

The Quest for Neuroprotection for Injuries in the Developing Brain

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ABSTRACT

The advancement in medical knowledge is not possible without interlocking and reinforcing the bedside-lab continuum. In the field of pediatric neurology, one of the best illustrative examples is our quest for finding neuroprotective therapies for neonatal hypoxic-ischemic encephalopathy. In this review, we discuss the careful clinical observations driven from the bedside dating back to the work of Frank Ford. We trace the relentless efforts to emulate the pathogenesis in animal models to testing potential therapies in clinical trials that made transfer of this knowledge back to the bedside possible.

Keywords: Neuroprotection, hypoxic-ischemic injury, neuroplasticity, inflammation, repair, term and preterm infants, encephalopathy, glutamate, excitotoxicity

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FRANK R. FORD (1892-1970): “THE JUDGE”

Ford was born in 1892 in Baltimore, USA. He graduated from Johns Hopkins School of Medicine in 1920. He then traveled to New York to commence his clinical neurology training only to come back to Johns Hopkins in 1923. He served as the chief of neurology from 1932 to 1958. His special interest in neurological disorders of children was apparent. Dr. Ford's astute clinical observations, “authoritative bedside finesse” [1] and “incisive thinking and ability to formulate diagnosis without particular effort” [99] earned him the title “the Judge”.

Ford was among the first clinicians who put together a concise review of clinical and pathological aspects of cerebral birth injuries in his first book *Cerebral Birth Injuries and Their Results*, which was published in 1927 [100]. In his book, the clinical description of neonates with presumed birth injury is clear and encompasses motor and non-motor symptoms including lethargy, unresponsiveness, restlessness, feeble cry, failure to nurse, irregular respiration, slow heart rate, local or generalized convulsions, rigidity, retraction of the head, unequal pupils, and bulging fontanelles in the setting of increased intracranial pressure. At the end of part 1, he highlighted the long-term results of neonatal brain injury including epilepsy and psychomotor retardation and emphasizes the existence of “normally developing” children despite a history of birth trauma and/or asphyxia.

In contrast to the common trend of approaching diseases of the nervous system from an anatomical basis, Dr. Ford was a pioneer in introducing etiological classifications in child neurology. In his second textbook titled *Diseases of the Nervous System in Infancy, Childhood, and Adolescence*, published in 1937, he grouped neurological disorders based on etiology and pathogenesis. Of interest to this review is his overview on cerebral palsy: “*Thus, five principal theories have been advanced to account for cerebral diplegia; that is due to asphyxia, to meningeal hemorrhage, to an arrest of development resulting from prematurity, to a degenerative process in utero, and congenitally defective development of the brain.*” (Ford, 1927) [101].

This early appreciation for categorizing disorders by etiology created a paradigm shift in the pursuit of deciphering the pathogenesis of neurological disorders of the developing brain.

In this manuscript, we will provide a brief overview of hypoxic-ischemic encephalopathy in term and preterm infants. We then discuss the major “tipping points” that deepened our understanding of hypoxic-ischemic encephalopathy and redefined our therapeutic targets. Finally, we conclude by highlighting “on the horizon” neuroprotective therapies for injury in the developing brain.

INTRODUCTION

“All true scientific research in medicine stems from the bedside” Bernard Sachs (1858-1944). [1]

The field of pediatric neurology in North America emerged as a stand-alone specialty in its modern form in the early 20th century by the hard work and futuristic vision of the “founding fathers” [2] including Bernard Sachs, Bronson Crothers and Frank Ford to whom this review is dedicated. In this review, we will discuss the continued quest for neuroprotective therapies for injury of the developing brain in the setting of hypoxic-ischemic insult.

1. ENCEPHALOPATHY OF THE DEVELOPING BRAIN: A GLOBAL CONCERN

Neonatal encephalopathy (NE) is a clinical syndrome of abnormal neurological function in the first few days of life in infants born at or older than 35 weeks of gestation [3]. Emphasis on strict gestational age represents, in theory, an effort to differentiate neonatal encephalopathy from encephalopathy of prematurity. A recent study by Lee and colleagues estimated that worldwide, approximately 1.15 million newborns develop encephalopathy that is associated with intrapartum events. The overwhelming majority of these newborns resides in low- and middle-income countries (around 96%) and is subjected to underdeveloped obstetric care and/or neonatal resuscitation capacities. Neonatal deaths related to encephalopathy secondary to intrapartum events were estimated to be around 287,000 cases. Among those who survived, 181,000 newborns had mild neurodevelopmental impairment while 233,000 newborns had moderate to severe neurodevelopmental impairment. In the 2010 Global Burden of Disease report, intrapartum-related conditions had one of the highest disability-adjusted life years (DALY) estimated to be at 50.2 million [4]. Thus, it is not surprising that resources should be funneled into researching this complex clinical entity with urgency to dissect the clinical elements, isolate the earliest biological markers and develop targeted therapies that improve survival and long-term neurological outcomes.

Hypoxic-ischemic encephalopathy is a common cause of neonatal encephalopathy in term infants. The criteria for diagnosis are based on exposure to low oxygen levels for a significant period and metabolic markers of acidosis (cord pH < 7 and base deficit > 16). While advancement in neuroimaging techniques has allowed for better understanding of this condition, hypoxic-ischemic encephalopathy is primarily a clinical diagnosis. In 1976, Sarnat and Sarnat proposed the first clinical-electrographic staging system for neonates with “perinatal asphyxia” [5]. The staging system is heavily based on six cardinal aspects of examination: level of consciousness, neuromuscular control, complex reflexes, auto-

Table 1: The original Sarnat and Sarnat staging system, which was published in their seminal paper in Archives of Neurology in 1976.

	Stage 1	Stage 2	Stage 3
Level of consciousness	Hyperalert	Lethargic or obtunded	Stuporous
Neuromuscular control			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreases or absent
Segmental myoclonus	Present	Present	Absent
Complex reflexes			
Suck	Weak	Weak or absent	Absent
Moto	Strong; low threshold	Weak; incomplete; high threshold	Absent
Oculovestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic function	Generalized sympathetic	Generalized parasympathetic	Both systems depressed
Pupils	Mydriasis	Miosis	Variable; often unequal; poor light reflex
Heart rate	Tachycardia	Bradycardia	Variable
Bronchial and salivary secretions	Sparse	Profuse	Variable
Gastrointestinal motility	Normal or decreased	Increased; diarrhea	Variable
Seizures	None	Common; focal or multifocal	Uncommon (excluding decerebration)
Electroencephalogram findings	Normal (awake)	Early: low-voltage continuous delta and theta; Later: periodic pattern (awake); Seizures: focal 1-1½Hz spike-and-wave	Early: periodic pattern with isopotential phases. Later: totally isopotential
Duration	Less than 24hr	Two to 14 days	Hours to weeks

nomic functions, clinical seizures and EEG findings. This staging system takes into consideration the normal neurodevelopmental trajectory of full-term newborns. Based on the findings of clinical examination infants can be categorized into three stages with ascending severity of encephalopathy. The stage of encephalopathy predicted death or neurodevelopmental disabilities (NDD) in the form of cerebral palsy and intellectual disabilities. The original Sarnat staging table is provided here for reference (see table 1) [5]. Infants categorized in stage 1 had no long-term

NDD on follow up at 6-12 months. On the other hand, infants in stage 2 had severe NDD reported in 30-50%. Ninety percent of Infants in stage 3 had severe NDD on follow up. To date, this staging system continues to be a strong predictor of short-term neurodevelopmental outcomes following hypoxic-ischemic encephalopathy even when compared to biochemical markers such as cord blood metabolites [6]. Given the evolving plasticity of the developing brain, it is important to note that serial clinical examination and staging of infants during and after interventions, time spent in each stage and time needed to evolve from each stage to another are more valuable predictors than the initial staging score [7].

