TED PELLA, Redding, CA, USA), at

450 µM thickness. Brain slices were cultured in 1 mL of complete culture medium (containing 50% Dulbecco's modified Eagle's medium, 25% Hank's balanced salt solution, 25% equine serum (Gibco), 2.98 mg/L hydroxyethyl piperazine ethanesulfonic acid, 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin, and 6.5 g/L D-glucose) in 6-well culture plates (Costar, Vernon, CA, USA). Then, cultured slices were transferred to Millicell culture plates (Gibco) and incubated at 37°C in an incubator (Heraeus, Wetzlar, Germany) in 95% O_2 + 5% CO_2 and saturated humidity. Half of the culture medium was changed twice per week.

Establishment of oxygen-glucose deprived models
Brain slices cultured for 36 hours were respectively placed in 92% nitrogen + 8% oxygen, glucose-free culture medium, in a 37°C incubator for the oxygen-glucose deprivation group, hypoxia-inducible factor 1α transfection group and oligodendrocyte lineage gene-1 transfection group. Oxygen and glucose supply was restored 60 minutes later[50]. Hematoxylin-eosin staining was applied to observe nuclear pyknosis, karyorrhexis, and fusion.

Target gene transfection
Brain slices were transfected with 2 × 10^7 IU Ad5-oligodendrocyte lineage gene-1 and Ad5-hypoxia-inducible factor 1α virus (Vector Gene Technology Co., Ltd., Beijing, China). In brief, 2 × 10^7 IU virus was diluted to 400 µL complete culture medium and transfected for 4 hours. Then, virus was discarded and fresh normal medium was added to Millicell culture plates[51]. The blank control group and oxygen-glucose deprivation group received no treatment.

Morphology of brain slices
Cultured brain slices were observed twice a week under an inverted microscope (Olympus, Tokyo, Japan), to detect brain slice viability, growth and structure.

Frozen sections of brain slices
The cultured brain slices were fixed with 10% formaldehyde and incubated with 30% sucrose overnight. Each brain slice was then cut into 2–3 frozen sections at 20 µm thickness.

Hematoxylin-eosin staining for brain slices and histological changes under light microscope
After 4 hours of transfection, brain slices were stained with 10% Harris hematoxylin for 3 minutes and differentiated with 0.5% hydrochloric acid alcohol. Blue dye was developed with saturated lithium carbonate

water. Slices were rinsed with distilled water for several seconds and stained with 0.5% eosin for 1 minute. Afterwards, slices were dehydrated in graded ethanol, cleared in xylene, and mounted with gum. Cell morphology and nuclei were observed under an optical microscope (Olympus).

Electron microscope observation

Rat cerebral cortex, hippocampus and lateral ventricle tissue were fixed with 2.5% glutaraldehyde for 2 hours, rinsed with PBS three times, and fixed with 1% osmic acid for additional 2 hours, and finally dehydrated in gradient alcohol, for epoxy propane/resin replacement. Slices were embedded in pure resin, trimmed, and cut into semithin sections. Sections were stained with azure-methylene, and positioned under light microscope. Then semithin sections were further sliced into ultrathin sections and stained with uranyl acetate-lead citrate, finally observed under an H-7650 electron microscope.

Expression of hypoxia-inducible factor 1α and oligodendrocyte lineage gene-1 in rat brain tissues as detected by immunohistochemical staining

Immunohistochemical analysis was performed at 4 hours, 8 hours, 1 day, and 3 days after transfection. Frozen sections were soaked in distilled water for 10 minutes and treated with 3% H_2O_2 for 10 minutes. Then, sections were rinsed with distilled water twice for 5 minutes each; citrate buffer was added and sections were microwaved for 1–2 minutes, to make the solution boiling. Afterwards, sections were soaked in cold water to cool them to room temperature, and rinsed with PBS twice for 5 minutes. Sections were blocked with 5% bovine serum albumin at room temperature for 20 minutes, and incubated with rabbit anti-oligodendrocyte lineage gene-1 polyclonal antibody (1:3 000; Chemicon, CA, USA) or rabbit anti-hypoxia-inducible factor 1α polyclonal antibody (1:100; Boster, Wuhan, China) at 4°C overnight. Sections were then rinsed with PBS three times for 5 minutes each time, and incubated with goat anti-rabbit IgG (Boster) at 37°C for 20 minutes. After three 5-minute PBS washes, sections were incubated with SABC at 37°C for 20 minutes, and again rinsed with PBS four times, for 5 minutes each time. Sections were developed with 3,3'-diaminobenzidine, to 50 seconds, and counterstained with hematoxylin for 40 seconds, followed by a series of procedures: dehydration, transparency and mounting. Five to ten visual fields (1 000 × magnification) randomly selected from each frozen section were used for image analysis. Immunohistochemical images were analyzed using Image Pro Plus 6 software (Media,

Cybernetics, Baltimore, MD, USA), and the average absorbance value was measured.

Statistical analysis

Measurement data are expressed as mean ± SD and were statistically analyzed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). The mean differences between groups were compared using the Mann-Whitney U test. A *P* value less than 0.05 was regarded as indicating a significant difference.

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