Neuroprotective Strategies in Neonatal Brain Injury

Pratik Parikh, MD, and Sandra E. Juul, MD, PhD

The developing brain responds to injury in a gestational age–dependent manner. During the third trimester, white matter injury is most common, whereas in infants born at term, gray matter injury predominates. Together, prematurity and hypoxic ischemic encephalopathy (HIE) account for 50% of global neonatal mortality, as well as significant neurodevelopmental impairment in survivors. In fact, the neonatal mortality from birth asphyxia is under-reported, as many affected infants in resource-limited settings are not resuscitated and are reported as stillborn. In this review, we will focus primarily on HIE, a devastating condition characterized by a combination of hypoxia (lack of adequate oxygen) and ischemia (a lack of perfusion of essential nutrients). HIE is a clinically defined syndrome of disturbed neurologic function manifested by difficulty with initiating and maintaining respiration, depression of tone and reflexes, and altered level of consciousness in the absence of other causative factors such as anatomic malformations, stroke, or genetic causes. HIE may present with seizures in the first 24 hours after birth in infants born near-term and term; it is a major cause of neurodevelopmental disability and mortality in infants born at term.

HIE occurs in 1.5-2 per 1000 births in developed countries and in as many as 26 per 1000 live births in resource-limited settings.[1] The presence of infection/inflammation can sensitize the brain, resulting in more severe brain injury.[2,3] The coexistence of inflammation with HIE may stimulate different mechanisms of brain injury with a resulting difference in response to therapeutic approaches. Without treatment, between 20% and 50% of newborns with HIE die within the newborn period, and up to 25% of the survivors will exhibit permanent neuropsychological handicaps, including intellectual disability, cerebral palsy (CP), epilepsy, or sensorineural hearing loss or vision loss.[4] HIE is estimated to be the underlying cause of 23% of the 2.8 million neonatal deaths that occur annually, most of which occur in low-resource settings. This difference in outcomes based on care in the pre- and postnatal periods underscores the fact that medical intervention can make an enormous difference in outcomes.[5]

The use of animal models has facilitated our understanding of the pathophysiology and progression of HIE, and these studies have laid the groundwork for neuroprotective interventions. One of these interventions, therapeutic hypothermia, has improved outcomes of neonates with HIE and has become standard of care in the developed world.

CP	Cerebral palsy
Darbe	Darbepoetin
Epo	Erythropoietin
EpoR	Erythropoietin receptor
FA	Fractional isotropy
HIE	Hypoxic ischemic encephalopathy
IL	Interleukin
MCS	Mesenchymal stem cells
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NAA	N-Acetyl aspartate
NAC	N-Acetyl-L-cysteine
NMDA	N-Methyl-D-aspartate
UCB	Umbilical cord blood
Xe	Xenon

Pathophysiology

Brain injury after hypoxia ischemia occurs in multiple phases. Immediately after hypoxia, anaerobic metabolism leads to decreased adenosine triphosphate production and primary energy failure with lactic acidosis, failure of cell membrane pumps leading to an influx of sodium and calcium, cell swelling, and death. Secondary energy failure occurs after reperfusion when there is accumulation of excitotoxic glutamate, activation of N-methyl-D-aspartate (NMDA), alpha-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid, and kainite receptors, free radical formation, nitric oxide production, oxidative stress, and delayed cell death. The progression of injury continues in the tertiary phase with ongoing inflammation, impaired neurogenesis, and alteration in synaptogenesis and axonal growth. The mechanism of cell death changes from necrosis early on to apoptosis later, with a continuum of phenotypes emerging in the developing brain (the apoptosis–necrosis continuum).[6]

Diagnosis

Clinical

The diagnosis of HIE is made by a combination of clinical and laboratory findings. Generally, one or more of the following signs must be present after birth: 10-minute Apgar score <5, prolonged resuscitation (>10 minutes) after birth, umbilical cord or first neonatal blood gas pH <7.0, or cord or base deficit >12 (**Table**).[7] In addition, the physical examination must show evidence of encephalopathy: decreased level of consciousness, decreased spontaneous activity, abnormal posture, decreased tone, decreased primitive reflexes, and abnormal

From the Department of Pediatrics, Division of Neonatology, University of Washington, Seattle, WA

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Table. Criteria for defining an acute intrapartum hypoxic–ischemic event as a cause of CP*

Essential criteria	Suggestive criteria
• Evidence of metabolic acidosis in fetal umbilical cord arterial blood at delivery (pH <7 and base deficit >12 mmol/L) • Early onset of severe or moderate neonatal encephalopathy in infants born after 34 weeks' gestation. • CP of the spastic quadriplegic or dyskinetic type • Exclusion of other identifiable causes	• A sentinel or hypoxic event occurring immediately before or after the onset of labor. • Sudden and sustained fetal bradycardia or the absence of fetal heart rate variability, in the presence of persistent, late, or variable decelerations after a previously normal fetal heart rate pattern • Apgar scores of 0-3 beyond 5 min • Onset of multisystem organ involvement within 72 h of delivery • Early imaging showing evidence of acute nonfocal cerebral abnormality

*American College of Obstetricians and Gynecologists.[7]

autonomic function. Amplitude-integrated electroencephalography sometimes is used to augment the diagnosis, with progressively severe abnormalities, including (1) discontinuous activity (minimum amplitude predominantly <5 μV, maximum amplitude >10 μV), (2) burst suppression, (discontinuous activity with minimum amplitude <5 μV and bursts predominantly with amplitude ≥25 μV), 3) continuous low voltage (maximum amplitude <10 μV and minimum amplitude <5 μV), or 4) flat trace (primarily isoelectric trace with both maximum and minimum background activity <5 μV).

