NEURAL DYSFUNCTION AND NEURAL REGENERATION, A NEW WINDOW INTO THE NEONATAL HYPOXIC-ISCHEMIC BRAIN DAMAGE

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ABSTRACT

Neonatal hypoxic-ischemic (H/I) brain damage is a serious complication of intrauterine asphyxia during perinatal period, eventually leading to severe long-term neurodevelopmental disability or even death. Survival babies would experience cerebral palsy, epilepsy, mental retardation, cognitive, sensory and motor dysfunctions. However, there has no proven effective treatment available to protect the brain against injury after H/I occurs, because the exact timing of the hypoxic-ischemic event is unknown and we hardly identify the phase of injury or recovery in an individual patient precisely. In recent years, much effort has been made on the understandings of the H/I damages in the brain and underlying mechanisms of neural dysfunction, expecting the intervention of targeted neuroprotection in the newborn stage. We briefly summarize recent findings of the pathogenesis of hypoxic-ischemic injury with an emphasis on the disturbed neurogenesis process in the brain; the potential role of neural regeneration in basic and clinical research, including the endogenous stem cells mobilization and cell transplantation aiming to enhance the brain function.

Key words: Hypoxic-Ischemic, Neonate, Brain Injury, Neurogenesis, Regeneration.

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Introduction

Hypoxic-ischemic (H/I) damage of brain in neonates is an important clinical issue for both preterm and term infants, occurs in 1-6 of every 1000 live term birth. Approximately 55% of insults occur prior to or during birth (1), Neonatal H/I refers to exposure to low oxygen (hypoxia) and decreased blood flow (ischemia) prior to, during, or after birth and is regarded as a dominant cause of neurologic deficits in term and preterm infants, the primary cause of the neonatal encephalopathy and cerebral palsy. Typical neurologic symptoms include early and often refractory seizures, hypotonia, respiratory depression and apnea, and a depressed level of consciousness (2, 3). Pathological patterns of the injured brain in selected areas rather than the entire brain are peri-ischemic lesions and cortico-subcortical lesions, especially in the senso-motor cortex and the parasagittal region, and deep gray matter lesions of basal ganglia and thalamus in the near-term and term newborn (1, 4). This selective vulnerability of the brain to H/I and short/long-term prognosis are largely dependent on the severity and length of the initial insult (5), which currently have no promising therapy.

Hypoxia-ischemia damages selected regions of the immature brain occurring during the prenatal/neonatal stages of critical cellular or tissue differentiation process which have a serious impact on brain maturation. Thus the gestational and perinatal age of the infant is one of the main variables in determining the neuropathological picture of brain injury. This H/I encephalopathy is not a single event but an evolving process. It begins with the initial insult, progresses into a latent phase, and finally occurs as a secondary injury phase. The initial insult causes neural cellular death and apoptosis due to metabolic activity failure, increased levels of excitatory neurotransmitters and free radicals, inflammation, and intracellular pump failure. During the latent phase, the infant may appear stable; however, electroencephalography (EEG) activity remains suppressed, and hypoperfusion and
reduced oxygen consumption continue\textsuperscript{(6)}. The latent phase is considered a window of opportunity for initiation of neuroprotective strategies designed to interrupt the process of cellular death that occurs during the final phase, which is currently accepted as from 6 hours to 3 days and would be initiated as soon as possible after the initial insult.

Experimental studies suggest that traumatic brain injury involves a primary injury that includes direct disruption of brain parenchyma and a secondary injury that is characterized by a cascade of biochemical, cellular and molecular events similar to those associated with ischemia, excitotoxicity, energy failure, and resultant cell death cascades. Neuroimaging methods, such as magnetic resonance imaging (MRI) and diffusion-weighted MRI have supplemented postmortem studies that demonstrate patterns of selective vulnerability and neuronal loss in different brain areas at different stage and age during which the H/I insult has occurred. MRI has also revealed a special pattern of symmetric injury to the putamen, thalamus and cerebral cortex after severe or near-total asphyxia\textsuperscript{(7)}.