2. THE “TIPPING POINTS” IN UNDERSTANDING NEONATAL HYPOXIC-ISCHEMIC ENCEPHALOPATHY

A. EXPERIMENTAL ANIMAL MODELS

The clinical-pathological partnership often reported in case series of newborns and children with hypoxic-ischemic injury (HII) was not sufficiently sophisticated to decipher the complex brain dynamics following the insult. Animal models, therefore, were crucial for depicting and studying the temporal-spatial evolution of injury at structural, functional and molecular levels. They also served as mediums for targeted pre-clinical experimental therapies.

In their quest for modeling neonatal asphyxia, Rice, Vannucci, and Brierley developed the first neonatal rodent model of hypoxic-ischemic injury (HII) in rat pups in 1981. The original model was created using unilateral ligation of the common carotid artery of 7-day-old rat pups (age equivalent to 32-34 weeks gestation human fetus). This was followed by 1-2 hours of recovery, after which 60-100 minutes of hypoxia (8% oxygen/nitrogen balance) at 37°C was delivered [8]. The histopathological profile of this model successfully recapitulated some of the findings in humans including infarction and necrosis involving the cerebral cortex, basal ganglia, thalamus and white matter (in the ipsilateral hemisphere) as early as 14-15 days after recovery [9]. With a model in hand, the scientific community scrutinized the Vannucci model for reproducibility and reliability, and along with that a better understanding of the associated physiological changes and temporal evolution of the injury was attained [10]. However, it was apparent that the rodent model was not suitable to study brain development or neurobehavioral phenotype after injury, as the rodent's brain circulation, cardiovascular system, and metabolism are significantly different from the human infant.

Other animal models were developed to overcome the shortcomings of the rodent model including piglets, rabbits, sheep and non-human primates such as Rhesus monkeys and Baboons. In their comprehensive review of animal models of HII, Huang and colleagues emphasize the importance of recognizing the equivalent time-scale of brain development across species in comparison to human brain development [11]. For example, the brain of a postnatal rat pup at ten days of life is developmentally equivalent to human term infant, while for a sheep model; sheep fetus at 135 days of gestation is equivalent to human term infant. Another important factor to be considered is the research question being asked. For example, understanding pathogenesis and molecular alterations following injury are better studied using the rodent model, while therapeutic interventions and impact

on survival and development are better studied in non-human primate who share a similar cardiovascular system compared to humans. To date, the non-primates term and preterm model of HII are the most biologically plausible and physiologically compatible to humans given similarities in the cardiovascular system, placental function, and age-specific brain development. However, this model is limited by high set-up and maintenance costs as well as ethical concerns. For a detailed review of advantages and disadvantages of animal models in neonatal HII, the reader is referred to the review by Huang and colleagues [11].

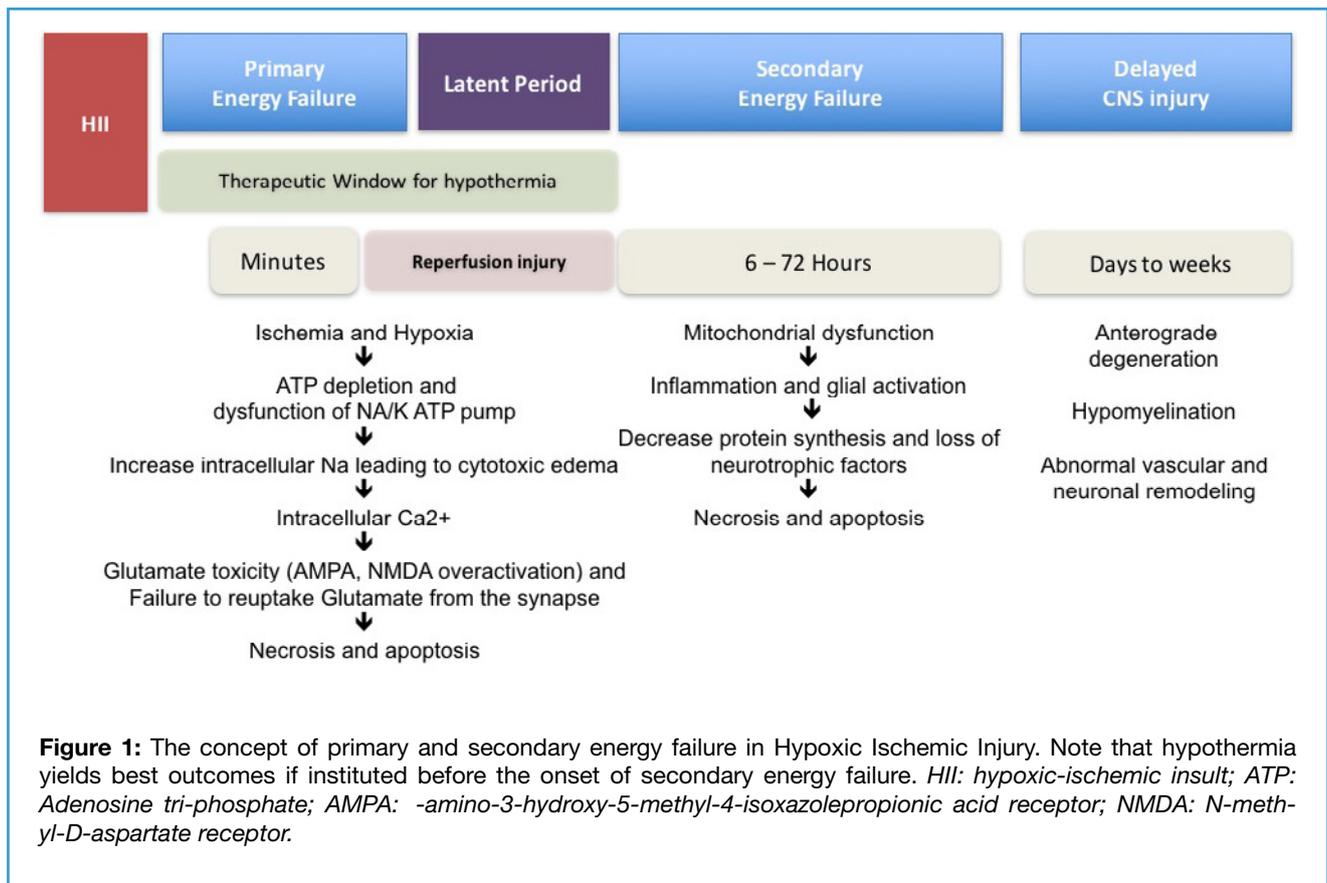
Developing animal models of HII allowed deciphering fundamental principles and identifying key mechanistic pathways that lead to clinically relevant translational applications. In the following paragraphs, we will focus on selected studies that added significantly to our understanding of the aftermath of perinatal HII.