Neuroimaging

Brain injury detected by magnetic resonance imaging (MRI) evolves over time, so evaluating patients at a consistent time is important. Early evaluation is more sensitive, and later evaluation is more specific. Many sites choose to study infants with HIE after rewarming, but before discharge, around days 4-6. Two main patterns of injury can be seen with moderate-to-severe HIE.[8-10] The first, deep nuclear gray matter injury (25%-75% of cases), involves the deep gray nuclei and perirolandic cortex, extending further into the cortex when severe. The second, watershed injury (15%-45% of cases), involves injury at the watershed areas between the anterior and middle cerebral arteries anteriorly and the middle and posterior cerebral arteries posteriorly. Deep gray matter injury often is associated with more severe neurodevelopmental impairments, and when there is abnormal signal in the posterior limb of the internal capsule, the risk of long-term motor deficits is high.[11] The watershed pattern of injury most often is associated with predominantly cognitive impairments, with fewer functional motor deficits.[12]

Magnetic resonance spectroscopy (MRS) can provide additional prognostic information. N-acetyl aspartate (NAA) is found primarily in neurons, so a reduction in NAA peak is thought to reflect neuronal injury or loss. Choline is a cell membrane component and also is present in the neurotransmitter acetylcholine. Lactate may be elevated after asphyxia and anaerobic glycolysis. Ratios of NAA/choline or NAA/lactate may therefore provide important prognostic information regarding extent and severity of brain injury.

Biomarkers

Biomarkers that accurately reflect the timing, severity, evolution of injury, and response to therapy have the potential to improve the clinical management of newborns with HIE. Circulating candidate biomarkers in neonatal HIE can be subdivided into 4 categories: neural injury–specific, neuroinflammatory, oxidative byproducts, and metabolism-related markers. Each of these markers peaks in a different pattern after an injury, so the timing of when they are studied is critical. Clinical studies evaluating neonatal biomarkers of brain injury generally have been small, single-center studies with differences in the timing of assessing biomarkers and in primary outcomes. Neuron-specific cytoplasmic proteins considered to be potential biomarkers include neuron-specific enolase, spectrin breakdown products, and ubiquitin carboxyl-terminal hydrolase isoenzyme L1. Axon-related proteins include neurofilament light polypeptide, contained in large-caliber myelinated axons, and total tau, a biomarker of injury to thin nonmyelinated axons. Amyloid precursor protein and amyloid-β are produced in axon terminals and might be involved in synaptic activity and plasticity. Astrocyte-specific proteins include S100-B and glial fibrillary acidic protein, so astrocytic injury is reflected by increased concentrations of these proteins in both cerebrospinal fluid and blood. Neuroinflammation and astrocytosis also can lead to increased production of interleukins (ILs), particularly IL-6, IL-8, and IL-1beta. At this time, study results remain inconsistent, and no biomarker has the capability to diagnose the severity of disease in timely fashion, so no biomarkers have been established for clinical use. An ongoing prospective study is evaluating the predictive value of a combined biomarker panel (including glial fibrillary acidic protein, S100B, neurogranin, brain-derived neurotrophic factor, metallothionein 3, neuron-specific enolase, intracellular adhesion molecule 5, and beta synuclein) for diagnosing traumatic brain injury.[13] Novel biomarkers like microRNA have been investigated in brain injury. Recent studies have shown upregulation of microRNA 21 as a marker of severe traumatic brain injury, neonatal HIE, and predictor of 6-month outcomes.[14,15]

Metabolomics is the quantitative analysis of low-molecular-weight metabolites, including intermediates, signaling molecules, and components of metabolic pathways. Metabolomics has created an opportunity to assess the net biochemical changes occurring HIE. In nonhuman primate studies of HIE, arachidonic acid, butanoic acid, citric acid, fumaric acid, lactate, malate, propanoic acid, and succinic acid correlated with early and/or long-term neurodevelopmental outcomes.[16,17] Very few studies in humans have been performed looking at metabolites in cord blood samples and urine of newborns for evaluation of HIE.[18] Reinke et al studied umbilical cord serum from newborns with HIE by using nuclear MRS and showed 18 of 37 reproducibly

detectable metabolites were altered significantly between study groups.[19] Multiple-linear regression modeling using 4 metabolites (3-hydroxybutyrate, glycerol, O-phosphocholine, and succinate) predicted HIE severity with an adjusted R^2 of 0.4. A metabolic index of the aforementioned 4 metabolites correlated with clinical outcomes at 3 years of life.[20]

State of the Art: Current Therapy

Therapeutic hypothermia is the standard of care used to treat neonates with moderate-to-severe HIE in developed countries. Six large randomized controlled trials have established the effectiveness of therapeutic hypothermia in infants ≥36 weeks of gestation when initiated within 6 hours of birth. A recent meta-analysis including 11 randomized controlled trials showed that therapeutic hypothermia resulted in a statistically significant reduction in the combined outcome of mortality or major neurodevelopmental disability at 18 months of age (typical risk ratio 0.75; 95% CI 0.68-0.83) with a number needed to treat for an additional beneficial outcome of 7 (95% CI 5-10; 8 studies, 1344 infants). Hypothermia resulted in significant reduction of mortality alone with number need to treat of 11 (95% CI 8-25; 11 studies, 1468 infants). Beneficial effects of hypothermia persisted in both the CoolCap trial and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Whole Body Cooling trial at 7-8 years.[21,22] The Total Body Hypothermia for Neonatal Encephalopathy (TOBY) trial long-term outcomes showed significant reductions in the risk of CP (21% vs 36%, $P = .03$) and the risk of moderate or severe disability (22% vs 37%, $P = .03$) with improved motor function scores in the hypothermia group.[23] Hypothermia as a treatment for infants with HIE born preterm has not been studied extensively, but a recent retrospective study of 34- to 35-week gestational age infants showed increased mortality and morbidity compared with cooled infants with HIE born at term. No infants with HIE born preterm who were not cooled (control group) were included in the publication.[24]

