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# Neuroprotection Strategies for term encephalopathy

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## Abstract

Brain injury in the full-term and near-term neonates is a significant cause of mortality and long-term morbidity, resulting in injury patterns distinct from that seen in premature infants and older patients. Therapeutic hypothermia improves long-term outcomes for many of these infants, but there is a continued search for therapies to enhance the plasticity of the newborn brain, resulting in long-term repair. It is likely that a combination strategy utilizing both early and late interventions may have the most benefit, capitalizing on endogenous mechanisms triggered by hypoxia or ischemia. Optimizing care of these critically ill newborns in the acute setting is also vital for improving both short and long-term outcomes.

## INTRODUCTION

Brain injury in the full-term and near-term gestation neonate is a significant contributor to mortality and long-term morbidity, secondary to the vulnerability of the developing brain to injury. Causes of early brain injury include stroke, birth trauma, metabolic or genetic disorders, neonatal-onset epilepsies, and a variety of perinatal events that lead to decreased blood flow or oxygen delivery to the brain. This last cause is the most common cause of perinatal brain injury[1]. It usually presents with neonatal encephalopathy, or an abnormal neurological exam, and is estimated to occur in 3 to 5 in 1000 live births[1]. This is referred to as hypoxic-ischemic encephalopathy (HIE), and currently this diagnosis can only be confirmed by magnetic resonance imaging (MRI). Stroke is also common, with an estimated incidence of 1 in 2000 live births[2], and is most commonly arterial-ischemic in origin, with clots or emboli likely originating from the placenta. It also shares many of the same risk factors as HIE[3]. While some suffering from perinatal brain injury die during early life, the majority of survivors exhibit neurological deficits that persist, such as cerebral palsy, intellectual disability or epilepsy[4]. Therapeutic hypothermia is the only proven therapy for HIE, but it must be initiated early and provides only partial benefit to newborns with HIE. Aside from therapeutic hypothermia, no established therapies exist, and treatment and care for the sequelae of early brain injury requires significant resources. In addition, diagnosis of early brain injury is often delayed, making the identification of delayed therapeutic options

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or strategies crucial[5]. Neonates that present with encephalopathy following a sentinel event are more likely to benefit from hypothermia, whereas those who suffer from a more remote or more prolonged insult may not improve, even with immediate initiation of hypothermia after delivery[6]. Full-term neonates with underlying congenital cardiac disease are also at increased risk of white matter injury and cortical volume loss more commonly associated with premature brain injury[7]. For infants that present with more remote hypoxic injury or other etiologies of impaired development, delayed therapeutic strategies present the only option.

Injury to the immature brain involves a number of different mechanisms that lead to cellular damage and death, as well as altered cell fate characterized by changes in neural precursor cell proliferation, differentiation and migration. Even in cases where the etiology of hypoxia or ischemia is brief or acute, injury continues to evolve over a period of days to weeks, and even months[8, 9]. The initial period following an insult is characterized by primary energy failure, where decreased ATP and increased lactate production lead to loss of cell membrane integrity and calcium entry, excitotoxicity and necrosis within the core of damaged tissue. This is followed by a latent phase, where restoration of blood flow and oxygen delivery lead to initial recovery of cells within the penumbra, or the damaged tissue may survive the initial insult but is still susceptible to further damage. This is why it is critical that acute therapies such as hypothermia are initiated early, as there is a brief window to rescue these cells prior to secondary energy failure. Left untreated, a number of cells are then overwhelmed by an influx of inflammatory mediators, free oxygen radicals, and further excitotoxicity that lead to mitochondrial failure and programmed cell death. The mechanisms leading to programmed death patterns include classically described apoptosis, as well as other caspase-dependent and independent processes such as ferroptosis and necroptosis[10].

Endogenous repair involves mechanisms that enhance neurogenesis, gliogenesis, vasculogenesis/angiogenesis, and remyelination[11]. Many of these endogenous repair processes are mediated through stabilization of neuronal transcription factors, including hypoxia-inducible factor (HIF)-1α, which increase expression of downstream cytokines and growth factors[12]. Despite these endogenous processes, significant deficits often persist following early brain injury. Therefore, new post-injury strategies are necessary to increase the therapeutic window for treatment and further improve long-term outcomes. For those reasons, a search for therapies that can prevent injury progression or enhance repair of the immature brain continues, with the goal of improving long-term motor and cognitive outcomes. Since the neonatal and adult brain respond differently to insults, with different responses to hypoxia in regards to gene regulation and vulnerability to excitoxicity and oxidative stress, alternate therapies and strategies must be sought[13]. While some treatments that manipulate injury pathways show promise, not all neonates will benefit from treatment, depending on the etiology and timing of the insult, as well as the particular mechanisms of injury progression and repair involved[14].