B. UNFOLDING THE PATHOPHYSIOLOGICAL CASCADE OF HII

Integral to the pathogenesis of HII is the concept of primary and secondary energy failure and the latent period in between (see figure 1). Following HII, a time-sensitive excitotoxic cascade takes place. This cascade is kindled by primary energy failure and glutamate toxicity; and propagated by secondary energy failure causing mitochondrial dysfunction, inflammation and glial activation leading to cell death (see figure 1).

Within minutes of HII, primary energy failure ensues leading to failure of neuronal sodium/potassium ATPase (Na⁺/K⁺ ATP) pump function which causes accumulation of sodium intracellularly and prolonged depolarization leading to an abnormal surge in intracellular calcium which leads to excessive release of glutamate into the synapse.

Glutamate plays a major role as a trophic neurotransmitter that allows synaptic communication and therefore, neural network functioning and development [12]. However, its role in HII appears even more critical [13]. Studies have shown age-dependent selective vulnerability for glutamate-mediated excitotoxicity in the setting of HII [14-18], which is heightened in glutamate containing pathways, namely the posterior putamen, ventrolateral thalamus and peri-rolandic cortex [19,20]. Damage due to glutamate hyperactivity in these pathways has been associated with electrographic and clinical seizures in newborns with HII [15]. Excitotoxicity mediated by glutamate is the result of pathological hyperactivity of its receptors, namely α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-Methyl-D-aspartate (NMDA) and metabotropic glutamate (mGluR) receptors following hypoxia-ischemia. The opening of NMDA receptor-operated channels is especially important, as this leads to the entry of calcium into neurons and activation of neuronal nitric oxide synthase to form nitric oxide, which is toxic for mitochondria [12,13]. The overflow of excitatory neurotransmitters (glutamate, aspartate) was reported in the setting of HII [21,22] due to the failure of glutamate reuptake mechanisms in the synapse. Interestingly, the concentration of cerebrospinal fluid (CSF) glutamate correlates with the severity of hypoxic-ischemic encephalopathy [23,24]. Studies have also shown that cholinergic neurons are more sensitive to hypoxia-ischemia compared to dopaminergic and GABAergic-specific neurons [10]. The selective heightened vulnerability of cholinergic neurons is thought to be the result of their biological immaturity in relation to dopaminergic and GABAergic neurons at



the time of injury emphasizing selective heightened vulnerability as a function of maturation.

Following primary energy failure, if reperfusion occurs, a latent period of metabolic recovery takes place. At 6-72 hours following injury, a secondary energy crisis occurs characterized by mitochondrial dysfunction, inflammation and glial activation (see figure 1).

Astrocytes are one of the major supporting cells for intact neuronal function. Astrocytes create a microenvironment of balanced pH and ion concentration and support neuronal function by reuptake of excess neurotransmitters from the synapse [25]. Hypoxia and energy failure in astrocytes lead to a disturbance of neuronal ionic and pH homeostasis and a decrease in glutamate re-uptake from the synapse, contributing to glutamate excitotoxicity. Moreover, astrocytes express angiogenic factors such as vascular endothelial growth factor (VEGF) leading to increase vascular permeability and risk for cerebral edema and hemorrhage [26]. Glial fibrillary acidic protein (GFAP), which is abundant in astrocytes, increases following acute brain injury and thus proposed as a potential biomarker for severity of HII. Interestingly a recent prospective clinical study umbilical cord blood of infants with HII did not show elevated levels of GFAP; which questions its use as a potential early biomarker of injury [27].

Hypoxia also leads to a decrease in the number of oligodendrocyte progenitor cells, alters the maturation of oligodendrocytes and induces apoptosis [28]. Moreover, inflammation compromises microglial cell function, setting the stage for ongoing brain injury and compromised myelination [29,30]. White matter injury following HII is a common finding in surviving infants [31].

Inflammation has been increasingly recognized as a major culprit in brain injury in the setting of HII. CSF pro-inflammatory cytokines, namely IL-6 and IL-8 are elevated in human infants with perinatal asphyxia and proportionally correlate with the degree of encephalopathy [32]. Systemic inflammation as seen in fetal inflammatory response syndrome (FIRS) in the setting of chorioamnionitis [33], neonatal sepsis and necrotizing enterocolitis [34], has been reported to aggravate brain injury. Placental biomarkers of inflammation are linked to neonatal brain injury supporting the notion that early identification of "stress-related biomarkers" might provide a better prediction of clinical outcomes [35].

The functional and developmental state of cerebral vasculature determines acute response to alterations in metabolic demand and capacity for autoregulation [36,37]. Oxygen regulates angiogenesis via hypoxia-inducible factor alpha (HIF-alpha) dependent genetic expression [38]. Following HII, transient compensatory increase in cerebral blood flow occurs, followed by vasoconstriction and later loss of cerebral autoregulation augmenting metabolic compromise and injury. Hypoxia stimulates the expression of VEGF, among others, promoting angiogenesis in the face of energy crises. However, excessive VEGF can also compromise the quality of newly formed blood vessels and integrity of blood-brain barrier, culminating in increased risk for edema and hemorrhage. Hypoxia and ischemia can also affect the contractility of blood vessels allow ungated transmission of changes in systemic blood vessels to cerebral blood vessels setting up the stage for cerebral injury at extremes of fluctuation in blood pressure. An excellent review of the vascular response to HII in the developing brain is referenced here for interested readers (Baburamani et al., 2012, [26]).

Hypoxic-ischemia injury can impact the normal developmental trajectory of epigenetic modification via modulation of DNA (de) methylation, histone modification and biogenesis of microRNAs, which are necessary for proper expression of genetic determinants of neuronal and glial cells and vascular bed development. This can cause heightened vulnerability to the insult as well as impairment in appropriate neuroplastic response and remodeling following injury [17,39]. Further, the heightened vulnerability of the developing brain to long-lasting damage from HII is related to the effect of hypoxia on neurogenesis and plasticity. HII can induce apoptosis of neural progenitor cells in the subventricular zone affecting neurogenesis, migration, and proliferation [40], which can lead to alerted synaptic connectivity and disruption of normal excitatory/inhibitory balance of networks function [41]. HII can also induce neurogenesis in certain brain regions such as the hippocampus promoting functional recovery [42]. One proposed mechanism is modulation of neurogenesis by dopamine via D2 receptors [43].