A recent trial of prolonged hypothermia or deeper hypothermia concluded that, among neonates who were born at full term with moderate or severe HIE, longer cooling, deeper cooling, or both compared with hypothermia at 33.5°C for 72 hours did not reduce mortality.[25] Futility analysis determined that the probability of detecting a statistically significant benefit for longer cooling, deeper cooling, or both for death in the neonatal intensive care unit was <2%, and thus the enrollment in the trial was stopped. The evaluation for the primary outcome of death or disability at 18-22 months is ongoing. Preemie Hypothermia for Neonatal Encephalopathy (NCT01793129) is a randomized, controlled trial to assess the safety and efficacy of whole-body hypothermia for 72 hours in infants born preterm 33-35 weeks of gestation who present at <6 hours of age with moderate-to-severe encephalopathy. This study will enroll infants with signs of HIE at 18 Neonatal Research Network sites and randomly assign them to either receive hypothermia or normothermia (standard care).

Therapeutic hypothermia as a treatment for moderate/severe HIE has decreased death or significant neurodevelopmental impairment from approximately 65% to 40%-50%. This is proof of principal that outcomes can be improved with appropriate therapy. However, 40%-50% death or disability is still unacceptable. Thus, additional treatments are being sought to reduce morbidity and mortality.[26] As therapeutic hypothermia has not been shown to provide benefit in low- and middle-income countries in which HIE is most common, these alternative or supplementary therapies might provide a pathway to healing that is otherwise unavailable to children in these countries.[27] Next, we will highlight promising pharmacologic agents for neonatal neuroprotection that are either in clinical trials or the preclinical phase of research.

Clinical Trials of New Therapies

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine), a neurohormone derived from the amino acid tryptophan and secreted by the pineal gland, is a strong antioxidant capable of scavenging free radicals and stimulating several antioxidative enzymes including glutathione, glutathione reductase, peroxidase, and superoxide dismutase.[28] Melatonin exerts its effect through both receptor-mediated and receptor-independent mechanisms. Melatonin can directly stimulate cellular membrane G protein–coupled high-affinity melatonin receptors that activate numerous second messenger cascades, which vary in cell, tissue, and species-specific ways. Melatonin also can induce receptor-independent intracellular activities by targeting calcium-binding proteins, cytoskeletal and scaffold proteins, and components of mitochondrial signaling.[29] Melatonin's safety profile, antioxidant, anti-inflammatory, and antiapoptotic properties have made it an attractive neuroprotective candidate for treating neonates with HIE.[30,31]

Melatonin has been studied in animal models of neonatal HIE with promising results; it has been shown to decrease infarct area, improve neuronal survival, reduce reactive gliosis, and improve white matter injury.[32] Melatonin reduces oxidative damage with pretreatment (10-20 mg/kg) and also reduces free radicals directly with reduced free radical–mediated lipid peroxidation and increased glutathione levels in models of neonatal brain injury.[33,34] Long-term neurodevelopment also is improved in a neonatal rat model of hypoxia–ischemia when doses of 5 and 15 mg/kg are used.[35,36] Melatonin also has been studied as an adjuvant to hypothermia. In piglets, combined melatonin and hypothermia showed greater neuroprotective effects than hypothermia alone.[37]

Phase I and II trials are ongoing to test melatonin neuroprotection for HIE. Aly et al showed feasibility and the potential efficacy of melatonin as an adjuvant to therapeutic hypothermia in infants with moderate-to-severe HIE, showing reduced oxidative stress and improved survival and neurodevelopmental outcome at 6 months of age.[38] Currently a dose-escalation study to evaluate efficacy of enteral melatonin in infants with neonatal HIE is underway (NCT02621944). The primary outcome is to identify the maximum tolerated dose, and the secondary outcome is to look

at neurodevelopmental outcome. Melatonin also holds potential as an antenatal therapy that could be administered to pregnant mothers with at-risk fetuses because it appears safe, crosses the placenta,[39] and crosses the blood–brain barrier.[40] Further research is needed to clarify the mechanisms by which melatonin may regulate neuronal cell survival, brain tissue homeostasis, and neuroprotection in neonates with HIE.

Xenon

Xenon (Xe), a noble gas that crosses the placenta and the blood–brain barrier, binds to NMDA glutamate receptors to inhibit function, thus decreasing neuronal apoptosis.[41,42] Significant benefit was demonstrated in preclinical studies of HIE,[43] so several phase I and II trials were undertaken. The Total Body hypothermia plus Xenon (TOBY-Xe) trial was conducted in the United Kingdom. Ninety-two infants (36-43 weeks of gestation) were enrolled, 46 of whom were assigned randomly to therapeutic hypothermia only and 46 to 30% Xe plus hypothermia. The primary outcomes were MRI/MRS-based: preserved fractional anisotropy (FA) in the posterior limb of the internal capsule determined within 15 days of birth and MRS assessment of thalamic lactate to NAA ratios. The TOBY-Xe trial was powered adequately to detect changes in FA but underpowered to detect changes in the lactate to NAA ratios. No significant differences were detected between groups. Based on these results, early hypothermia plus 30% Xe for 24 hours begun by 12 hours of age was deemed by the authors as unlikely to improve clinical outcomes compared with hypothermia alone, so enrollment was stopped early.[44]

