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Hypoxic ischemic brain injury: Potential therapeutic interventions for the future

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Abstract

Perinatal hypoxic-ischemic brain injury is a common problem with potentially devastating impact on neurodevelopmental outcomes. While therapeutic hypothermia, the first available treatment for this disease, reduces the risk of death or major neurodevelopmental disability, the risk of major neurologic morbidity following HI remains significant. Basic research has identified cellular mechanisms that mediate neuronal death. This article reviews the cellular processes induced that lead to brain injury following HI, and identify treatments currently under investigation for potential translation to clinical trials.

Keywords

excitotoxicity; lipid peroxidation; oxidative stress; erythropoietin; nitric oxide; poloxamer 188; F-68; melatonin; topiramate; xenon; allopurinol

INTRODUCTION

Perinatal hypoxic-ischemic (HI) brain injury is an important clinical problem in the neonate, leading to cerebral palsy and developmental delay, and affecting one out of every 1000 live term births in the US (1). Brain hypothermia, induced by externally cooling either the head or the whole body in the first six hours following perinatal HI, is the only treatment currently employed to reduce death and disability, and only in term infants (2, 3). Cooling decreases the incidence of the combined outcome measure of death or disability at 18 months to 2 years following HI (2, 4-6). Disappointingly, however, long-term follow-up studies of neurodevelopmental outcomes in survivors from two large trials (2, 5) have not found cooled infants to have lower incidences of moderate or severe disability at school age compared with those who had not been cooled (7, 8). These studies highlight the need for new therapies to rescue injured neurons following HI and improve neurodevelopmental outcomes. Current research suggests that achieving these goals may be possible with new

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approaches to treatment. To put these approaches in context, we will first briefly review the outcomes and presentation of HI brain injury.

PRESENTATION OF HYPOXIC-ISCHEMIC BRAIN INJURY

Encephalopathy – the neurologic syndrome comprised of abnormalities of consciousness, tone, and autonomic control - is the hallmark of acute HI brain injury in the newborn (9). In the absence of clinically obvious encephalopathy over the first 24 hours after birth, subsequent development of abnormal neurodevelopmental outcomes cannot be ascribed to perinatal hypoxia-ischemia. The stage of encephalopathy depends on the timing and severity of the HI, as well as the genetic endowment of the infant, so that encephalopathy severity can differ widely between infants who have experienced apparently similar insults. The relationship between clinical presentation and HI severity has been encapsulated in the commonly used (10, 11) Sarnat staging system (9). Thus, infants with stage I encephalopathy demonstrate hyperalertness, increased sympathetic autonomic outflow, and normal to increased tone. Infants exhibiting stage II encephalopathy can present with a variety of consciousness levels, ranging from lethargy to obtundation, accompanied by increased parasympathetic autonomic function, mild hypotonia, and, commonly, seizures. Finally, infants with stage III encephalopathy exhibit stupor, flaccid tone, and depressed sympathetic and parasympathetic function (9). Importantly, the sequelae of HI evolve over time: in stage I and II encephalopathies, end-organ injury resolves and consciousness improves; with stage III encephalopathy, infants can die, progress to a chronic vegetative state, or survive with severe impairments. Understanding this natural history can be helpful in determining when an intra-uterine period of HI has taken place: an infant experiencing HI shortly before birth will demonstrate acute encephalopathy. In contrast, an infant who has experienced HI days prior to birth will exhibit signs consistent with the evolving sequelae of HI.

CELLULAR MECHANISMS OF NEURONAL DEATH FOLLOWING HI

Neurons require a continuous supply of metabolic substrates, particularly glucose and oxygen. HI brain injury arises from the inter- and intra-cellular processes during and after an imbalance of availability and consumption of these substrates in the brain. In animal models, neuronal death following HI occurs in two phases (12): immediately following HI, neurons begin to die rapidly, likely due to necrosis, a process of cell death characterized by acute loss of plasma membrane integrity and loss of ATP (13). In the second phase, neurons die over hours to days (14), primarily via apoptosis (15), an active, tightly-regulated cascade of intracellular events. In human infants, neuropathologic evidence of classic neuronal apoptosis following HI is less clear than in animal models (16). However, it is unquestionable that immediate and delayed neuronal death underlie the neurologic injury following HI in infants. The comparatively long duration of the second phase of HI-induced neuronal death, and its persistence following birth suggest that the severity and extent of this delayed death is more likely to be modified by therapy than is the early, immediate phase.