To maximize the efficacy of current post-injury treatment, we need to be able to quickly identify those patients that will benefit from therapy; however, identification of sufficiently accurate and timely biomarkers of brain injury remains elusive[15]. For that reason, a number of clinical and metabolic predictors are used to identify term infants at risk for hypoxic brain

injury. These include low Apgar scores at 10 minutes of life or prolonged resuscitation at birth, significant cord blood or early arterial blood acidosis, and the presence of encephalopathy on neurological examination[16]. Cerebral function monitoring using bedside amplitude-integrated EEG (aEEG) has provided an efficient means for identifying abnormal background patterns and concern for brain injury, but the aEEG is somewhat prone to artifact, is not available at all centers, and inferior to continuous video EEG[17, 18]. Brain imaging with magnetic resonance imaging (MRI), including spectroscopy (MRS) and diffusion-weighted imaging (DWI), provides the most accurate assessment of injury and currently represents the gold standard for diagnosis[19, 20]. This can help determine both the severity and evolution of brain injury, with specific injury patterns being associated with poorer outcomes, such as loss of gray/white differentiation or basal ganglia/thalamus injury[20]. However, early imaging in neonates is difficult secondary to scanner availability, patient instability and difficulty in transporting and monitoring critically ill newborns. There is also controversy regarding the benefit of current therapy with different severities of underlying injury or encephalopathy – including the uncertain benefit of therapeutic hypothermia in newborns with mild encephalopathy[21]. Serum and urine biomarkers of injury are being studied but are currently of equivocal value in identifying early neonatal brain injury. Given all of the available evidence, a combination of encephalopathic exam, metabolic or resuscitation criteria, and early EEG or aEEG monitoring provide the best predictors of those at risk that may benefit from treatment[16].

The term “neuroprotection” is frequently used to describe the treatment goals after brain injury, but the aims with treatment are threefold. The first is to protect the brain or prevent injury from occurring. The second is to repair the injured portions of the brain that have suffered an initial insult but may potentially recover. The third is to repair the injured brain by increasing proliferation, migration of differentiation of neural precursor cells to replace injured tissue, and enhance underlying angiogenesis and restore blood flow to injured regions. Optimizing therapy for early brain injury requires capitalizing on multiple pathways that not only prevent cell death, but also enhance cell growth, differentiation, and long-term integration into neural networks. Preventing injury is particularly difficult in the population of term/near-term infants because of the difficultly in quickly identifying those patients at risk. By targeting the different mechanisms of injury and enhancing the endogenous response to hypoxia, hopefully selected pharmacotherapies can salvage cells that would otherwise die, protect cells from becoming injured by increasing tolerance, and regenerate injured brain tissue. The ultimate answer may be in combination therapies or strategies that target each of these different mechanisms of injury and repair at different time points, to maximally enhance these protective and reparative responses. This review will focus primarily on previous and ongoing research in the full-term human brain, and therapies with potential to benefit humans in the near future.

## ACUTE THERAPIES

### HYPOTHERMIA

Therapeutic hypothermia is standard of care for neonatal HIE[14]. Current evidence suggests that hypothermia must be initiated within 6 hours of birth, and continued at a goal

temperature of 33.5 degrees C for 72 hours. Multiple pre-clinical animal models of perinatal brain injury demonstrate histological and functional benefit with this early initiation of hypothermia[22–25]. Brief hypothermia provides partial neuroprotection, while prolonged moderate hypothermia (total body or selective brain) to 32-34°C for 24-72 hours results in sustained improvement in behavioral performance in both newborn and adult animals[26, 27, 25, 24]. The mechanisms underlying protection with hypothermia appear to be multifactorial, including modifying apoptosis and interrupting early necrosis, reducing cerebral metabolic rate, and reducing release of excitotoxins, oxygen and nitrogen free radicals[28, 29]. Initiation prior to the period of secondary energy failure can help suppress the influx of these damaging mediators and help rescue mitochondria from overwhelming injury, preventing cell death processes[21].