The CoolXenon 1 trial was a feasibility trial for use of Xe in HIE. The first 6 infants received increasing Xe concentrations from 25% to 50% for 3-12 hours, and infants 6-12 received 50% Xe for 12 hours. Fifty percent Xe was sedative, but there was no hypotension or change in cardiac output.[45] The other ongoing trials are CoolXenon 2 and 3. These trials combine 50% Xe for 18 hours (started within the first 5 hours of birth) with 72 hours cooling to 33.5°C (started within 3 hours of birth). The primary outcome for CoolXenon 2 (NCT01545271) was early biomarkers (MRI and amplitude-integrated electroencephalography) of HIE. CoolXenon 3 (NCT02071394) is currently recruiting patients, with the primary outcome of death and moderate or severe disability at 18 months of age. Multiple factors may impact inhaled Xe treatment outcomes, including the timing, dose, and duration of treatment. Potential reasons for lack of efficacy in the TOBY-Xe trial include a greater number of patients with severe HIE in both groups, and, possibly, the concentration of Xe used was outside the therapeutic window. The question of therapeutic window will be answer by comparing the CoolXenon and TOBY-Xe trials.

Limitations of Xe. The inhalation of Xe reduces the available fractional concentration of oxygen; thus, it cannot be used in infants who require high fraction of inspired oxygen. Another disadvantage of Xe is its high cost and consequent need for a recycling system.

Argon is another noble gas that has been tested for neuroprotective properties. Recent preclinical studies have shown its neuroprotective effects in neonatal brain injury.[46] A preliminary study in hypoxic-ischemic piglets showed cardiovascular stability of argon during therapeutic hypothermia.[47] Argon is cheaper than Xe and may be an alternative neuroprotective agent.

Stem Cell Therapy

This is an established first-line or adjunctive therapy for a variety of neonatal diseases such as inborn errors of metabolism. Different types of stem cells ranging from neural stem cells, embryonic stem cells, umbilical cord blood (UCB) stem cells, bone marrow–derived mesenchymal stem cells (MSCs), and inducible pluripotent stem cells have been used as treatment for neonatal brain injury. Stem cell treatments have been effective in providing significant neuroprotection and improvement in functional outcomes in animal models of neonatal hypoxia–ischemia, CP, and stroke.[48-50] In recent years, focus has shifted to MSC, especially UCB-MSCs. Preclinical studies have shown that MSCs do not engraft but rather respond to signals of local injury by secreting trophic factors, thereby increasing progenitor cell proliferation and survival of neurons and neuronal stem cells.[51-53] MSC treatment also causes reduction of inflammation.[52,53] Along with an increase in neuronal progenitor cells, MSCs also stimulate axonal sprouting and proliferation of oligodendrocyte precursors and promote mature oligodendrocytes fate.[52,54,55] The time of delivery and number of doses affect the nature of protection. It has been shown that 2 doses given 3 and 10 days after injury provides better neuroprotection than a single dose at 3 days after injury. No neuroprotective effect was seen when MSC given 17 days after injury. Recently a combined treatment of human UCB-derived MSC transplantation and hypothermia in a severe neonatal hypoxia–ischemia model showed improvement in infarction, behavioral testing, and reduction in inflammation, which was better than either therapy alone.[56]

Several open-label clinical studies have studied effects of stem cell therapy in children with CP. A randomized, double-blinded, placebo control study by Min et al divided children with CP aged 10 months to 10 years in 3 groups: intravenously UCB potentiated with erythropoietin (Epo), Epo only, and controls.[57] Compared with the Epo and control groups, the UCB group had significantly greater developmental scores at 6 months. Diffuse tensor imaging revealed significant correlations between the gross motor performance measure increment and changes in FA in the UCB group. To date, no clinical trials evaluating effects of MSC from UCB have been reported. A study of 23 neonates by Cotten et al assessed the safety and feasibility of intravenous administration of noncryopreserved, autologous UCB cells. No significant adverse reactions, cardiopulmonary compromise, or infections were observed with the transfusion of up to 4 doses of $1\text{-}5 \times 10^7$ cells per dose. A total of 74% of UCB recipients were alive at 1 year with Bayley scores >85, compared with only 41% in the concurrent cooled infants.[58] Currently, there are ongoing phase I and phase II trials, including autologous cord blood and human

placental–derived stem cells in neonates with severe HIE (NCT02434965), umbilical cord milking for neonates with HIE (NCT02287077), and neural progenitor cell and paracrine factors to treat HIE (NCT02854579). These studies will help us understand the optimal dose, duration of therapy, therapeutic window, and dose response to the treatment. Further work is required to assess the long-term outcomes of stem cell therapy.

Erythropoietin

Epo and its receptor (EpoR) are required for normal brain development. Astrocytes are the main source of brain Epo, and many cell types express the receptor, including neurons, oligodendrocytes, and epithelial cells. The EpoR is expressed in hippocampal and cerebral cortical neurons, and expression of both Epo and EpoR decline postnatally in brain but remain present at lower levels throughout adulthood.[59,60] Prolonged hypoxic exposure leads to upregulation of EpoR gene expression. In the absence of Epo to bind these up-regulated EpoRs, neurons and oligodendrocytes have a greater risk of apoptotic cell death.[61,62] Mechanisms of Epo neuroprotection include phosphorylation of Janus kinase 2, activation of mitogen-activated protein kinase, extracellular-related kinase, phosphatidylinositol 3-kinase, and signal transducer and transcriptional activator 5 pathways, which are critical for cell survival (**Figure 1**).[63] Epo also stimulates the production of other growth factors, including vascular endothelial growth factor and brain-derived neurotrophic factor, which contribute to its angiogenic and neurogenic properties. Epo effects are dose-dependent, with multiple doses being more effective than single doses.[64,65] Studies in animals have shown that Epo reduces neuronal loss and learning impairment after brain injury even when initiated as late as one week after injury,[66] emphasizing the importance of the regenerative and reparative effects of Epo.