New approaches to rescuing neurons following perinatal HI have leveraged current understanding of the mechanisms of HI-induced brain injury in order to specifically target

central mechanisms of neuronal death. In order to place these treatments in the context of the cellular mechanisms they target, we will briefly review the processes of excitotoxicity, free radical toxicity, and inflammation.

Excitotoxicity

Glutamate is a ubiquitous excitatory neurotransmitter in the brain. Under pathologic conditions, including HI, neuronal receptors for glutamate are over-activated due to pathologically high concentration of glutamate in the extra-neuronal space. This high concentration arises as a result of pathologically increased synaptic release of glutamate, dysfunction of glutamate uptake mechanisms, and release of glutamate from the intracellular metabolic pool. Glutamate receptor over-activation results in neuronal death, hence, excitotoxicity. Over-activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor has been highly implicated in neuronal death following HI. NMDA receptor over-activation allows intracellular calcium to rise to toxic levels and activates cytotoxic phospholipases, proteases, lipases and endonucleases, leading to cell death. Calcium is also taken up by the mitochondria, leading to loss of ATP synthesis, oxidative stress, release of pro-apoptotic factors, and activation of the apoptotic cascade.

Free Radical Toxicity

Free radicals are molecules containing one or more unpaired electrons, which allow them to have increased inter-molecular reactivity. The primary oxygen free radical generated in cells is superoxide anion (O_2^-). Superoxide is an important intracellular signaling molecule, as is its metabolite hydrogen peroxide (H_2O_2). Together with the highly reactive hydroxyl radical, O_2^- and H_2O_2 are the main oxygen-derived free radicals in the cell. Oxidative stress refers to increased levels of these radicals. Oxidative stress contributes to neuronal death following HI (17), by degrading cellular proteins and DNA.

In addition to oxidative stress, increased production of a nitrogen free radical, nitric oxide (NO), is a central mechanism of HI-induced neuronal death (18). Increased NO production is mediated by a neuron-specific NO synthase (nNOS) activated by HI- (and excitotoxicity-) induced elevations of intracellular calcium concentrations. A second isoform of NO synthase, endothelial NOS (eNOS), controls vascular resistance in all organs including the brain. Preserving eNOS activity during and following experimental HI improves cerebral blood flow and neuronal survival (19), so treatments aimed at reducing neuronal NO production must specifically target nNOS and preserve eNOS activity. In addition to its direct effects, NO interacts with O_2^- to form the highly reactive and toxic radical, peroxynitrite (20). Peroxynitrite-mediated peroxidation of lipid constituents of cellular membranes (21) and oxidative modification of mitochondrial proteins (22) are important mechanisms of neuronal injury. In particular, lipid peroxidation alters cellular membrane structure and function, inducing cellular necrosis or trigger apoptosis.

Inflammation

Improved outcomes in animal models of HI following inhibition of inflammation (23) demonstrate that inflammation is an important mechanism of HI-induced neuronal death. Following HI, microglia are activated (24), producing pro-inflammatory cytokines, e.g. IL-1

and TNF-alpha. In addition, microglia-derived chemokines acutely increase (25) recruiting peripheral immune cells to the brain. HI activates the complement cascade within the brain (26). Complement activation results in the formation of membrane attack complexes, which form pores within plasma membranes, leading to cell lysis (27). Thus, following HI, a coordinated inflammatory response in the brain arises that makes a significant contribution to HI-induced neuronal death.

NEW, POTENTIAL TREATMENTS FOR HI BRAIN INJURY

With increasing understanding of the mechanisms of HI-induced neuronal in the newborn, new approaches to neuroprotection have shown promise in pre-clinical studies and early clinical trials. Below, we review some of the most promising approaches, at different stages of development from early stage research to clinical studies and FDA approval. Because these treatments may address mechanisms different from those mediating hypothermia-mediated neuroprotection, these new therapies may also provide additive neuroprotection to that available from hypothermia treatment.

