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Neuroprotective Interventions: Is It Too Late?

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Abstract

In most cases of neonatal hypoxic-ischemic encephalopathy, the exact timing of the hypoxic-ischemic event is unknown, and we have few reliable biomarkers to precisely identify the phase of injury or recovery in an individual patient. However, it is becoming increasingly clear that for neuroprotection in neonates to succeed, an understanding of the phase of injury is important to ascertain. In addition, in utero antecedents of chronic hypoxia, hypoxic preconditioning, intrauterine infection, and fetal gender may change the expected time course of injury. Neuroprotective interventions, such as hypothermia and N-acetylcysteine, currently have efficacy in human and animal studies only if instituted early in the inflammatory cascade. While these cascades are currently being investigated, molecular mechanisms of recovery have received little attention and may ultimately reveal a window for therapeutic intervention that is much longer than current paradigms.

Chorioamnionitis, an ascending infection of the fetal membranes, is associated with significant white and grey matter brain injury in newborns and is particularly important in the pathogenesis of periventricular leukomalacia (PVL) and cerebral palsy. Cytokine release and neutrophil and macrophage activation by bacterial products cause far-reaching damage in fetal brain parenchyma and vasculature, altering blood-brain barrier permeability and fetal blood flow and resulting in inflammatory brain injury. In addition to causing direct injury, the inflammatory state induced by chorioamnionitis predisposes the newborn to hypoxic-ischemic injury as well, with cellular reserves too low to withstand the interruption of blood flow during the relatively hypoxic-ischemic process of birth. Fetal well-being is severely compromised with heart rate decelerations and bradycardia episodes, and this poor tolerance for labor may further attenuate cerebral perfusion, resulting in white matter injury and permanent consequences for the developing neonatal brain.

Very little translational research has been attempted in this area of brain injury, because protecting the fetal brain through the maternal/placental/fetal circulations poses extraordinary challenges. Consideration must be given to placental metabolism and permeability different pharmacokinetics in the fetus compared with the mother. Considerable safety concerns in this population with unique physiology and vulnerabilities also exist.

N-acetylcysteine is a promising antioxidant therapy that has shown effective neuroprotection in an animal model of chorioamnionitis. It has a favorable safety profile with known side effects, which appear to be limited and manageable. Extensive clinical and safety data from research involving pregnant mothers and preterm infants, established from acetaminophen overdose and European studies of N-acetylcysteine for prevention of chronic lung disease of prematurity, are available. As neuroprotective compounds that can be used in this population

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are quite rare, the case for further evaluation of N-acetylcysteine is compelling. The National Institute of Neurologic Disorders and Stroke is sponsoring a pilot trial of intravenous N-acetylcysteine given to mothers antenatally and their infants postnatally who present with the diagnosis of chorioamnionitis, to evaluate safety and pharmacokinetics.

Hypoxia ischemia is a common pathway of neonatal brain injury in both extra- and intrauterine environments. The resulting inflammatory cascade leads to well-described phases of injury and recovery, including reperfusion, latency, and secondary injury, with fairly predictable time courses for each.[1] Reperfusion typically lasts 30 minutes to 60 minutes, followed by a latent phase of 6 hours to 12 hours, before resumption of cerebral oxidative metabolic failure and delayed cell death at 6 hours to 24 hours, lasting for days or weeks.[2,3] These phases of injury and recovery may not be strictly sequential or may have significant overlap, and the time course may be changed by severity of injury or other conditions.[4]

Neuroprotective interventions would ideally occur prior to reperfusion, when interruption of oxidative metabolism leads to adenosine triphosphate depletion and necrosis. However, this is generally not possible in neonatal hypoxic-ischemic injury, except in neonatal cardiac surgery cases that require prolonged low-flow or interruption of cardiopulmonary bypass. A window of therapeutic opportunity lasting several hours opens during the latent phase, when high-energy phosphate stores in surviving cells return to normal.[1,5] The secondary injury phase is characterized by oxidative stress and glutathione depletion, loss of glial function and neurotrophic support, and apoptosis and selective necrosis. Although this phase is quite prolonged and therapeutic intervention is not ruled out, neuroprotection becomes more difficult to attain in animal models, if delayed after onset.[6] The difficulty in achieving neuroprotection during the secondary injury phase has been assumed to be due to amplification of inflammatory signals or cascades extending past a point of no return for mitochondrial adaptation and caspase activation. However, it is also likely that mediators of injury are also triggering recovery mechanisms that overlap with the continued apoptosis in a manner that makes targeting neuroprotective interventions in this secondary phase particularly challenging. The timing of different recovery mechanisms has barely begun to be investigated and may reveal a window of intervention that is much wider than current paradigms.

Most Neuroprotective Strategies Target the Latent Phase

The latent phase in hypoxic-ischemic injury is marked clinically by improving physiologic stability after resuscitation and bedside circumstances that make the neonate amenable to intervention during this period to prevent or decrease secondary injury. Although designated the “latent phase,” it is not a biochemically quiet period. Inflammatory cascades are starting or ongoing during this time, including induction of Nuclear Factor-κβ, inducible and neuronal Nitric Ooxide Synthase. Vascular endothelium is activated, leading to chemokine and cytokine production, blood-brain barrier permeability, and impaired cerebral autoregulation. Secondary cell death begins when enough of these and other intra- and extracellular processes lead to adenosine triphosphate depletion and induction of apoptosis.[7]