Six major randomized controlled trials of hypothermia in term or near-term human neonates have been published[14]. Overall, they show a reduction in mortality and long-term neurodevelopmental disability, with follow-up ranging anywhere from 12 months to 8 years of age[30, 31], with most benefit seen in moderately encephalopathic infants[32–35]. Sustained protection does depend on the dose of hypothermia, with maximum benefit obtained with cooling to 33-34°C, as well as limited delay to treatment initiation[24, 36]. Mild hypothermia to this level is well tolerated without serious adverse effects[33, 37, 38], with the most common complications being transient effects on heart rate and blood pressure[39]. There also appears to be a mild increased risk of pulmonary hypertension in cooled infants, though generally not severe[40]. In addition to severity of encephalopathy, larger infants appear to be more responsive to hypothermia and at more risk for injury if hyperthermic at any point[41–43]. While both head and whole body cooling have been shown to be effective, whole body cooling may be more effective in reducing temperature of deep brain structures[44], and may be more feasible in certain clinical settings by providing access for EEG monitoring[45].

While early hypothermia has demonstrated benefit in this subset of patients with moderate or severe encephalopathy and underlying HIE, there are still many infants that do not benefit from this therapy[14].This may be secondary to delayed recognition of injury, more remote injury, other underlying etiologies, or individual metabolic or genetic differences in the response to injury and treatment. Other studies have sought to expand treatment options when it comes to cooling therapies in these neonates. While active cooling during transport is safe and effective in getting babies to target temperature sooner[46, 47], deeper and longer cooling, down to 32 degrees and for as long as 120 hours did not improve outcomes[48, 49]. More recently, initiation of cooling between 6 and 24 hours of age was found to have possible benefit, with a 71% chance of improving outcomes by 2% in a Bayesian analysis; however, there was also a risk of worse outcomes as babies were cooled for a longer period of time (96 hours)[50]. There is also scant preclinical evidence of benefit with delayed initiation of cooling[51]. Finally, while two of the randomized cooling trials cooled babies < 36 weeks gestation, the evidence for cooling babies at younger gestational age is lacking. The NICHD Neonatal Research Networks is currently enrolling patients in a randomized controlled trial that cools babies born between 33+0 and 35+6 weeks gestational age ().

## ANTI-EXCITOXICITY AGENTS: *MAGNESIUM SULFATE*

Glutamate plays an important role in progenitor cell proliferation and fate, including neural circuitry development. Excitotoxicity has long been known to play a part in the progression of hypoxic-ischemic brain injury, and differences in receptor expression contribute to the vulnerability of the developing brain[52]. Excitotoxicity refers to excessive glutamatergic activation that leads to cell injury and death[53]. Following hypoxia-ischemia, vesicular release and reversal of glutamate transporters results in rapid accumulation of glutamate in the brain[54–57]. Glutamatergic receptors include N-methyl-D-aspartate (NMDA), alpha-3amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate. NMDA receptor activation is important in synaptic plasticity and circuit formation during development[58], but following injury overactivation increases intracellular calcium accumulation and pro-apoptotic signaling pathways[59, 60].

There has long been a search for agents that decrease brain injury by decreasing excitotoxicity. Magnesium sulfate has shown some benefit in preventing white matter damage in animal models[61–63], likely mediated through its function as an NMDA receptor antagonist, thereby limiting excitotoxicity[64]. While benefit has been demonstrated in reducing cerebral palsy when given antenatally to mothers at risk for preterm delivery[65], there is less evidence in the full-term population. For example, magnesium administered to asphyxiated term neonates did improve aEEG background patterns, but when given in larger doses was associated with profound hypotension[66, 67]. In pre-clinical studies of full-term injury, magnesium has also shown equivocal benefit[68], although more recent evidence suggests that the mechanism of benefit includes gene upregulation and preconditioning, which may explain the benefit in antenatal administration to pregnant mothers at risk for premature delivery[69]. In small-scale human trials there is a suggestion of a possible benefit with postnatal treatment, but studies are hindered by a heterogeneous population, the lack of cooling, and no long-term follow up [70]. Rapidly identifying full-term infants at risk of hypoxic-ischemic injury to enable early/pre-treatment would require better early biomarkers or predictors of injury.