Finally, an interesting arena of research is the effect of sex differences in vulnerability and response to HII in neonates. Pre-clinical and clinical data support the notion that male infants are more vulnerable to HII and interestingly, they also display more long-term neurobehavioral deficits [44].

C. ASSESSING THE INJURY PROFILE WITH NEUROIMAGING

With advancement in neuroimaging techniques over the past few decades, it became possible to diagnose, estimate the timing of insult and, to a certain degree, prognosticate neonatal brain injury following hypoxic-ischemic insult. Se-

lecting the appropriate neuroimaging modality to examine suspected hypoxic-ischemic encephalopathy is important. In neonates and children, the use of Computerized Tomography (CT) is generally discouraged due to radiation effects and low resolution to pick up subtle parenchymal abnormalities. However, Head CT can be useful to characterize bony abnormalities or identify bone fracture related to birth trauma if needed. The modalities of choice for neonatal encephalopathy, in general, are cranial Ultra Sound (US) and brain Magnetic Resonance Imaging (MRI).

Cranial US can be a powerful bedside tool in the hands of the expert especially for infants who are not clinically stable enough to undergo MRI evaluation. The Cranial US can help estimate the gestational age based on the morphology of brain development and identify abnormalities such as germinal matrix hemorrhage, intraventricular hemorrhage, atrophy, and hydrocephalus. In hypoxic-ischemic encephalopathy, the typical findings include white matter hyper-echogenicity, increased cortico-medullary differentiation, edema and slit-like ventricles. We refer interested readers to the review by Orman and colleagues on the application of cranial US in neurological disorders of neonates [45]. The limitation of US is its inability to provide high-resolution images of white matter and subcortical structures and its significant operator-dependent technique. If the clinical picture does not match the finding on the cranial US, brain MRI is recommended.

Brain MRI gives the highest imaging resolution and is radiation-free. However as mentioned earlier, it cannot be performed in critically ill infants. If planned, sedation is usually not needed for infant brain MRI protocol. Timing the MRI around the infant sleep-wake cycles and adequate feeding and swaddling before imaging are usually sufficient. In

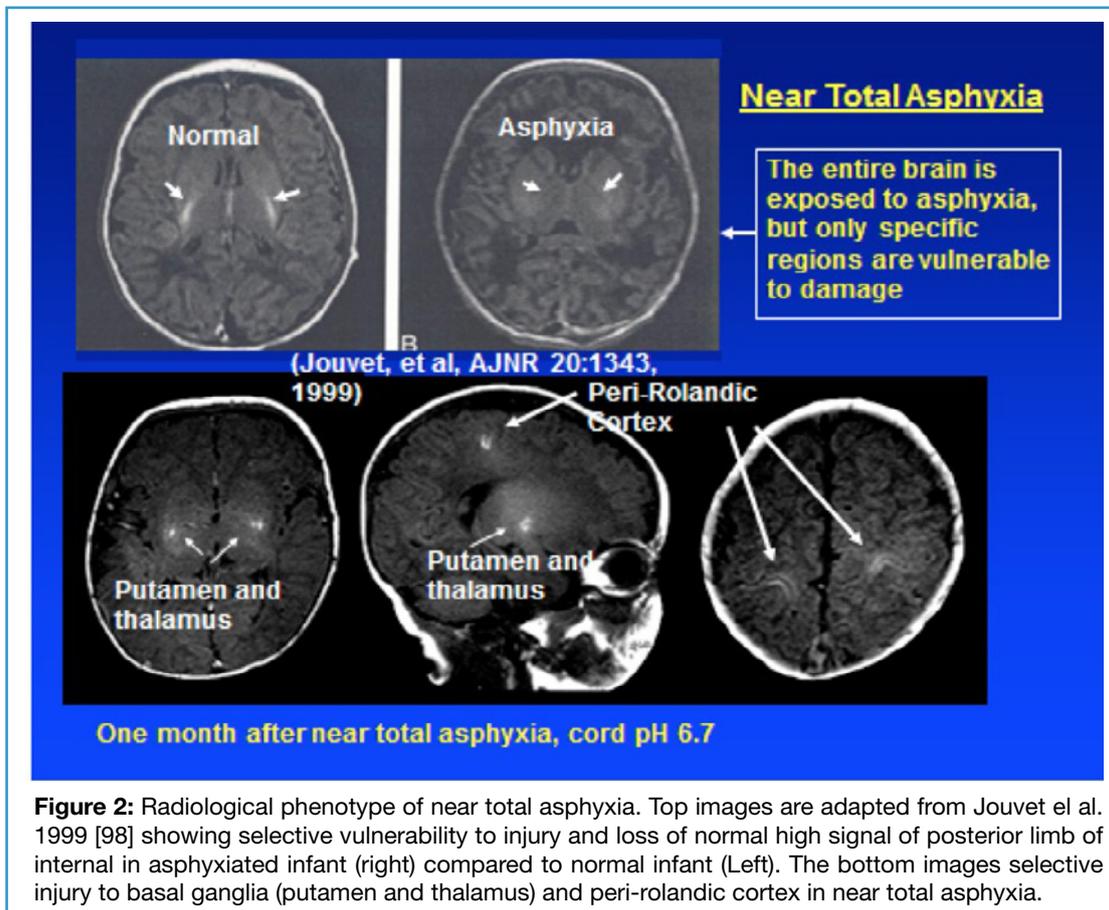
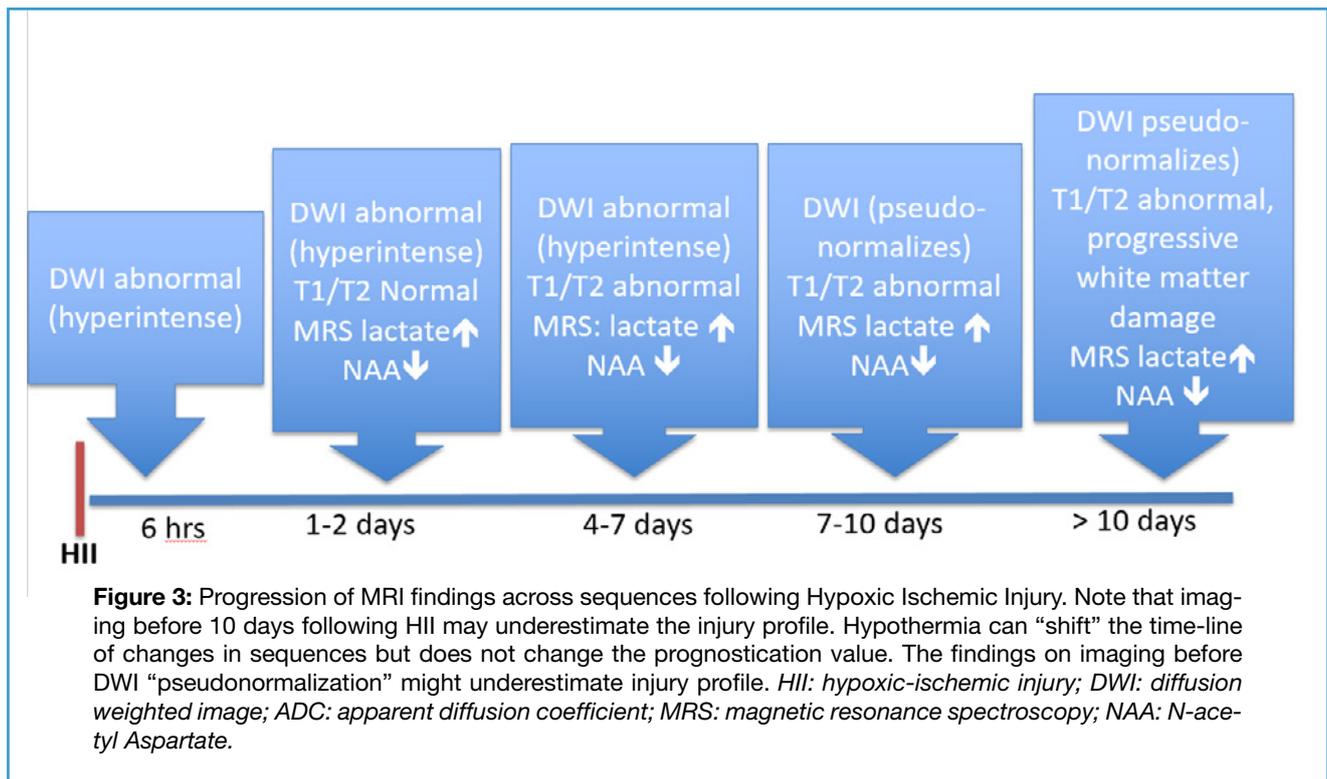