The striatal subventricular zone (SVZ) adjacent to the lateral ventricle is a forebrain region in which neurogenesis persists postnatally\textsuperscript{(8)}. Under stress or injury circumstances, the neural stem cells(NSCs) pool would be mobilized and the normal neurogenesis process is altered. The growing interest in cell therapy has resulted in much knowledge about the details of NSCs, helping to determine the therapeutic effects that makes NSCs useful in the treatment. Advances in regenerative medicine and exploration of neurogenesis lead to new ways of recovering neuronal dysfunction after birth.

**Altered Neurogenesis In Neonatal Hypoxic-Ischemia**

The brain consists of nerve cells known as neurons that form tracts throughout the brain. Neurogenesis arising from the progenitor populations that persist in the neurogenic niches of subventricular zone(SVZ) and subgranular zone(SGZ) is now an established evidence both in animal models and humans\textsuperscript{(9)}. Neurons are generally viewed as amongst the most anoxia-sensitive of all cells within the central nervous system(CNS). Every neuron in the brain is very sensitive to oxygen and changes its activity in response to hypoxia. Because the brain has limited oxygen reserves and limited ability to utilize anaerobic processes, most neurons reduce their metabolic requirements by decreasing their activity due to hypoxia.

The mechanisms for sensing hypoxia need to be considered with regard to whether the hypoxic exposure is acute, sustained, chronic or intermittent, with the latter capable of inducing long-term adaptive responses. The neuronal responses to hypoxia likely reflect neurophysiological changes due to changes in the function of ion channels, oxygen sensors (heme proteins), signaling pathways, neuromodulators and genomic processes\textsuperscript{(10, 11)}. In this respect, hypoxic insults have been associated with delayed selective neuronal apoptosis that involves the active participation of specific gene products. Among the various adaptive responses of the brain to the injury, it has been reported that new neurons can be generated through the proliferation of progenitor cells, and thus might help to repair the damaged CNS\textsuperscript{(12)}.

Neurogenesis after H/I injury presents two possible alternatives: 1) ischemic injury directly affects committed neuroblasts, which then expand and mature into neurons, or 2) ischemic injury affects unrestricted precursors that subsequently produce new neuroblasts. Studies have proved that experimental ischemia in the brain can trigger neurogenesis as a compensatory mechanism to neural cell death\textsuperscript{(13, 14)}.

Perinatal H/I increases the proliferation of cells that are positionally and phenotypically NSCs. Increased proliferation within the stem cells niche occurs at 2 d after perinatal H/I. Of those stem cell-related genes that change, the membrane receptors Notch1, gp-130 and EGF receptor, as well as the downstream transcription factor Hes5, which stimulate NSCs proliferation and regulate stem cells, are induced before NSCs expansion\textsuperscript{(15)}.

Plane et al. examined the effects of H/I on SVZ cell proliferation and neurogenesis of neonatal mouse. Postnatal day 10 (P10) mice underwent right carotid artery ligation followed by 10% O2 exposure for 45 min. H/I significantly enlarged the ipsilateral SVZ at P18, P24 and P31, and increases in the SVZ area correlated directly with the degree of hemispheric damage. H/I also stimulated cell proliferation and neurogenesis in the SVZ and peri-infarct striatum\textsuperscript{(16)}. Another similar research\textsuperscript{(17)} proved that the postnatal day 7 (P7) rats underwent right carotid artery ligation followed by 8% O2 exposure for 90 min. The effects of H/I injury on SVZ cell proliferation and neurogenesis were examined 1-3 weeks later by morphometric mea-
measurement of dorsolateral SVZ size; H/I injury resulted in enlargement of the ipsilateral SVZ at P14-28 and a corresponding increase in BrdU cell numbers both in the ipsilateral SVZ and striatum at P21. H/I injury also stimulated SVZ neurogenesis, based on increased doublecortin immunostaining in the SVZ ipsilateral to lesion at P14-28. H/I injury in the P7 rats resulted in decreased survival of new cells in the granular cell layer and dentate hilus(18). Given H/I treatment at P7, BrdU-labeled cells in the mouse brain were increase chronically in the hippocampus and double labeling indicates an increase of new neurons and decrease in oligodendrocytes(19).