## OTHER ANTI-EXCITOXICITY AGENTS

Other therapies targeting the excitotoxic cascade have been studied in humans but have not yet shown clinical benefit. Topiramate is an AMPA-kainate receptor antagonist that is FDA-approved for seizure treatment for patients greater than 2 years of age, and inhibits glutamate function while also increasing inhibitory signaling through the GABA pathway. It has also been shown to reduce brain injury and cognitive impairment in newborn rodents when administered within two hours of the insult[71, 72]. It has yet to demonstrate efficacy in newborns with HIE[73]. Dizocilipine (MK801) is a NMDA receptor antagonist that is poorly tolerated in humans and may actually increase injury in some models[74]. Memantine is a low affinity noncompetitive NMDA receptor that is well tolerated in adults[75] and has shown some benefit in pre-clinical models[76–78]. Cannabinoids have also shown promise as a treatment for both neurodegenerative disorders[79] and adult ischemia[80, 81]. They are involved in control of synaptic transmission, and their receptors (CB1 and CB2) are expressed on neurons and glia[82, 83]. In the immature brain, cannabinoids have effects on excitotoxic lesions[84], and the agonist WIN 55,212-2 reduces short-term brain injury when administered

after neonatal rodent HI[85]. While cannabinoids are being studied for pediatric epilepsy (), there are no clinical studies specific to neonatal brain injury.

## ANTI-OXIDANTS: *ALLOPURINOL*

Oxidative stress also plays a critical role in injury progression following hypoxia-ischemia[86], resulting from excess formation of free radicals (FR) (reactive oxygen species (ROS) and reactive nitrogen species (RNS))[87, 88]. While antioxidant defenses such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, and compounds such as vitamins A, C, E, beta-carotene, glutathione and ubiquinones scavenge FRs under normal conditions, damage occurs when there is an imbalance between pro- and anti-oxidants. Newborns are at the greatest risk for oxidative stress secondary to this imbalance[87, 89].

A number of strategies have been tested to improve this balance and reduce underlying oxidative stress. For the acute phase of injury, allopurinol is a xanthine oxidase inhibitor that has shown initial promise in pre-clinical and human studies. Allopurinol reduces free radical production by inhibiting xanthine oxidase-derived superoxide and $H_2O_2$ free radical production, while also scavenging free hydroxyl radicals. High dose allopurinol given 15 minutes after HI in newborn rats decreases acute edema and long-term infarct volume[90]. Short-term benefits have also been seen in neonates undergoing cardiac surgery for hypoplastic left heart syndrome [91]. Early administration of allopurinol to encephalopathic infants improved short-term neurodevelopmental outcomes, with the greatest benefit seen in moderately encephalopathic infants[92]; however, there may only be a brief window for benefit, as no improvement in outcomes was seen with later treatment after a hypoxic-ischemic insult[93].

## MELATONIN

Another antioxidant strategy that may have benefit as either an acute or delayed therapeutic option is melatonin. Melatonin is an indoleamine that easily crosses the blood-brain barrier and has a number of protective roles, including scavenging ROS, anti-inflammatory and anti-apoptotic functions[94]. It provides long-lasting neuroprotection in pre-clinical HI and focal cerebral ischemic injury models[95, 96], and human neonates treated with melatonin were found to have decreased pro-inflammatory cytokines[97, 98]. Melatonin levels are relatively deficient in newborns (MINT; ISRCTN15119574), and studies are ongoing to identify optimal treatment doses (MELIP; and MIND, ). There is also an ongoing trial evaluating melatonin to prevent brain injury in unborn growth restricted babies, where a composite neonatal outcome will be evaluated ().

## XENON

Xenon is an inhaled anesthetic approved for use in Europe that appears to have potent neuroprotective effects in pre-clinical models. This is mediated through NMDA antagonism, as well as the upregulation of pro-survival proteins BDNF, Bcl-2, HIF[99]. It appears to be superior to other NMDA antagonists, possibly through additional inhibition of AMPA and kainite receptors, reduction of neurotransmitter release, or effects on other ion channels[100–102]. While it has demonstrated some benefit as a monotherapy in animal studies, it is likely that combination therapy with hypothermia would provide the most

benefit (see below). Other antioxidant strategies, including deferoxamine, vitamin E, and selective inhibition of nitric oxide synthase have demonstrated limited benefit that have not translated to human neonates.