Figure 2: Radiological phenotype of near total asphyxia. Top images are adapted from Jouvet et al. 1999 [98] showing selective vulnerability to injury and loss of normal high signal of posterior limb of internal in asphyxiated infant (right) compared to normal infant (Left). The bottom images selective injury to basal ganglia (putamen and thalamus) and peri-rolandic cortex in near total asphyxia.



some cases, rectal midazolam can be used. Brain MRI can be helpful in confirming the diagnosis of hypoxic-ischemic encephalopathy and ruling out common mimickers including neuro-metabolic diseases, neonatal stroke, congenital hydrocephalus, congenital infections and structural abnormalities. With sophisticated protocols such as diffusion tensor imaging (DTI) and magnetic resonance spectroscopy (MRS), risk stratification and prognostication is possible. Choosing the imaging modality should always be done after consulting the neuroradiologist to optimize the yield of imaging.

The definition of the radiological phenotype of hypoxic-ischemic encephalopathy has been pursued for a long time. Generally speaking, there are three patterns of HII in term infants [46]. The first is near total asphyxia pattern, which results from acute umbilical cord compression and placental abruption. In this pattern, the MRI shows hyperintensity on T2 weighted images in the basal ganglia, thalamus, peri-rolandic cortex and posterior limb of internal capsule, indicating selective vulnerability in which asphyxia damages structures that are connected by excitatory pathways (see figure 2). Infants with this pattern grow to develop dyskinetic cerebral palsy (CP) in the majority of cases. The second pattern is predominant cortical injury (sparing the basal ganglia). This pattern results from prolonged partial asphyxia, secondary to intermittent cord compression or long-standing placental abnormalities. Spastic quadriplegia, intellectual disabilities, and seizures are common long-term comorbidities. The third pattern is the watershed pattern of injury affecting cortical wedges between anterior cerebral and middle cerebral arteries and between middle cerebral and posterior cerebral arteries secondary to severe hypotension. The neurological and developmental outcomes in this pattern are less severe than the first two patterns. Perinatal and postnatal events associated with prominent hypotension are usually reported. For a detailed review of neuroradiological patterns, interested readers are referred to De Vries and Groenendaal [47].

In the preterm infant, MRI pattern of injury is different and is thought to reflect the age-dependent vulnerability of the immature brain. Germinal matrix hemorrhage in the caudothalamic notch is more common in immature infants. Periventricular leukomalacia (PVL) is a pathognomonic pattern of preterm brain injury, which is characterized by cortico-thalamic injury and axonal destruction with micro and macrocytic degeneration representing coagulation necrosis and inflammatory microglial and astrocytes response. For a comprehensive review on the mechanisms underlying the peculiar imaging pattern of hypoxic ischemic encephalopathy in preterm infants, we refer the reader to the work of Joseph Volpe [48,49].

The role of different MRI sequences in prognostication has been evaluated by many studies. The scoring system of Barkovich and colleagues based on examining the basal ganglia and watershed areas using T1- and first and second Echo T2-weighted sequences accurately discriminated between good and poor neurological outcomes at 3 and 12 months following hypoxic-ischemic encephalopathy [50]. Additionally, low mean apparent diffusion coefficient (ADC) in the vermis, pons, medulla and left hemisphere during the first week of life correlates with poor motor outcomes or death [51]. Similarly, in infants treated with hypothermia, low ADC values in the posterior limb of internal capsule (PLIC), thalamus and cortical white matter predict poor outcomes [52]. By employing the newer MRI sequences such as DTI and MRS, studies found that low fractional anisotropy (FA) on DTI [53,54] and low (N-acetyl aspartate (NAA; a marker of neuro-axonal integrity) with high lactate on MRS [55] in the basal ganglia and thalamus [56] and low choline [57] correlated with poor neurodevelopmental outcomes. The brain MRI signature of HII changes with time (see figure 3). Due to the evolving nature of the injury, images obtained after the first 1-2 weeks of life had better prognostic values [58].

D. REAL-TIME EVALUATION OF BRAIN DYNAMICS

Technological advances in electrophysiological and blood flow monitoring allowed for real-time evaluation of how the infant brain reacts and adapts to injury. Near-Infrared Spectroscopy (NIRS) is a non-invasive, bedside tool that measures tissue oxygenation based on regional blood flow in the cortex. Under normal conditions, the limits of auto-regulation of cerebral blood flow are carefully regulated in the face of fluctuation in blood pressure. Theory of impaired auto-regulation secondary to brain injury secondary to trauma or stroke is well established in animal and human subjects. The integrity of auto-regulation as a function of maturity is a vital compensatory mechanism in the setting of the hypoxic-ischemic event and severe fluctuations in systemic blood pressures. In a study by Massaro and colleagues, higher pressure passivity index (PPI) using continuous mean arterial pressure (MAP) and certain NIRS measurements correlated with adverse outcomes in term infants with hypoxic-ischemic encephalopathy [54].

In a pilot study of 28 term infants with HII by Burton and colleagues, optimized MAP at which vasoreactivity is greatest was found to be superior to the standard gestational age based MAP (GA + 5) in decreasing motor and cognitive impairment at 21-32 months of age. This highlights the importance of individualizing the MAP goal for patients based on the status of cerebral autoregulation regardless of gestational age [59]. Deviations below optimized MAP were associated with moderate to severe injury to paracentral gyri, basal ganglia, and thalamus [60].