Studies of the impact of H/I on the immature hippocampal neurogenesis are relatively limited. As a well studied region, the hippocampus has played key role in learning and memory, as well as other behavioral performance. It has been demonstrated that neonatal H/I rats with hippocampal injury perform poorly on the task of Spontaneous alternation. Deficit in T-maze and passive avoidance have been shown to be affected by changes in hippocampal neurogenesis(20, 21). Hypoxic stress induces apoptosis of hippocampal CA1 neurons while selectively sparing those in CA2-3. Proliferation and differentiation of local stem cells may potentially replace lost neurons. Miller lab(22) examined MAP kinase signaling regulation of these dual responses. Rat organotypic hippocampal cultures were exposed to hypoxia for up to 6 h followed by reoxygenation, and found that JNKs and ERKs were maximally activated by 4 h, returning approximately to basal levels by 6 h, and proved the hypoxia concurrently triggered both JNK and ERK signaling, and with reoxygenation, ERK1 activation and stem cell proliferation followed by neuronal progenitor cell differentiation and targeted migration to the site of pyramidal neuronal loss.

In Daval’s study(23), when rats are exposed to 100% N2 for 20 min at 36°C, within 24 h after birth, temporal changes in the vulnerable CA1 hippocampus were monitored. Cell density measurements revealed delayed cell death in the pyramidal cell layer reflecting apoptosis, as shown by levels of Bcl-2, Bax, and caspase-3. Neuronal loss was confirmed by reduced density of neuron-specific enolase (NSE)-labeled cells, and peaked by 1 week post insult, to reach 27% of total cells. BrdU analysis showed that newly divided cells expressing neuronal markers increased by 225% in SVZ, and they tended to migrate along the posterior periventricle toward the hippocampus. Therefore, transient hypoxia in the newborn rat triggered apoptosis in the CA1 hippocampus followed by increased neurogenesis and apparent anatomical recovery, suggesting that the developing brain may have a high capacity for self-repair. Recently, Zhao, et al(24) used the patch clamp, immunohistochemistry and Western blotting techniques to identify that a decrease in neuronal excitability and a significant increase in the frequency of spontaneous excitatory postsynaptic currents and the duration of EPSCs in the CA1 pyramidal cells of H/I brain damage rats of 7 days old. The glutamate transporter subtype 1 (GLT-1) expression level of the hippocampal CA1 area in the H/I group was decreased. These results revealed that changes of electrophysiological characteristics and synaptic functions occur instantly after H-I brain damage in the hippocampal pyramidal cells of neonatal rats. The failure to eliminate glutamate should be one of the important factors of excitotoxicity injury on hippocampal CA1 pyramidal cells, while neuronal excitation was not increased in the H-I brain injury model.

Therefore, under H/I condition, the neonatal brain would experience with the impaired neural development process, which would eventually result in the brain dysfunction. In the initial stage of H/I insult, the NSCs at the neurogenic region would be able to generate more new neurons to make up for the neonatal brain malfunction. However, if the insult continues, the brain function would not be reversible. These would potentially provide us with a time window as to rescue the H/I injured brain.

**Stem Cell-Based Regeneration Strategy**

Numerous researches have proved the pathophysiology and histopathology of H/I insults and proposed neuroprotective strategies. Several drugs have been applied in the clinical practice for the H/I patients, such as Barbiturates(25), Allopurinol(26), Erythropoietin(27), Hypothermia(28). However, the clinical trials after H/I show variable success in neonates. A variety of potential experimental therapies are being investigated in the animal models with some results being promising and some non-promising. It is hard to translate from animal models to human clinical trials due to possible differences in developmental age correlations and mechanisms. These potential therapies that have been studied in animal models with significant neuroprotection, are including but not limited to 1) Anti-
Excitotoxicity Therapy$^{(29,30)}$, 2) Antioxidation Therapy$^{(31,32)}$, 3) Anti-Apoptotic Therapy$^{(33)}$, 4) Stem Cell Therapy.