Epo is now in clinical trials for neuroprotection of term infants with HIE, and as a prophylactic neuroprotective treatment of extremely low gestation neonates. The anti-inflammatory, antiexcitotoxic, antioxidant, and regenerative effects are relevant to promoting brain development and minimizing brain injury in both age groups (**Figure 1**). Epo has many properties that make it appealing as a clinically useful

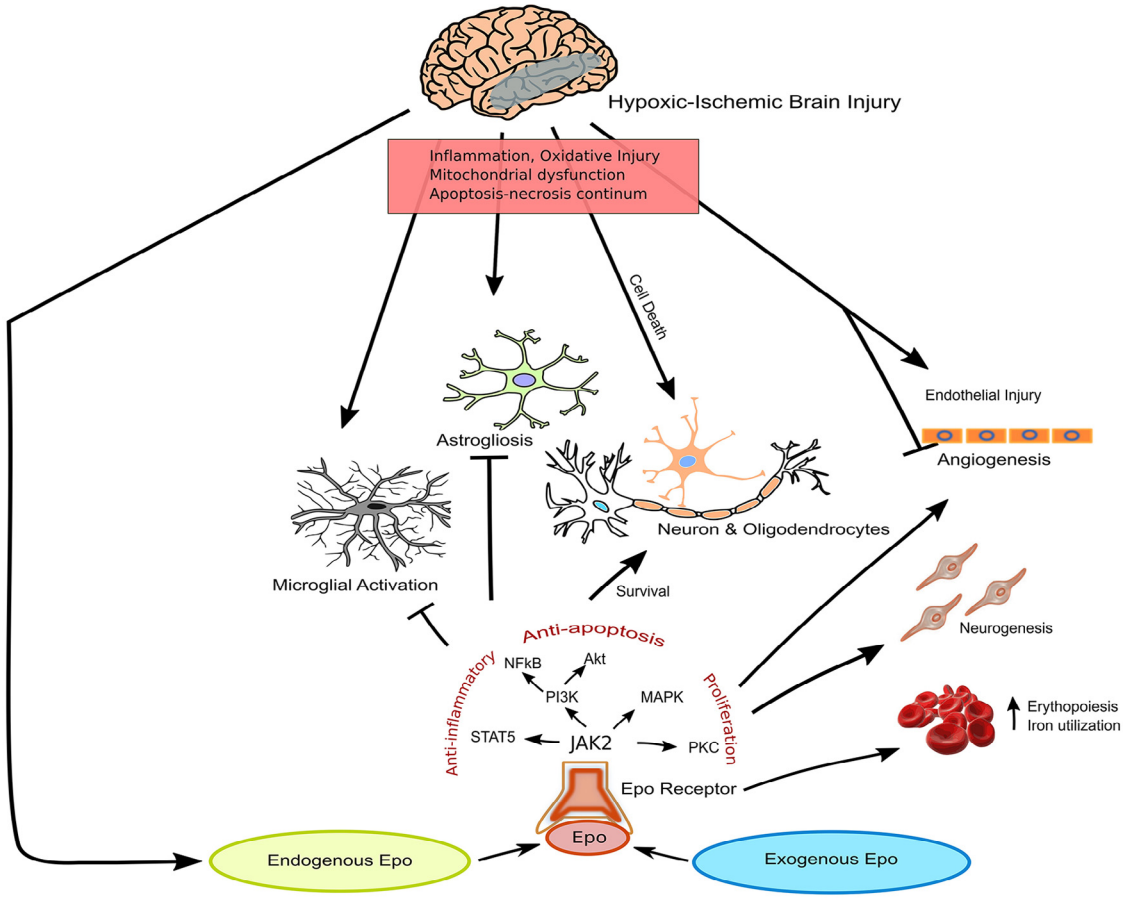

Figure 1. Neuroprotective effects of Epo in brain after hypoxic-ischemic injury. Brain injury initiates a cascade of inflammation, oxidative injury, mitochondrial dysfunction, and cell death. Microglia are activated, and astrogliosis occurs. Epo (endogenous or exogenous) binds to cell-surface EpoR with combined effects of decreasing local inflammation and apoptosis and enhancing neurogenesis, angiogenesis, and erythropoiesis. These combined effects decrease acute injury and enhance repair. *STAT5*, signal transducer and activator of transcription 5; *PI3K*, phosphoinositide 3-kinase; *JAK2*, Janus kinase 2; *MAPK*, mitogen-activated protein kinase; *PKC*, protein kinase C.

neuroprotective agent. It has been studied extensively in neonates, children, and adults and has a robust safety profile, it is approved by the Food and Drug Administration, and it is relatively inexpensive, costing approximately $50 per single-dose vial. Phase I/II trials have been done to establish safety and translational pharmacokinetics of Epo in neonates born preterm and at term.[67-69] These studies suggest that 1000 U/kg/dose provides an area under the curve most like a neuroprotective dose of 5000 U/kg in rodents.