Erythropoietin

Erythropoietin (EPO) is an endogenous, hypoxia-induced glycoprotein produced in the kidney, first shown to regulate hematopoietic function via EPO-specific receptors (28). Currently approved to increase erythropoiesis in anemia, recombinant EPO (r-EPO) has also been demonstrated in animal studies of HI to be neuroprotective (29-31). Activation of neuronal EPO receptors prevents HI-induced activation of NMDA receptors and increases expression of anti-apoptotic proteins, potentially reducing excitotoxicity and decreasing apoptosis (31, 32). EPO receptor activation also inhibits HI-induced increases of peroxynitrite (oxidative stress) and inflammatory cytokines, potentially reducing free radical toxicity and inflammation (32). Of particular relevance for neonatal HI, EPO receptor expression is abundant in the developing mammalian brain (33). Systemically administered r-EPO following HI has been shown to cross the blood-brain barrier (34); in one study, in babies who were given EPO following HI, the pharmacokinetics of EPO levels in cerebrospinal fluid paralleled that observed in serum (35), suggesting that r-EPO may cross the blood brain barrier in humans.

Clinical trials of r-EPO in infants following HI brain injury have begun. A phase I, multi-center, open label, dose-escalation trial recently showed that EPO is well-tolerated in term infants undergoing hypothermia as treatment for HIE, without serious adverse effects at plasma concentrations shown to be neuroprotective in animals (36). In the only prospective, randomized clinical trial to date, r-EPO reduced the risk of death or moderate/severe disability in term infants with HIE who were not treated with hypothermia. Notably, improved outcomes were restricted to infants in the moderate HIE subgroup (35). However, the lack of blinding in this study raises concerns for its validity. Furthermore, because the patient population was restricted to patients who could pay for r-EPO, the generalizability of these results to other populations is unclear.

Melatonin

Melatonin is a pineal gland hormone secreted in response to environmental light-dark cycles (37). Melatonin has multiple cellular effects, two of which directly target known mechanisms of HI brain injury. First, melatonin reduces free radical toxicity, scavenging hydroxyl radical and peroxynitrite by direct electron transfer (38). Melatonin also decreases O_2^- production in brain slices *in vitro* following hypoxic ischemic stress (39). Second, melatonin has anti-inflammatory activity. Thus, following umbilical cord occlusion in fetal sheep, melatonin decreased the production of 8-isoprostanes (40), potent mediator of HI-induced inflammation. Furthermore, melatonin, given immediately to rats following focal cerebral ischemia, decreased the extent of neutrophil emigration and macrophage/activated microglial infiltration 48 hours later, and only in the ischemic hemisphere (41). Finally, melatonin reduces NF- κ B binding to DNA, ultimately decreasing the production of pro-inflammatory cytokines including interleukin-2, interleukin -6, and tumor necrosis factor- α (42). These cellular effects have led to extensive investigation of melatonin as a treatment for HI brain injury.

In adult rat, melatonin, given after focal cerebral ischemia, improves short term evaluations of infarct size and neurobehavioral outcomes (41), suggesting that melatonin treatment may be applicable to global brain ischemia in the neonate. However, short term improvements may reflect only transient inhibition of death-inducing processes without altering the ultimate extent of neuronal death. More encouragingly, melatonin provided to neonatal mice before and after severe hypoxia significantly increased hippocampal neuronal survival at 3, 7 and 14 days as well as functional motor outcomes two weeks following insult (43). Some data suggest that antenatal treatment with melatonin may be beneficial in improving outcomes from birth asphyxia: antenatal melatonin provided to spiny mouse dams for 1 week prior to *in utero* global asphyxia of the fetuses improved cortical neuronal survival at 24 hours of life (44). Finally, melatonin effects may be additive to the neuroprotective effects of induced hypothermia. Following induction of global ischemia in neonatal pigs, melatonin combined with hypothermia decreased MR spectroscopic indices of impaired cerebral energy metabolism compared with hypothermia alone (45). Low levels of indices have high specificity in identifying asphyxiated infants who subsequently have normal neurodevelopmental outcomes at 1 year of age (46). In the only study of melatonin and asphyxiated infants to date, melatonin given in the first 6 hours of life decreased levels of malonaldehyde, a product of lipid peroxidation (47) in serum, the clinical importance of which is unknown. A randomized, double-blind placebo phase I study evaluating the effect of melatonin on infants undergoing hypothermia as treatment for HI brain injury is planned to begin in late 2013 (48).