## DELAYED THERAPIES: GROWTH FACTORS

### ERYTHROPOIETIN

There are a number of growth factors that are critical for development, maturation and function of the immature brain. Erythropoietin (EPO) is a glycoprotein produced primarily in the fetal liver, but also by multiple cell types in the central nervous system (CNS) during development, with its principal functions mediated through binding to its specific receptor (EPO-R)[103]. There is elevated EPO/EPO-R expression in the brain during gestation, which declines rapidly postnatally. EPO production in neurons and astrocytes and EPO-R expression in neurons, glia, and microglia are upregulated in a temporal specific manner, via upregulation by HIF after hypoxia[104, 105]. Numerous studies of EPO neuroprotection performed in rodents, sheep, and nonhuman primates, involving both global and focal hypoxic-ischemic brain injury, have consistently shown that exogenous EPO administration results in both histologic and functional benefit[106, 107]. High-dose EPO improves short-term histological and behavioral outcomes following neonatal stroke, but multiple dose treatment protocols resulted in the long lasting functional improvement[108, 109]. Studies have also demonstrated benefit with delayed initiation of EPO treatment. EPO therapy initiated 48 hours after neonatal hypoxia-ischemia in mice improved both behavioral outcomes and white matter injury, while enhancing neurogenesis[110], while EPO initiated 24-72 hours after adult rodent stroke enhanced neurogenesis, angiogenesis, and functional outcomes[111, 112, 113]. More recently, EPO therapy initiated one week following stroke improved brain volume and sensorimotor behavioral function in newborn rats[114].

EPO binding to EPO-R leads to phosphorylation and activation of a number of downstream pathways that limit inflammation, decrease apoptosis[115], promote neural precursor cell proliferation[116, 117], and preserve endothelial cell survival and stimulate their production *in vitro*[118]. In addition to its acute effects, EPO stimulates additional growth factor release, thus providing neuroprotective and trophic effects that last well beyond the acute period of injury. EPO enhances angiogenesis and improves white matter survival assessed by MRI and pathologic analysis[109, 113, 119–123]. EPO also interacts with VEGF and stimulates the production of growth factors such as brain-derived neurotrophic factor (BDNF) and glial cell derived neurotrophic factor (GDNF) that likely contribute to effects on cell survival and vasculogenesis[124, 125].

Pilot trials of exogenous EPO treatment for human brain injury have suggested benefit, with larger trials ongoing. Multiple doses of EPO administered daily for adult stroke reduced the size of infarct and improved short-term recovery of cognitive function and neurological deficits [126]. However, a follow-up study in adults with middle cerebral artery territory stroke saw increased mortality in treated patients, possibly secondary to interaction with, and timing of, TPA administration [127].

EPO monotherapy for neonatal HIE has been studied in a few small clinical trials, prior to hypothermia becoming standard of care for these infants. These studies found that high-dose EPO improved short-term neurodevelopmental outcomes[128, 129]. Zhu et al. studied 167 neonates with HIE randomized to 300-500 U/Kg of EPO every other day for two weeks, or placebo[128]. Infants in the EPO group were less likely to die or have moderate to severe disability at 18 months. Similarly, Elmahdy et al. studied 30 infants with HIE who were randomized to receive 5 daily doses of Epo 2500 U/kg or placebo[129]. The EPO-treated infants had improved EEG background activity, reduced biomarkers of oxidative stress at 2 weeks, and improved neurodevelopment at 6 months. Given the small sample sizes and prevalence of therapeutic hypothermia, EPO treatment for HIE must be studied within the context of concomitant hypothermia treatment, except for lower resource settings.

Darbepoietin is a long-acting formulation of EPO that may provide a potential therapy with less frequent dosing (). Animal studies that provide evidence of darbepoietin neuroprotection have been limited to traumatic brain injury and intracerebral haemorrhage in adult rodent[130, 131]. Further studies comparing the use of EPO with darbepoietin in HIE are warranted. EPO and darbepoietin is also being evaluated as a neuroprotective agent for other causes of full-term brain injury, including mild encephalopathy not qualifying for cooling, congenital heart disease[132] and perinatal stroke[133].