Several trials to evaluate safety and efficacy of Epo as a potential neuroprotective therapy for HIE have been done, some in the setting of therapeutic hypothermia, and some without. Zhu et al[70] compared Epo to supportive care in infants with moderate-to-severe HIE. Epo was dosed at either 300 or 500 U/kg and given immediately after injury and repeated every other day for 2 weeks. The authors demonstrated a decreased incidence of moderate-to-severe disability or death at 18 months of age in infants given either of the 2 doses of Epo, particularly in infants with moderate compared with severe HIE. In 2010, Elmahdy et al compared Epo with supportive care in infants with mild-to-moderate HIE.[71] Epo was dosed at 2500 U/kg subcutaneously, started within 4-6 hours of injury, and repeated daily for 5 total doses. These authors also demonstrated improved outcomes in infants treated with Epo, including decreased seizure activity, decreased endogenous nitric oxide production, and improved neurodevelopmental outcomes up to 6 months.

Malla et al randomized 100 neonates born at term with moderate or severe HIE to receive either 500 U/kg Epo or placebo given intravenously on alternate days for a total of 5 doses, with the first dose given by 6 hours of age.[72] No hypothermia was used. Death or moderate or severe disability occurred in 40% of neonates in the treatment group compared with 70% in the placebo group (risk ratio 0.57; 95% CI 0.38-0.85; $P = .003$). There was no difference in death rates. The risk of CP and seizure burden were lower among survivors in the treatment group, $P = .04$.

The phase I/II trial by Wu et al was a dose escalation study in infants with HIE born at term treated with hypothermia.[68] Doses ranged from 250 to 2500 U/kg and were administered intravenously, starting at <24 hours of age and continuing every 48 hours for up to 6 total doses. The authors showed that dosing at 1000 U/kg produced plasma concentrations comparable with neuroprotective doses in animals and was well tolerated. At mean age 22 months, infants treated with Epo exhibited a surprisingly low rate of death (none) and long-term, moderate-to-severe disability (one), even in the setting of moderate to severe brain injury at study entry.

The Neonatal Erythropoietin And Therapeutic Hypothermia Outcomes in Newborn Brain Injury (NEATO) study enrolled 50 subjects with moderate or severe HIE and randomized them to Epo or placebo in the setting of therapeutic hypothermia. Despite no differences in enrollment characteristics, severity of injury on early MRI was significantly lower in infants treated with Epo, and neurodevelopment at 1 year of age was improved in the Epo-treated group.[69] Other ongoing studies of infants with HIE born at term in the setting of therapeutic hypothermia include the NeuroEpo study in France (NCT#01732146), the PAEAN study in Australia (NCT#03079167), and the HEAL trial (NCT# 02811263) in the US. All these ongoing trials will test the effects of 1000 units/kg Epo, with multiple doses being given intravenously over the first week of age.

Darbepoetin (Darbe) is an Epo analog that contains 5 N-linked oligosaccharide chains, as compared with 3 N-linked chains on Epo.[73] The additional carbohydrate chains increase the approximate molecular weight of the glycoprotein from 30 kDa to 37 kDa, lower receptor binding, and confer a longer half-life while maintaining erythropoietic effects. There is great interest in using Darbe for neonatal neuroprotection because, if it is effective, less frequent dosing would be required as compared with Epo. It also could be used potentially as a monotherapy in low-income countries in which therapeutic hypothermia has not been shown to be effective.[27] The Darbe Administration in Newborns Undergoing Cooling for Encephalopathy (DANCE) trial measured the pharmacokinetics of 2 and 10 μg/kg doses of Darbe and demonstrated short-term safety in this population. The Mild Encephalopathy in the Newborn Treated with Darbepoetin (MEND) trial (NCT# 03071861) is a phase II, multicenter, placebo-controlled randomized, feasibility/safety trial for infants >34 weeks of gestation with mild neonatal encephalopathy. Participants will be receiving either one dose of Darbe or placebo within 24 hours of birth. Neurodevelopmental testing (Bayley III and Gross Motor Function Assessment) will be performed at 12-18 months of age.

Pharmacological Treatments in the Preclinical Stage of Development

N-Acetyl-L-Cysteine (NAC)

NAC is a potent thiol-containing antioxidant, and precursor of glutathione.[74] It is also a scavenger of oxygen free radicals. Neuroprotective effects of NAC include restoration of glutathione and reduction of apoptotic cell death, inflammatory cytokines, and inducible nitric oxide synthase.[75,76] NAC alone and as an adjuvant of hypothermia reduces hypoxic ischemic brain injury in neonatal rats.[77] The dose has ranged from 20 to 200 mg/kg of NAC. Park et al used NAC (100 mg/kg) intraperitoneally given 30 minutes before injury at postnatal day 7 and once a day up to postnatal day 44, which resulted in improvement of motor function and myelination of the corpus callosum.[78] When combined with hypothermia, NAC at 50 mg/kg reduces infarct volume and improves myelin expression and functional outcomes after hypoxic ischemic brain injury.[77] A recent study showed sex-specific treatment effects of NAC, with only female subjects responding with short-term improvement in infarct volumes compared with saline-treated animals. However, both male and female subjects showed benefit after prolonged treatment with NAC.[79] In neonatal piglets, after hypoxia-reperfusion, NAC (20 or 100 mg/kg/h) decreased cerebral oxidative stress with reduction in oxidized glutathione levels, hydrogen peroxide, and lipid hydroperoxide concentrations.[80]

Successful neuroprotection has been achieved using lower doses of NAC (10 mg/kg) that are linked to polyamidoamine dendrimers in a rabbit model of CP. The dendrimers direct the uptake of drug to microglia and astrocytes for cell-specific anti-inflammatory activity. Intravenous administration of dendrimer-NAC resulted in improvement in neuronal injury and motor function in CP rabbit compared with NAC (100 mg/kg) that was not dendrimer bound.[81] In summary, NAC is an important antioxidant with the potential to be neuroprotective in neonatal brain injury. More work is needed to evaluate the appropriate dose, duration, and delivery method of NAC in preclinical and clinical studies.