Allopurinol

Allopurinol is an inhibitor of xanthine oxidase, a source of cytosolic O_2^- during HI that has received interest as a potential neuroprotective agent, especially as it can cross the placenta to produce therapeutic levels in newborns (49). Animal models including *in vivo* and *in vitro* rat models and *in vivo* sheep models have shown allopurinol to be neuroprotective (50-53).

Neonatal trials following HI brain injury have been limited. One randomized, placebo-controlled trial enrolled 32 severely asphyxiated infants (overall mortality rate 72%), and found no outcome differences between the groups (54). However, in a larger randomized study of 60 babies having a range of asphyxia severities, allopurinol treatment significantly decreased death or severe disability at one year of age (55). While this single study demonstrates some potential for postnatal allopurinol treatment of affected infants, interest is currently more focused on prenatal treatment, as reactive oxygen species are produced during HI in utero. During intrauterine asphyxia in fetal lambs, maternal administration of allopurinol suppressed superoxide production during intermittent partial umbilical occlusion (56) and decreased fetal hippocampal injury (50), suggesting that providing allopurinol to fetuses at risk for HI may be helpful. In fact, in a randomized double blind placebo-controlled study of 53 pregnant women whose fetuses demonstrated evidence of hypoxia, arterial cord blood from infants of allopurinol-treated mothers exhibited lower levels of S-100B, a marker of brain injury, a very short-term outcome. A randomized double blind placebo-controlled trial of antenatal allopurinol treatment is ongoing with the goal of determining allopurinol effects on asphyxia-associated mortality and long term neurodevelopmental outcome (57).

Topiramate

Topiramate is a newer anti-epileptic drug that has attracted interest as a potential neuroprotective agent for HI brain injury. Topiramate prevents seizures by inhibiting neuronal excitability, including through blockade of glutamate receptors (58). This potentially anti-excitotoxicity effect suggests topiramate as a candidate therapy for HI brain injury. Indeed, following carotid artery ligation in rat, topiramate significantly reduces neuronal death through inhibition of glutamate receptor activity (59), decreasing HI-induced neuronal apoptosis (60). Of significant interest is the observation that topiramate has added neuroprotective effects in animal models when combined with hypothermia (61).

In a pilot study, topiramate, given in conjunction with whole body hypothermia to 27 asphyxiated infants, caused no adverse effects, short-term outcomes differences, or incidence of pathologic brain magnetic resonance imaging compared to 27 controls (62). Data from two phase I trials, one ongoing in the US (63) and one recently completed in Italy (64) are awaited. Additional large clinical trials are needed to evaluate the efficacy of topiramate in preventing HI injury. Ultimately, the limitation of oral administration to critically ill infants may restrict the scope of use.

Xenon

Xenon is a chemically non-reactive gas that has undergone intensive investigation in Europe as an general anesthetic (65, 66), due to its highly favorably safety profile. One of xenon's activities is against NMDA receptor activation, decreasing excitotoxicity. This decreased activity arises from xenon block of glycine binding to its regulatory site on the receptor (67). Following hypoxia or excitotoxicity in cultured murine neurons, increasing concentrations of xenon significantly increased neuronal survival (68). In neonatal rat, xenon inhalation improved both histologic and functional outcomes two months after global HI (69). Similarly, following global forebrain ischemia in neonatal pig, xenon inhalation markedly

improved neuronal survival 72 hrs following insult (70). Notably, xenon-induced neuroprotection in these models has been found to be additive to the neuroprotection afforded by induced hypothermia.

In infants, preliminary evidence from a pilot study employing 12 asphyxiated infants indicates that adding xenon (50%) inhalation to ongoing hypothermia does not significantly alter blood pressure, heart rate, or FiO₂ requirement (71, 72). Currently, two phase I trials are ongoing in the UK (73, 74), to further evaluate the safety and efficacy of Xenon paired with cooling in infants with hypoxic-ischemic encephalopathy. Even if xenon is determined in phase II-III trials to be effective in improving long-term neurodevelopmental outcomes following HI brain injury, the obstacles to its routine use for asphyxiated infants are significant, as its costs is very high, requiring a closed circuit delivery system.