NSCs are karyotypically normal, undifferentiated, possess extensive proliferative capacity, are capable of long-term self-renewal and are multipotent. They reside in neurogenic zones throughout life, such as the subventricular zone and subgranular zone of the dentate gyrus in rodent models, and help maintain cell turnover at baseline and replace injured cells by migrating to penumbral tissue. Cell therapy holds promise in various models of brain injury or disease. Advances in regenerative medicine may lead to new ways of recovering neuronal function lost or damaged during the perinatal period; such injuries are not amenable to conventional therapies. The developing brain is revealed to have considerable potential with respect to proliferation and migration to the injured site. However, the generation of fully differentiated neurons is extremely limited after brain injuries. Aggressive efforts to adjust the environment of the damaged brain where the neural regeneration occur or stem cell transplantation might be required for the successful treatment of developmental brain injury.

Endogenous Neural Therapy

Recently years, researches have been focusing on understanding the brain’s intrinsic potential to repair following cerebral injury. This research both investigates the potential for endogenous repair and also aims to learn more about the permissive and restrictive cues in the damaged brain that will be crucially important if cell replacement therapy is seriously considered in the future. It is now clear that injury to the CNS does indeed result in the proliferation of endogenous neural precursors, although the numbers are insufficient to enable fully functional recovery. It has been accepted that endogenous neural progenitor cells function as therapeutic target after spinal cord injury$^{(34)}$ and neurological insult and injury to the brain$^{(35,36)}$.

One of the pioneer studies of insult-induced neurogenesis showed that transient global ischemia causing the death of pyramidal neurons in the CA1 region in the hippocampus of the adult gerbil activates the proliferation of NSCs in the SGZ, increasing the number of new granule neurons in the GCL$^{(37)}$. In addition, after focal ischemia induced by middle cerebral artery occlusion (MCAO), the most common model for ischemic stroke that causes infarction of the lateral striatum and adjacent neocortex, a small number of striatal projection neurons are regenerated$^{(38)}$. NSCs in the SVZ provide new neurons with a remarkable migration capacity, which may compensate for neurons lost to insult, and help regenerate the neuronal circuitry. These findings further imply that the SVZ could be an important therapeutic target for various pathological conditions.

Neural cells, however, have a limited capacity to regenerate and the small population of endogenous NSCs seems unable fully to reconstitute and restore function after damage. Although ischemia-induced neurogenesis might contribute to the specific recovery of memory function lost following injury, a high proportion of the dividing cells are lost over the weeks following injury. the demonstration of the continued production, and survival of neural cell types following injury, has led to renewed interest in mechanisms of the endogenous cell response and whether this could be exploited further to instruct repair following injury. This has led to examine the potential of cell replacement therapy after cerebral injury. The main sources of cells for potential therapeutic replacement are: neural precursor cells from fetuses; NSCs from fetal brain; NSCs from adult brain; and neural cells derived from embryonic stem cells.

Exogenous Neural Therapy

Over the past two decades, lots of studies have evaluated the efficacy and/or feasibility of transplantation within the injured brain of stem cells or specialized progenitor cells of both neural and non-neural origin, to replace lost cells or to prevent damaged cells from dying. NSCs have been widely investigated in both adult and neonatal ischemia models for neuroprotective effects. In neonatal H/I models, intraventricular administration of NSCs after H/I showed migration of these cells to the area of injury and differentiating into neurons, astrocytes, oligodendrocytes and undifferentiated progenitors$^{(39)}$. NSC transplantation has shown potential as a therapeutic strategy in adult animal models of stroke and H/I. Implanted cells integrate into injured tissue, decreasing volume loss and improving behavioral outcomes$^{(40)}$. These stem cells differentiate into neurons, astrocytes, oligodendrocytes as well as undifferentiated progenitors. These cells not only promote regeneration, but non-neuronal phenotypes inhibit inflammation and scar formation,
while promoting angiogenesis and neuronal cell survival in both rodent and primate models\(^{(41)}\).

While no adverse effects have been noted, efficacy is dependent on time of implantation, and the therapeutic window is not known. More recent technology enables labeling of stem cells, which can then be tracked from their site of implantation through their migratory path into the ischemic tissue\(^{(42, 43)}\), which will make tracking of these cells in humans possible.