## VEGF

Other growth factors have also demonstrated benefit with delayed initiation of therapy in pre-clinical models. Vascular endothelial growth factor (VEGF) stimulates angiogenesis and vasculogenesis, and is a downstream effector of HIF-1α[134]. Following ischemia, the brain responds by increasing collateral vessel development and arterial perfusion to the ischemic penumbra. VEGF is upregulated following stroke in rats, with increased arteriogenesis and neurogenesis both *in vitro* and *in vivo*[135, 136, 25, 26]. In the early stages following ischemic injury, endogenous VEGF expression contributes to disruption of blood-brain barrier integrity, with increased vascular permeability and uncoupling of endothelial cell-cell junctions[137, 138]. For this reason, the timing of exogenous VEGF treatment is crucial as studies have shown that early administration increases edema and infarct volume, while later treatment reduces injury, increases blood vessel formation and myelin basic protein production in the injured penumbra[135, 139]. In humans, there is an increase in VEGF levels for 3 months following stroke, which correlates with functional outcome[140].

## BDNF

Brain-derived neurotrophic factor (BDNF) is also critical for cell survival and tissue repair in the brain following ischemia. Exogenous BDNF administration after ischemia reduces histological injury and improves behavioral outcomes, with increased oligodendrocyte differentiation and myelin formation with delayed injection of BDNF following stroke[141, 142], and increased neurogenesis and migration of neural precursor cells to injured regions of the brain[143]. BDNF injections for 5 days following photothrombolytic stroke has also been shown to improve sensorimotor outcomes in pre-clinical models[144].

## DELAYED THERAPIES; *STEM CELLS*

Stem cells have gained traction in recent years as a therapeutic option for a number of different CNS diseases in both the mature and immature brain, with the ability to enhance repair in some pre-clinical models. Neural stem cells (NSCs) are multi-potent precursors that self-renew and retain the ability to differentiate into a variety of neuronal and non-neuronal cell types in the CNS. They reside in neurogenic zones throughout life, such as the subventricular zone and subgranular zone of the dentate gyrus in rodent models, and help maintain cell turnover at baseline and replace injured cells by migrating to injured tissue. Implanted cells integrate into injured tissue[145], decrease volume loss[146–148] and improving behavioral outcomes[149, 150] in both neonatal and adult models of ischemia[147, 148]. These stem cells differentiate into neurons, astrocytes, oligodendroctyes, as well as undifferentiated progenitors. These cells not only promote regeneration, but non-neuronal phenotypes inhibit inflammation and scar formation, while promoting angiogenesis and neuronal cell survival in both rodent and primate models[151, 152]. While no adverse effects have been noted, the therapeutic window is not known.

In humans, mesenchymal stem cells (MSCs) are a particularly promising candidate to repair the ischemic brain damage because of their low immunogenicity, their availability and promising pre-clinical data. MSCs can be isolated from a variety of tissues types, and administration of MSCs reduces lesion volume and improves functional outcomes in neonatal rodent stroke[35]. In addition, this therapy appears to be effective with delayed administration, as there has been demonstration of short-term benefit with treatment times ranging anywhere from 3 hours to 10 days after the onset of injury[153]. These data indicate that stem cells have both neuroprotective and neuroregenerative properties. This role is also supported by results showing that there is decreased apoptosis after stem cell transplantation, with endogenous neurogenesis is enhanced, and with increasing benefit with multiple injections.

While it was originally thought that implantation and engraftment of exogenous cells may result in proliferation and differentiation to replace dead or dying cells as part of the repair process, systemic transplantation-induced effects after ischemia frequently occur in the absence of grafted cell survival. This suggests that transplanted cells may improve outcome via indirect mechanisms on local growth factor production or cellular differentiation, as opposed to replacement of damaged cells with transplanted cells. MSCs have been shown to secrete a number of factors involved in cell death, cell proliferation, cell fate, and functional incorporation, including BDNF, VEGF, and insulin-like growth factor-1 (IGF-1)[154]. These findings have now led to the alternative strategy of combining cell-based therapies and gene manipulation or delivery[155]. Transplantation of endothelial precursor cells (EPCs) over-expressing IGF-1 reduced apoptosis and increased blood vessel number in a model of cardiac injury. Stem cells that have been manipulated to over-express specific neurotrophic factors, such as BDNF, have also shown benefit in different pre-clinical models of brain injury[156, 157]. Administration of BDNF modified MSCs has shown benefit in small animal models of transient stroke, traumatic brain injury, and spinal cord injury. Intranasal administration of BDNF-overexpressing MSCs given three days after neonatal stroke reducing histological injury and improving short-term motor function[153].