nNOS inhibition

The central role played by NO in HI-induced neuronal injury and the availability of specific small molecule inhibitors of nNOS make nNOS inhibition a potentially attractive approach. With the discovery of the toxic role of NOS in HI, initial studies of NOS inhibitors produced conflicting results (75), due to the lack of isoform specificity of the early inhibitors. However, newer, specific nNOS inhibitors may have more promise (71). In preterm fetal sheep, prophylactic use of the highly specific nNOS inhibitor, JI-10, improved neuronal survival following profound asphyxia (76). In addition, a second highly selective nNOS selective inhibitor, JI-8, improved locomotion scores and muscle tone in a rabbit model of intrauterine hypoxia-ischemia-induced cerebral palsy (77). Although initial data for selective nNOS inhibitors is promising, the extent of off-target effects, such as inhibition of eNOS activity and any accompanying decrease in cerebral blood flow (19) will need to be explored before clinical trials can begin.

Pluronic co-polymers

Following HI, the functions of cellular membranes can become altered, due to lipid peroxidation and alterations of lipid signaling. Following severe HI, neuronal plasma membrane dysfunction leads to decreased membrane integrity, leakage of intracellular constituents into the extracellular space, and necrosis. When HI is not sufficiently severe to induce necrosis, HI-induced dysfunction of the intracellular membranes of mitochondria can trigger apoptosis (78). Recently, a class of synthetic molecules, the pluronics, has been used to address HI-induced dysfunction of injured neuronal membranes *in vitro* and *in vivo*. Plurionics consist of chains of poly[ethylene oxide] (PEO) and poly[propylene oxide] (PPO) arranged in a tri-block, PEO-PPO-PEO, structure. This structure enables plurionics to interact with cellular membranes (79, 80), and to restore plasma membrane integrity following injury. One member of the Plurionics, Pluronic F-68, has been shown to profoundly rescue neurons from death in *in vitro* models of HI through blockade of apoptosis (81, 82). Preliminary evidence also indicates that Pluronic F-68, provided to animals for one week following HI, markedly improves neuronal survival in the hippocampus, a brain region highly vulnerable to global HI, and rescues hippocampus-mediated behavior (83). The novelty of this membrane-targeted approach and its lack of

toxicity (84, 85) suggest that targeting membrane dysfunction may constitute a viable treatment for HI brain injury in the future.

CONCLUSION

Hypoxic-ischemic brain injury in the newborn arises from a simple imbalance between the brain's demand for energy and its supply in the perinatal period. Yet the cellular mechanisms that lead to neuronal death are complex and multi-factorial. The overall efficacy of induced hypothermia is relatively low, and the need for mechanism-directed treatments for HI brain injury is high. Basic research, in identifying the mechanisms underlying HI-induced neuronal death, can provide therapeutic targets for translational testing. The approaches discussed above target the cellular mechanisms of HI-induced neuronal death in vastly different ways. With continued research, one or more of these approaches, or derivatives of them, may ultimately become effective treatments for HI brain injury in the newborn.

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Educational Gaps

1. What are the pathophysiological mechanisms leading to neuronal death following hypoxiaischemia?
2. Hypothermia is the only treatment shown to reduce death or disability following hypoxiaischemia. What new therapeutic approaches are actively being studied?

Objectives

After reading this article, readers should be able to:

- Be familiar with the presentation of hypoxic-ischemic encephalopathy
- Understand the primary mechanisms leading to neuronal injury following perinatal hypoxia-ischemia
- Appreciate the variety of new approaches currently under investigation for treatment of newborns with hypoxic-ischemic brain injury

Figure 1.

The cellular mechanisms of HI-induced brain injury in the newborn. *Excitotoxicity*: Over-activation of glutamate receptors, represented here as the NMDA receptor (NMDAR), increases free intracellular calcium levels activation enzymes (lipases, endonucleases) that degrade key cellular constituents. *Free Radical Toxicity*: Reactive oxygen species, represented here as superoxide (O_2^-), is produced from multiple sites within the cell, induces apoptosis and, through generation of peroxynitrite ($ONOO^-$), damages cell membranes by lipid peroxidation. *Inflammation*: HI-induced activation of microglia releases pro-inflammatory cytokines that induce apoptosis in neighboring neurons. The sites of action of potential therapies discussed are shown. (*nNOS*: neuronal nitric oxide synthase; *EPO*: erythropoietin; *ROS*: reactive oxygen species)