Lithium

Lithium is a chemical element with the atomic number 3. It is present in plants, seafood, and vertebrates. Lithium salts have been used for treatment of bipolar and other psychiatric disorders. Adult preclinical data have shown neuroprotective and neurotrophic effects of lithium in stroke. It provides short-term and long-term improvement.[82,83] The underlying mechanisms of neuroprotection in adult ischemic models include prevention of NMDA receptor–induced excitotoxicity,[84,85] induction of the phosphatidylinositol 3-kinase/Akt cell survival pathway, inhibition of glycogen synthase kinase-3β, and increased expression of Bcl-2.[86] These pathways lead to inhibition of apoptosis and stimulation of neurogenesis.[83] A small, randomized controlled trial of lithium in poststroke motor recovery showed enhanced motor recovery in cortical stroke but did not provide significant improvement in all patients with stroke.[87]

In recent years, lithium has been investigated in neonatal brain injury. Preclinical studies by Li et al showed reduced infarct size and long-term improvement following brain injury. Lithium reduced neonatal brain injury by reduction in apoptosis, inflammation, and induction of neurogenesis.[88-91] When lithium was given 5 days after neonatal hypoxic ischemic injury, it reduced tissue loss by 38.7%, increased neurogenesis, and reduced motor hyperactivity and anxiety-like behavior after hypoxia ischemia. Lithium has potential side effects, such as drowsiness, tremors, and diarrhea; however, the dose used for neuroprotection is lower than that used in children with bipolar disorder, but its effects in developing brain are not well studied. Thus, a more thorough evaluation is needed for it to be used as neuroprotective agent.

Polyphenols

Polyphenols are natural molecules with variable phenolic structures that are enriched in vegetables, fruits, tea, wine, and other foods. They are classified into groups depending on the number of phenol rings and chemical groups bound to the rings. Most polyphenols have antioxidant, anti-inflammatory, and antiapoptotic properties. Many polyphenols have been studied for their role in neuroprotection.

Resveratrol. Resveratrol (3,5,4′-trihydroxystilbene) is a nonflavonoid polyphenolic compound consisting of 2 aromatic rings attached by a methylene bridge. Common dietary sources for resveratrol include grapes, soybeans, and pomegranates.[92] Resveratrol, a natural polyphenol, seems to play an important role in neuroprotection in models of ischemia, brain, and spinal cord injury. Resveratrol provides neuroprotection through multiple pathways (**Figure 2**). The antioxidative effects of resveratrol are due to its stilbene structure with 2 phenol rings, which allows it to scavenge a variety of free radicals and induction in the expression of several antioxidant enzymes through activation of nuclear factor (erythroid-derived 2)-like 2.[93] Resveratrol also reduces hypoxia inducible factor 1-α, Bax, and caspase-3 while increasing the levels of anti-apoptotic Bcl2 in PC12 cells.[94] Its anti-inflammatory effects are due to regulation of nuclear factor kappaB, cyclo-oxygenase 1 and 2, and inducible nitric oxide synthase. Resveratrol also modulates excitotoxicity by inhibiting activation of postsynaptic kainite and NMDA receptors.[95]

Several preclinical studies have demonstrated the efficacy of resveratrol in hypoxic–ischemic brain injury in neonatal rats, preserving neocortical and subcortical brain areas in the short term,[96,97] with long-term improvement in motor and behavioral functions.[98] Resveratrol also has been investigated as preventive treatment for neonatal brain injury, as it crosses the placenta and maternal supplementation can lead to reduction in tissue loss and decreased apoptosis in neonatal brains.[99,100]

Curcumin. Curcumin (diferuloyl methane) is a polyphenol present in the rhizomes of *Curcuma longa* (zingiberaceae). It possesses many therapeutic properties, including antioxidant,[101,102] anti-inflammatory,[103,104] and anticancer effects.[105] Curcumin treatment in an adult rat model of cerebral ischemia resulted in an improvement in infarct volume and neurologic scores.[106] The neuroprotective effect was the result of reduced neuronal apoptosis, oxidative stress, and proinflammatory cytokines (**Figure 2**).[106-108] Curcumin anti-inflammatory effects are due to inhibition of nuclear factor kappaB signaling, which leads to reduction in proinflammatory cytokines and enzymes.[104,108] In vitro and in vivo studies have shown that its antioxidative effects are due to direct scavenging with its phenolic structure and modulation of nuclear factor (erythroid-derived 2)-like 2 pathway.[102] Curcumin also inhibits its activation of NMDA, which is an important mechanism for excitotoxic brain injury.[109] Curcumin induces neurogenesis by modulating the canonical Wnt/β-catenin pathway, which leads to reversal of cognitive deficits in Alzheimer disease.[110] Thus, curcumin, like resveratrol and Epo, works on multiple pathways affecting brain injury and has a potential to be an effective treatment for neonatal brain injury. To date, most neuroprotection work with polyphenols has been done in adult models of injury.