Another advantage in the field of neonatal neurology is the early detection of abnormal electrical activity in newborns. The technology of amplitude-integrated electroencephalography (aEEG) and EEG made it easier to continuously monitor cerebral activity (continuity of electrographic pattern and sleep-wake cycles) and epileptiform activity to predict patients at risk for epilepsy and long-term neurological morbidities. Lack of recovery from abnormal aEEG background during hypothermia is considered a poor prognostic sign [7]. Abnormal EEG patterns such as burst suppression, low voltage and flat trace [61] and high electrographic seizure burden [62] are accurate predictors of long-term neurodevelopmental outcomes. Therefore, treating electrographic seizures is recommended.

3. THERAPEUTICS

As mentioned earlier, major therapeutic targets in hypoxic-ischemic encephalopathy are based on key mechanistic pathways of pathogenesis derived from animal models, including glutamate excitotoxicity via NMDA and AMPA receptors overactivation, inflammation and immune-mediated injury, oxidative stress and apoptosis pathways of secondary energy failure and induction of neuronal regeneration and remodeling [12,13]. For both preterm and term infants with hypoxic-ischemic encephalopathy, hemodynamic stability, correcting electrolyte imbalance, maintaining normoglycemia, adequate nutrition and promptly treating infections have been shown to improve survival and neurodevelopmental outcomes [63].

A. THERAPIES IN TERM INFANTS

The historical origins of the effect of hypothermia on asphyxiated infants can be dated back to the observation of Hippocrates that infants born with asphyxia in winter survived longer than those born in summer [64]. In animal models, the

severity of excitotoxic mediated brain injury is dependent on brain temperature [65]. The mechanisms for hypothermia-induced neuroprotection include reverting from aerobic to anaerobic metabolism, decreasing glutamate release, replenishing ATP, reducing nitric oxide (NO) production and free radical release, and inhibiting apoptosis [13,66].

The "window of application" of hypothermia in eligible infants is derived from the seminal work of Gunn and Thoresen in their sheep model of hypoxic-ischemic encephalopathy [64]. They demonstrated that hypothermia introduced within 1.5 hours after injury could "shield" the brain from HII yielding total protection. However, if hypothermia was applied at 8 hours following HII, no protective effect is achieved. Applying hypothermia 5.5 hours after HII yields moderate protection. Based on molecular studies, hypothermia provides the best neuroprotection before the onset of secondary energy failure, which is approximated to be 6 hours after the HII. Therefore, all clinical trials on human infants are based on this time frame.

Jacob and colleagues have published a Cochrane review on hypothermia in term and later preterm infants with hypoxic-ischemic encephalopathy [67]. The analysis of 11 randomized control trials (N=1505 infants) showed that hypothermia instituted within 6 hours after HII reduces mortality without increasing major disability in survivors. Moreover, hypothermia improves neurodevelopmental outcomes at 18-24 months. The number needed to treat for an additional beneficial outcome was 7 [67]. Modifying the cooling protocol such a lowering temperature to 32 degrees celsius and prolonged duration of 120 hours has been deemed futile in a recent RCT [NCT01192776].

Despite the clear benefit of hypothermia in mitigating the aftermath of HII for eligible infants (discussed later), the financial cost of setting up hypothermia cooling system in developing countries curtailed disseminated use in low-resource settings globally. To make hypothermia treatment more accessible worldwide, Kim and colleagues developed a low-cost hypothermia whole-body cooling device that utilizes evaporation and endothermic reaction to achieve hypothermia and controlled rewarming. In piglet model of HII, this device had lowered core temperature to 33.5 °C with a 1 °C margin of error [68]. Clinical trials will be needed for more rigorous assessment for clinical application.

B. THERAPIES IN PRETERM INFANTS

In preterm infants, administration of antenatal steroids and surfactant to boost fetal lung maturity, and caffeine and nitric oxide have been shown to improve cardiopulmonary and hemodynamic stability [45]. Postnatal steroid use has been viewed as a double-edged sword in preterm infants. While studies have shown the shorter duration of ventilator support and decreased risk of bronchopulmonary dysplasia, significant comorbidities such as infections, hypertension, hyperglycemia and GI perforation were reported. Postnatal steroids continued to be used for a specific subpopulation of preterm infants. A comprehensive review on the preferential effect of post-natal steroids regarding the timing of administration and route has been recently published [70].

The therapeutic application of hypothermia in preterm infants has been investigated in small studies with unfavorable results. For example, in a study of 31 preterm infants born at 34-35 weeks of gestation, hypothermia resulted in higher rates of complications and death compared to term infants [71]. Since hypothermia has not been investigated systematically in early preterm infants, adjunct neuroprotective ther-

apies are being actively investigated with promising results. Our early laboratory study of magnesium treatment of immature rat pups showed that it was protective against brain lesions caused by injection of the glutamate-receptor agonist, NMDA [72]. Based on this evidence, Nelson and Grether conducted a retrospective case-control study of babies born to mothers in which magnesium sulfate was used as a tocolytic for threatened premature delivery, which indicated that the incidence of CP was reduced in the magnesium group [73]. Numerous subsequent studies have replicated this effect. The French PREMAG, randomized control trial, reported a significant decrease in death, CP and cognitive dysfunction at two-year follow-up in pre-term infants born less than 33 weeks of gestation who received a single dose of magnesium sulphate antenatally compared to controls [74]. This has also been supported by meta-analysis review [75]. However, a trial of magnesium sulfate for term babies with asphyxia was not continued due to an unacceptable incidence of systemic hypotension [76].

C. ADJUNCT NEUROPROTECTIVE THERAPIES

Despite the revolutionary role of hypothermia in improving clinical outcomes, 30-50% of newborns eligible for this therapy continues to suffer from sequelae of brain injury. The need for further adjunct neuroprotective therapies remains. The quest for neuroprotection continues for two major reasons: i) in many regions of the world, the health system for care of women and infants is simply not adequate to provide modern obstetrical care, and so millions of babies are born with asphyxia - this is a resource and public health problem that needs to be addressed; 2) while cooling is very effective if applied within a few hours of acute asphyxia, many insults are not acute but are partial and prolonged, and the cooling is not effective once the neuroexcitatory cascade has become refractory to cooling. Better therapies are needed to address the events that are triggered by asphyxia but unfold over hours to days after injury.

Dating back to 1959 and inferring from his study of cortical spreading depression, Van Harreveld speculated on the pathogenic role of glutamate in HII [77]. As mentioned earlier, subsequent studies investigated the pathophysiology of glutamate-mediated injury and identified glutamate as a potential therapeutic target (reviewed in Rothman and Olney [21]). Systemic administration of NMDA blockade using dizocilpine (MK-801) was shown to be protective against HII. However its therapeutic window was short (< 3 hours) [78].