There has been a recent report that early (4 hours) and late (72 hours) neurosphere-derived precursor cell implantation significantly reduced brain lesion size in this neonatal model\(^{(44)}\). The implanted cells, modified in vitro prior to transplantation toward the oligodendrocytic lineage, were capable of migrating toward the lesion site even when implanted contralaterally to the lesion, a feature similar to the long-distance migration of NSCs seen in a H/I model of brain injury\(^{(45)}\).

### Clinical Trials with NSCs

Human NSCs are becoming very attractive cells for transplantation, because of their stable expansion and in vitro differentiation into neurons and oligodendrocytes. Fetal tissue-derived NSCs have been used in preclinical mouse studies. When transplanted intracerebrally in mice with a disorder resembling infantile neuronal ceroid lipofuscinosis (Batten disease), these NSCs showed robust engraftment, extensive migration, and production of sufficient enzyme levels to alter host neuropathology\(^{(46)}\). Human NSCs were transplanted into 6 patients in the advanced stages of Batten disease, directly into the brain parenchyma. The safety profile was favorable, and long-term survival of the transplanted cells was documented. This is the first FDA-approved phase I clinical trial with Human NSCs was completed in January 2009 (http://clinicaltrials.gov/ct2/show/NCT00337636), followed by the second phase I clinical trial in patients with Pelizaeus-Merzbacher disease (PMD), of which the expected primary completion date is December 2012 (http://clinicaltrials.gov/ct2/show/NCT01005004)\(^{(47)}\).

Umbilical cord blood (UCB) cells, which are readily available at birth, have been shown to reduce sensorimotor and/or cognitive impairments in several models of brain damage, representing a promising option for the treatment of neurological diseases. Autologous UCB is a possible, but unproven, treatment for acute neonatal brain damage. Mesenchymal stem cells (MSCs), which are present in UCB, are likely to be the treating cell type. UCB is effective in the treatment of neonatal rodent H/I injury and other types of brain injury when the cells are delivered acutely. The most likely mechanisms of action are participation in blood vessel regeneration, improvement of survival of intrinsic cells, perhaps via neurotrophic factors, or suppression of the release of inflammatory cells from the spleen. UCB is widely used for the purpose of hematopoietic stem cell replacement therapies. This function is mainly related to its high content of CD34+ cells. Several studies have reported benefit with UCB treatment of experimental acute neonatal rodent H/I\(^{(46, 49)}\). Current clinical trials with UCB are in progress, but there are no peer-reviewed reports as yet. A multicenter trial with specific inclusion criteria is needed.

The reported success of the clinical trials, not limited to these two, proved the promising future of stem cell-based therapy applied in patients. Based on animal models of H/I encephalopathy, human umbilical cord blood mononuclear cells(HuCBCs) and human mesenchymal stem cells (HuMSCs) may be the most promising stem cell sources, as they are effective and potentially available for human studies. Being easier harvested, better proliferative, less mature and less immunogenic, HuCBCs have advantages over MSCs that may support their use for neonatal insults. Those merits are associated with a lower risk of graft-versus-host disease in recipients of transplants from unrelated donors, even when there is some degree of human leukocyte antigen mismatch.

### Unsolved Questions

Stem cell transplantation may minimize the effect of H/I injury by replacing damaged cells, promoting cell regeneration, inhibiting inflammation, and releasing trophic factors that heal and improve cell survival. After intraventricular implantation in neonatal H/I animal models, stem cells migrated to the area of injury in the brain\(^{(50)}\). Nevertheless, lots of research need be further conducted regarding to the cell engraft, such as the Delivery Routes and Methods, Cell Dose, Timing of Transplantation, Monitoring, Immune Response, Tumors risk. Furthermore, the supply of fetal neural tissue is limited and consequently only small num-
bers of neurons are available. This could partially overcome by in vitro expansion, but fetal tissue contains a heterogeneous population of cells, many of which are post-mitotic. A further barrier is the poor survival of grafted neural cells: it has been estimated that as many as 95% of transplanted neural precursors die by apoptosis. So, although stem cell therapy for neonatal H/I appears promising, a significant amount of research is required before it can be considered for use in neonates.

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