## COMBINATION THERAPY

Monotherapies that address any one of the underlying mechanisms or pathways of injury may result in only mild improvement. Therapies that potentially address multiple pathways, such as hypothermia, erythropoietin, or melatonin, have demonstrated more benefit and may provide more potential for long-term repair. Hypothermia has become the standard of care since showing benefit in moderate to severely encephalopathic newborns, resulting in long-lasting improvement for many children; however, it does not completely protect or repair all injured brains[158, 159, 160, 161]. For this reason, there is a continued search for adjuvant or synergistic therapies that may provide more long-lasting neuroprotection and repair. Specific to HIE, these are often referred to as “Cooling Plus” therapies, and include early hypothermia combined with other therapies that include EPO, xenon, melatonin, allopurinol, magnesium sulfate, topiramate, and autologous cord blood, which will be discussed below. While it is difficult to study comparative effectiveness of combination therapies there may be a cocktail or sequence of therapies that provide the most benefit. Once adequate biomarkers are identified that can quickly and accurately determine those at risk and those that may benefit from particular strategies, we can take advantage of specific endogenous repair processes at different time points following injury can be craft a strategy to maximally repair the injured brain and improve outcomes.

In the phase I, dose escalation NEAT trial, infants cooled within 6 hours of birth then received up to EPO doses every 48 h (up to 6 doses), with the first dose given within 24 hours after delivery[162]. Of the 4 different EPO doses tested, a dose of 1000 units/kg/dose in cooled babies achieved plasma serum concentrations that most closely approximated optimum neuroprotective levels in different animal models[163]. Several larger clinical trials of hypothermia/EPO therapy are currently underway, in hopes of providing additional information on both safety and efficacy. The multicenter phase II double-blinded, randomized controlled NEAT O trial also demonstrated safety with multiple doses of 1000 units/kg/dose over a one-week period, and suggested short-term imaging and neurodevelopmental improvement, but was not powered to demonstrate efficacy [164]. Three large phase III randomized controlled trials will assess neurodevelopmental outcomes at age 2 years in cooled infants with HIE. A French study () began enrolling patients in 2013, and the Australian PAEAN study () and the HEAL trial in the United States () are currently enrolling. All three trials will test the administration of EPO 1000 U/kg, with multiple doses given intravenously over the first week of life.

Xenon and hypothermia have also been studied as combination therapy for moderate to severe encephalopathy and concern for HIE. In pre-clinical studies, combination xenon/hypothermia initiated 4 hours after neonatal HI provided synergistic histological and functional protection when evaluated at 30 days after injury[165]. Hypothermia reduces glutamate and glycine release[166], and NMDA receptor antagonism may explain these effects. An additive effect was also shown after neonatal HI in rats cooled to 32°C that received 50% xenon, with improvement in long-term histology and functional performance that exceeded the individual benefit of either[167]. In humans, a phase I trial where 50% xenon was administered for 18 hours, starting by 18 hours of age, was found to be safe while also suppressing seizures[168]. The TOBY-Xe trial in the UK administered 30% xenon by 12 hours

of age in cooled infants, continued for 24 hours, and similarly found a reduction in seizures, but no difference in neuroimaging biomarkers[169, 170]. Neither of these studies examined long-term neurodevelopment.

Other human combination trials include a randomized controlled pilot trial combining melatonin with cooling in term infants with HIE. The melatonin/hypothermia group had fewer seizures, less evidence of white matter injury on MRI, and a lower rate of mortality without developmental or neurological abnormalities at 6 months[171]. There is a phase II, melatonin escalation study ongoing () and larger ongoing trials are necessary. Allopurinol is also being studied in combination with hypothermia for HIE. Since pre-clinical evidence suggests it must be given early for HIE [90], the ongoing ALBINO trial will give two doses of Allopurinol, with the first dose within 30 minutes of birth (). Finally, there are ongoing phase I and phase II trials combining autologous cord blood or nucleated blood cells in combination with hypothermia for term HIE (; ).

There are other “Cooling Plus” strategies that have demonstrated benefit in animal models, but have yet to be systematically studied in humans. N-acetylcysteine (NAC) is a medication approved for neonates that is a scavenger of oxygen radicals and restores intracellular glutathione levels, attenuating reperfusion injury and decreasing inflammation and NO production in adult models of stroke[172, 173]. Adding NAC therapy to systemic hypothermia reduced brain volume loss at both 2 and 4 weeks after neonatal rodent HI, with increased myelin expression and improved reflexes[174]. Inhibition of inflammation with MK-801 has also been effective when combined with hypothermia in neonatal rats post HI injury[175]. In neonatal rats who underwent HI followed by early topiramate and delayed hypothermia, improved short-term histology and function was seen[73, 176, 177]. This may provide a window for protection if hypothermia is delayed.