Other NMDA antagonists such Xenon (noble synthetic gas) have been experimented as adjunct agents to augment the effect of hypothermia. In preclinical studies, the neuroprotective effect of xenon in HII has been debated. In two studies, Xenon combined with hypothermia showed histopathological neuroprotective effects and translated into a long-term functional improvement in the rat model of neonatal HII [79], even when administered late during injury [80]. However, another study by the same group did not show similar effect [81]. The TOBY-Xe trial (Total Body hypothermia plus Xenon) which was a proof-of-concept study, showed that 30% inhaled xenon application in infants with HII was feasible and safe. However, no change in neuroimaging injury profile was observed between treatment and control groups [82]. An ongoing study is investigating combining Xenon (8h xenon inhalation at 50% concentration) with hypothermia for 72 hours in the treatment of term infants with HII [CoolXenon3; NCT02071394] is actively recruiting.

The general theme of overt excitotoxicity in HII identified many potential therapeutic agents. Phenobarbital (gamma-Aminobutyric acid (GABA) agonist) and Topiramate (AMPA-receptor antagonist) were considered to potentially augment the therapeutic effects of hypothermia and prolong the temporal window of protection, respectively in animal models of HII [83]. Clinical trials of Topiramate (10mg/kg/day) for three days as an adjunct therapy to hypothermia for term infants with HII have been recently conducted. The clinical feasibility and safety of administration were reported by Filipi and colleagues in the NeoNATI Trial [84]. However, the efficacy in reducing mortality and improving neurological outcomes was not proven. Another trial investigating reduced dose of Topiramate (5mg/kg/day) for five days in near-term infants with HII has been completed recently [NCT01765218]. Despite its promising pre-clinical results, Bumetanide (loop diuretic that blocks NKCC1 modifying GABA receptor response) failed to treat acute HII-induced neonatal seizures as an add-on therapy to phenobarbital in the NEMO Trial [85].

Epigenetic modification after HII using Histone deacetylase inhibitors (HDACi) such as uridine and sodium butyrate has been investigated in pre-clinical models of HII. Uridine application in neonatal HII decreased Caspase-3 level and reduced infarct volume size [86]. Similarly, Sodium butyrate has been shown to exert neuroprotective properties following neonatal HII by curbing the rise in inflammatory cytokines [28]. Additionally, the neurogenic effect of sodium butyrate including protection against ischemia-induced loss of neuroblasts and oligodendrocyte precursor cells has been demonstrated [28].

Reducing production of oxygen free radicals following ischemic insult using allopurinol (Xanthine oxidase inhibitor) showed promising results in animal models of HII. A randomized clinical trial using allopurinol as an adjunct therapy to hypothermia in near-term and term infants is underway [NCT03162653]. Preclinical data supported the role of melatonin as an anti-inflammatory and anti-oxidant agent [87,88]. Given the known safety profile and wide clinical availability, melatonin (0.5 mg/kg enteral dose) within 12 hours of life following neonatal HII is being investigated in an early phase 1 dose escalation clinical study [NCT02621944].

Erythropoietin is showing promising clinical potential. Erythropoietin had been reported to decrease glutamate-mediated toxicity, reduce inflammation and oxidative stress, decrease oligodendrocytes injury and neuronal apoptosis and induce oligodendrogenesis and stimulate brain-derived growth factor (BDNF). Safety and efficacy in a small clinical trial support its use in infants with hypoxic-ischemic encephalopathy [89]. Randomized clinical trials of erythropoietin in infants at risk for brain injury are emerging. Monotherapy with erythropoietin (500 U kg⁻¹ per dose on alternate days for a total of five doses) has been shown reduce mortality and neurological disability at 19 months [90]. Erythropoietin as an adjunct agent to hypothermia has been shown to improve motor outcomes at one year [91]. Other clinical trials in the pipeline that are investigating higher doses of erythropoietin > 1000 IU/kg birth weight include [PAEAN trial NCT03079167; NEATO trial, NCT01913340; Neurepo Trial, NCT01732146]. The Preterm Erythropoietin Neuroprotection Trial (PENUT) trial is a Phase III study of Erythropoietin in Extremely Low Gestational Age infants (24-27 weeks of gestation) has recently completed enrollment with 940 subjects [NCT01378273].

Microglial and astrocyte-mediated inflammation plays a major role in the pathogenesis of HII as mentioned earlier. On the frontiers of adjunct therapies in HII is the precise delivery of anti-inflammatory medications via nanotechnology. For example, anti-inflammatory drugs delivered by carbon dendrimers which cross the blood-brain barrier and seek out activated microglia have been used to reduce cerebral palsy phenotype in rabbits with white matter injury [92]. Similarly, Nance and colleagues showed that D-NAC improves myelination and reduces white matter injury during a therapeutic window up to 9 days following white matter injury [93].

Cell-based therapies for brain injuries are also being investigated. Pre-clinical studies have shown that glial-restricted progenitor cells (progenitor cells that can differentiate into oligodendrocytes and astrocytes) provide trophic and immunomodulatory support in the face of HII [94]. Implantation of neural stem cells at day 10 post HII in mice reduced inflammation and lesion size, improvement in synaptic integrity and sensorimotor deficits [95]. In a sheep model of HII, Granulocyte-colony stimulating factor (G-CSF) to mobilize endogenous stem cells and enhance neural stem cells proliferation has been reported to reduce microglial activation. However, no effect on seizure control was observed [96].

Translating preclinical findings to the clinical arena is being actively pursued. A recent clinical study of infusing autologous cord blood cells in infants with HII within 14 days following injury was proved to be feasible and safe [97]. Additional trials addressing feasibility and safety [NCT02455830; NCT02256618], neuroprotective mechanisms [NCT01649648, NCT00375908], and effect on development [NCT02551003; NCT02612155] are in the pipeline. Intrathecal administration of neural progenitor cells and concentrated paracrine factors of human mesenchymal stem cell is being investigated [NCT02854579]. See table 2 for a detailed description of the individual clinical trials mentioned in this section.

CONCLUSION

Hypoxic-ischemic encephalopathy is a global health and economic burden because of the high mortality and morbidity rate involved. In our quest to protect the developing brain from the aftermath of injury, it is important for researchers in the clinical and basic science fields to take on a leading role in bringing clinical observations and data to the lab and back to the bedside in the form of translational therapeutics.

Although hypothermia is thus far the most validated and best-supported therapeutic tool we have, the advances in our understanding of the pathophysiology of this clinical entity in the context of brain development and plasticity will

allow for the next breakthrough in neuroprotection and remodeling. The ultimate goal of enhancing neuroprotection in infants with HII should focus on improving long-term neurodevelopmental outcomes. The dynamic change in the clinical and neuro-radiological profile of HII over time warrants serial neurological, developmental and neuropsychological evaluation to improve prediction of clinical outcomes and institution of early developmental interventions. While majority of clinical trials have focused on outcomes at 12-24 months of age, longer follow-up into school age is needed to delineate impact on higher cognitive functions such as attention, executive function, learning, memory and language (verbal, writing, higher-order processing), social, emotional and internalizing behaviors such as (anxiety and depression) commonly seen in this patient population.