Drug Delivery. One major limitation of polyphenols and other potentially protective compounds is poor bioavailability. Alternative delivery methods are being studied to circumvent this issue. Nanomedicine has emerged as an important field for delivering drugs to specific targets. Nanoparticles, such as

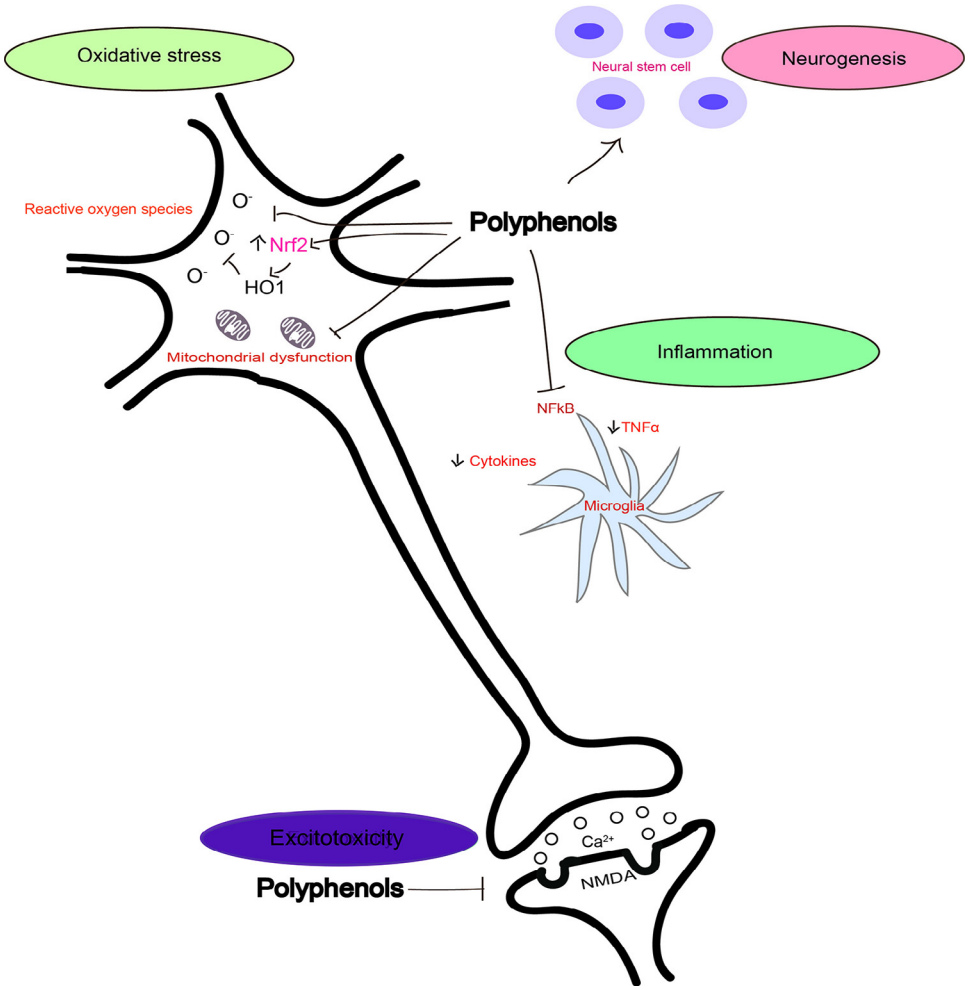

Figure 2. Overview of neuroprotective effects of polyphenols in preclinical studies. Polyphenols play an important role in reducing neuroinflammation by modulating the nuclear factor kappaB pathway, thus reducing cytokine production. Polyphenols also play an important role as a direct reactive oxygen species (O^-) scavenger and indirectly by modulating the nuclear factor (erythroid-derived 2)-like (Nrf2) pathway, thus inducing production of antioxidative enzymes. These combined effects reduce mitochondrial dysfunction and reduce apoptosis. Excitotoxicity is mitigated by reducing Calcium (Ca^{2+}) influx in neurons. Simultaneously polyphenol induces proliferation of neural stem cells by modulating the canonical Wnt/β-catenin pathway. *TNF*α, tumor necrosis factor-α.

polymeric dendrimers, can bind drugs, target their uptake by specific cell types, and modulate drug delivery by modifying speed of release. This can increase the bioavailability of drugs and decrease the dose needed for effect, thereby decreasing dose-dependent side effects. Although this has been studied intensively for delivery of cancer therapeutic agents, attention is now turning to using these nanoparticles for other purposes such as neuroprotection. Curcumin-loaded nanoparticles have been shown to be more effective than free curcumin in ameliorating cerebral ischemic reperfusion injury in rats.[111] Polyamidoamine dendrimers–NAC, which localize in activated microglia and astrocytes, have been used to treat newborn rabbits with CP.[80] Nanomedicine has great potential to deliver drugs across the blood brain barrier targeting the site of injury and repair to improve neurologic outcomes.

Summary

Therapeutic hypothermia is currently our only proven neuroprotective intervention for HIE. With this treatment, up to 50% of infants with moderate-to-severe HIE still die or suffer severe neurodevelopmental impairment. There is therefore an ongoing search for additional treatments. Ideal neuroprotective treatments should be safe, readily available, inexpensive, and effective when given after an injury occurs. The evidence for Epo neuroprotection is most promising to date, with positive data from both preclinical and clinical trials. Phase III trials

are ongoing and will answer this question soon. Epo is approved by the Food and Drug Administration, relatively inexpensive (approximately $50 per vial), and readily available, so if proven to be effective, it can be put into clinical use immediately. ■

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Reprint requests: Sandra E. Juul, MD, PhD, Department of Pediatrics, Division of Neonatology, University of Washington, Box 356320, Seattle, WA 98195-6320. E-mail: sjuul@uw.edu

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