## CONCLUSIONS

Many pre-clinical and clinical studies have focused on singular mechanisms of injury, such as oxidative stress, inflammation, or excitotoxicity. Evidence suggests that injury continues to progress and therapies may need to be administered over much a much longer period of time than had previously been appreciated [Figure 1]. While hypothermia and single pharmacotherapies show promise, combination therapy may be necessary to increase the therapeutic time window for protection and enhance reparative processes, making recovery possible.

Neonatal critical care has grown over many decades, with improved care of lung disease, congenital cardiac disease, improved ventilation strategies and ECMO. Despite these improvements, neurodevelopmental outcomes continue to suffer. The immature brain is unique in its developing complexity, and response to hypoxic and/or ischemic insults. While the above-mentioned therapeutic strategies show promise as future therapies, attention must be paid to a neonate’s physiological status, including their blood pressure, carbon dioxide and glucose levels, and the presence of seizures to prevent progression of brain injury or secondary brain injury from occurring. Neonatal neurocritical care, involving expertise from neonatologists, child neurologists, neuroradiologists, neonatal nurses, and developmental

specialists will provide the most appropriate acute care, identify those at risk who can benefit from therapy, and follow up to optimize long-term outcomes[178, 179].

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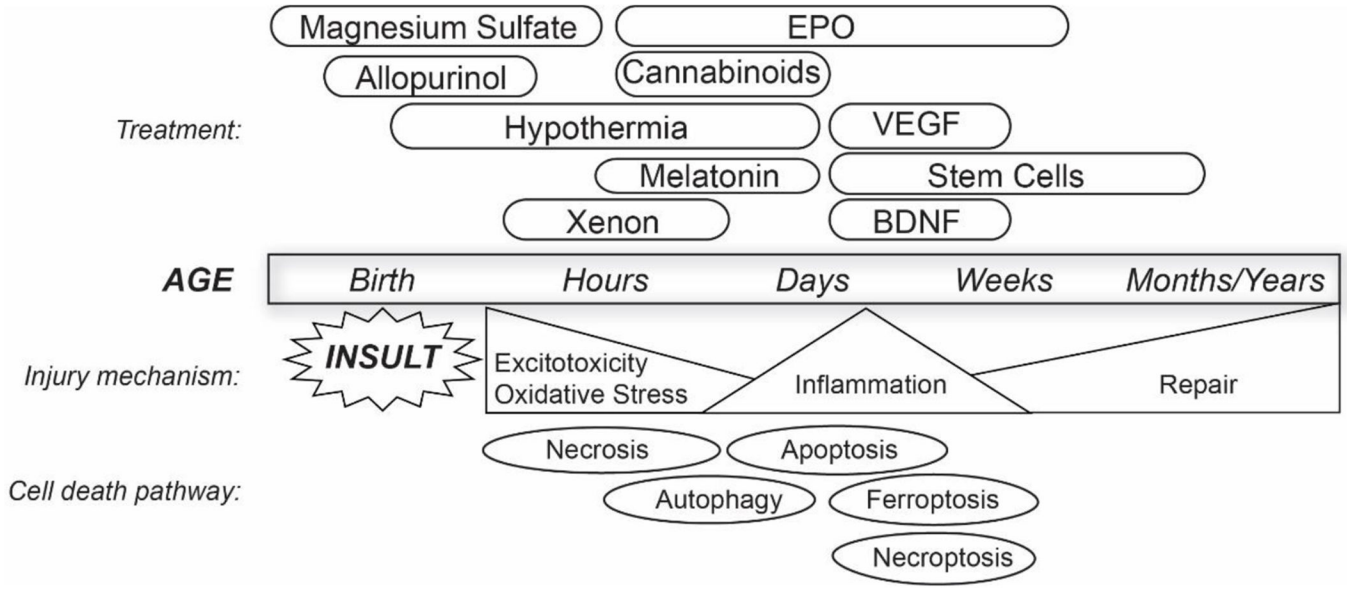


**Fig 1. Mechanisms of injury and potential therapies following perinatal insult.**

A number of injury mechanisms result in primary and secondary energy failure, leading to a variety of cell death pathways. Potential therapeutic strategies can alter these early pathways to limit cell injury and death, or enhance long-term repair.