Finally, it is important to recognize that advances in neuroprotection of the neonatal brain are mainly reported in academic institutions with rigorous experimental set up and abundant resources. However, the highest impact of such advances is only recognized when it can be translated to where it is needed most. As mentioned earlier, 96% of HII in neonates occurs in developing countries and thus, collaborative effort is needed to transcend economic barriers to bring cost-effective neuroprotective strategies to low-resources setting. The cost-effective hypothermia treatment device developed by Kim and colleagues [68] is a promising step towards globalizing medical discoveries to improve global healthcare.

Competing interests

The authors have no financial or non-financial competing interests.

Author Contributions

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Table (2): Description of active clinical trials pursuing new neuroprotective strategies for neonates with Hypoxic Ischemic Injury. h: hour; MRI: Magnetic Resonance Imaging; aEEG: amplitude integrated EEG. S100-Beta: marker of neuronal injury; CP: cerebral palsy; MTD: maximum tolerated dose; GMA: generalized motor assessment; AED: anti-epileptic drugs; PMA: Post menstrual age; SDF-1, TNF-alpha and IL-1: Biomarkers for oxidative stress, inflammation and immune response.

Therapeutic Target	Study (Clinical Trials.gov ID)	Participants	Experimental Protocol	Primary Outcomes	Secondary Outcomes	Status
Xenon	CoolXenon3 [NCT02071394] UK	Infants with HIE > 36 weeks	cooling + 18h xenon inhalation at 50% concentration	Death and moderate to severe disability at 18 months	Brain MRI (within 2 weeks of birth) aEEG grading within 1 week of birth Developmental Outcomes at 18-24 months	Phase 2: Recruiting
Topiramate	[NCT01765218] USA	Infants with HIE >=34 weeks	Cooling + Topiramate (5 mg/kg/day) for total of 5 doses	Seizures at 4 weeks or at discharge	HIE score at 4 weeks or at discharge Normalization of aEEG at 4 weeks or at discharge S100-beta levels at 1, 3, 7 days MRI score at 5-7 days Developmental Outcomes at 9, 18, 27 months	Phase 1, 2: Completed
Allopurinol	ALBINO [NCT03162653] Europe	Infants with HIE >=36 weeks	Cooling + Allopurinol (20mg/kg) within 30 minutes of birth and (10mg/kg) 12 h thereafter	Death and moderate to severe disability at 24 months	Incidence of Death and CP at 24 months GMFCS-score at 24 months Developmental outcomes at 24 months	Phase 3: Not yet recruiting
Melatonin	[NCT02621944] USA	Infants with HIE >=36 weeks	Cooling + melatonin (0.5mg/kg) within 12 h after birth	MTD Pharmacokinetics of escalated doses Adverse events Developmental outcomes at 18-20 months	Developmental outcomes subscales at 18-20 months GMA at 3 and 23 months Brain MRI 7-12 days after birth	Phase 1: Recruiting

Therapeutic Target	Study (Clinical Trials.gov ID)	Participants	Experimental Protocol	Primary Outcomes	Secondary Outcomes	Status
Erythropoietin	PAEAN [NCT03079167] Australia and New Zealand	Infants with HIE >=35 weeks	Cooling + erythropoietin (1000 IU/kg BW), on days 1,2,3,5,7 of age	Death and moderate/severe disability at 24 months	Death and CP at 24 months Developmental outcomes at 24 months Epilepsy at 24 months Cost of healthcare Frequency of AED	Phase 3: Recruiting
	NEATO [NCT01913340] USA	Infants with HIE >=36 weeks	Cooling + erythropoietin (1000 IU/kg BW) total of 5 doses	Markers of organ function for 2 weeks	Developmental and functional outcomes at 12 months	Phase 1, 2: Not yet recruiting
	Neurepo [NCT01732146] France	Infants with HIE >=36 weeks	Cooling + erythropoietin (1000-1500 IU/kg BW) total of 3 doses	Survival without neurological sequelae At 2 years	Death and Moderate/severe disability at 24 months Brain MRI within 6-12 days Tolerance at 2 years	Phase 3: Recruiting
	PENUT [NCT01378273] USA	Preterm infants 24-27 weeks of gestation	Cooling + erythropoietin (1000 IU/kg BW) for total of 6 doses followed by 400 IU/kg until 32 6/7 weeks of gestation	Neurodevelopmental outcomes at 24-26 months	Safety at term PMA Brain MRI at 36 weeks PMA Inflammation biomarkers at 24-26 months	Phase 3: Not yet recruiting

Therapeutic Target	Study (Clinical Trials.gov ID)	Participants	Experimental Protocol	Primary Outcomes	Secondary Outcomes	Status
Cell-Based	[NCT02455830] Japan	Infants with HIE >=36 weeks	Cooling + Autologous cord blood cell infusion (3 doses within 72 h)	Changes in cytokines and trophic factors level for 10 days	Brain MRI at 12 months Developmental and functional outcomes at 18 months Correlation with cytokines profile	Phase 1: Recruiting
	[NCT02256618] Japan	Infants with HIE >=36 weeks	Cooling + Autologous umbilical cord blood cells (3 doses within 72 h)	Adverse events at 30 days	Neurodevelopmental function at 18 months Brain MRI at 12 months	Phase 1: Recruiting
	[NCT02551003] China	Infants with HIE >=34 weeks	Cooling + Autologous umbilical cord blood cells (divided doses within 72 h)	Death and neurodevelopmental disability within 18 months	Neurodevelopmental outcomes at 12 and 18 months Brain MRI at 7, 28 days and 12 months Adverse events within 72 h Serum SDF-1, TNF-alpha, IL-1 at 4 and 14 days	Phase 1,2: Recruiting
	[NCT02612155] USA	Infants with HIE >=35 weeks	Cooling + Autologous umbilical cord blood cells (2 doses)	Survival at 1 year Neurodevelopmental outcomes at 1 year	Mortality rate, seizures and AED, Need for iNO use, ECMO and G tube feeding at 12 months	Phase 2: Recruiting
	[NCT02854579] China	Infants with HIE >=34 weeks	Neural progenitor cells and/or paracrine factors intrathecal infusion	Neurodevelopmental outcomes at 14 and 28 days Adverse events at 7 days	Neurodevelopmental outcomes at 1 2 months Death within 12 months Treatment-related CNS tumor within 5 years	Phase 1: Recruiting

Table (2): Description of active clinical trials pursuing new neuroprotective strategies for neonates with Hypoxic Ischemic Injury. h: hour; MRI: Magnetic Resonance Imaging; aEEG: amplitude integrated EEG; S100-Beta: marker of neuronal injury; CP: cerebral palsy; MTD: maximum tolerated dose; GMA: generalized motor assessment; AED: anti-epileptic drugs; PMA: Post menstrual age; SDF-1, TNF-alpha and IL-1: Biomarkers for oxidative stress, Inflammation and immune response.

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