For targeted neuroprotection to be effective during the latent phase, a better understanding of time courses for both mechanisms of injury and recovery is required. This is elegantly illustrated in work by Nijboer and Kavelaars in postnatal day 7 rats. NF-κβ was shown to have a biphasic induction after hypoxic-ischemic injury:[8] NF-κβ mRNA levels peaked at 30 minutes, normalized by 12 hours, then peaked again at 24 hours, again returning to normal by 48 hours. When inhibition of NF-κβ was used as a neuroprotective strategy, the importance of understanding this time course became apparent. Infarct volume was

dramatically decreased when the NF-κβ inhibitor was given at 0 and 3 hours after hypoxic-ischemic injury, but was worse if given at 0, 6, and 12 hours after injury. These data followed from the time course of NF-κβ induction, indicating that inhibition of the early expression of NF-κβ was protective, while inhibition of early *and* later NF-κβ expression peaks aggravated injury. Investigation of some mechanisms of downstream effects of NF-κβ may explain these surprising findings. Cytokines and caspases were decreased after hypoxia ischemia with early but not late NF-κβ inhibition. The early induction of NF-κβ expression occurred in the reperfusion phase and lasted into the latency phase, whereas the later induction of NF-κβ expression occurred in the secondary injury/recovery phase. Inhibition of NF-κβ expression during recovery led to significantly worse infarct size, leading the authors to conclude, as in interleukin-6, nitric oxide, reactive oxygen species, and other dual-role mediators, that *late expression of NF-κβ is necessary for repair*.[9–10] Elucidation of the expression pattern and specific time course of mechanisms of repair are proving to be essential in using targeted therapies effectively and understanding results.

Therapeutic Windows in Neonatal Neuroprotection

Therapeutic windows in neuroprotection are being established in neonatal hypoxia ischemia through extensive animal work and seem to be prolonged by hypothermia.[11] Gluckman and Gunn's investigations of hypothermia in near-term fetal sheep showed significant neuroprotection if hypothermia was initiated before 6 hours after injury.[12] In term infants who have moderate to severe hypoxic-ischemic encephalopathy, both selective head cooling with systemic hypothermia (CoolCap trial) and systemic hypothermia (NICHD and NINDS trials) resulted in improvement in moderate to severe disability or death if initiated less than 6 hours after birth.[13–14] The latent phase has been shown to be prolonged by moderate hypothermia in animal models, but to date this treatment is only in use clinically in term and near-term infants.

In preterm infants, the time course of hypoxic-ischemic injury may be shorter,[15,16] and the therapeutic window is unknown. [17] Each neuroprotective agent will likely have its own therapeutic window(s) based on the primary mechanism(s) affected. In addition to the phase of injury, the developmental stage of oligodendroglial precursor cells and other cell types will need to be taken into account, suggesting that neuroprotection in this vulnerable group will be a complex and delicate undertaking.

Importance of Pre-existing Inflammation in Neonatal Brain Injury

In utero events, such as chorioamnionitis, may trigger a significant inflammatory response in the fetus and dramatically worsen the fetal injury after hypoxic-ischemic stress. Chorioamnionitis is a common condition, present in approximately 5% to 10% of all deliveries, and in 45% of women who have premature rupture of membranes and labor.[18–19] However, chorioamnionitis may also be dramatically underdiagnosed in the preterm population, and may be causative in most spontaneous premature deliveries.[20]

Chorioamnionitis in the preterm infant imparts a significantly increased risk of periventricular leukomalacia (PVL), intraventricular hemorrhage (IVH) and seizures, and is the major perinatal risk factor for white matter injury by magnetic resonance imaging (MRI).[21,22] PVL occurs predominantly in 23- to 32-week infants and affects both motor and cognitive development.[23] However, the overall incidence of PVL is much less common in preterm infants (11% to 14%) than more diffuse hypomyelination (68% of all preterm infants, detected in MRI with diffusion tensor imaging).[24] In fact, only 5% of white matter lesions detected by MRI in preterm infants were cystic PVL. [25,26]

Fetal Inflammatory Response and Neurological Sequelae

Considering long-term motor outcomes, exposure to intrauterine inflammation accounts for 11% to 22% of cases of cerebral palsy in near-term and term infants, with an odds ratio (OR) of 9.3 for otherwise-unexplained cerebral palsy.[27,28] In a meta-analysis of chorioamnionitis, the relative risk for cerebral palsy is 4.7 for term infants and 1.9 for preterm infants.[29] In Yoon's study of histological chorioamnionitis among preterm infants ≤35 weeks' gestation, the presence of funisitis (inflammation of the umbilical cord) carried an OR of 5.5 for cerebral palsy at 3 years of age. [30]

There are compelling animal data indicating that chorioamnionitis leads to fetal brain inflammation as a primary mechanism of injury. In the fetal lamb, systemic inflammation is evident within 5 hours of lipopolysaccharide injection of the mother, at the same time as local chorioamnionitis.[31] Elevated interleukin-1b, interleukin-6, and interleukin-8 in cord blood are indicators of this fetal inflammatory response and good predictors of IVH, PVL, and cerebral palsy in the preterm and term infant.[32 –33] These inflammatory cytokines have been shown to induce nitric oxide synthetase in the fetal rat brain and activate microglial, gliosis, and apoptosis in oligodendroglial precursor cells, resulting in a decrease in myelin basic protein.[34,35] In particular, immature oligodendroglial cells are more susceptible to cytokine-induced injury compared with mature, myelin-producing cells.[36]

Lipopolysaccharide Sensitizes Fetal Animals to Subsequent Hypoxic-Ischemic Injury

Cytokine activation in utero sets up an inflammatory milieu, permitting and augmenting fetal and neonatal brain injury by other factors.[37] In the 7-day-old rat, administration of endotoxin within 4 hours to 6 hours of hypoxia-ischemia resulted in significant sensitization of the immature brain to hypoxic-ischemic injury compared with animals without endotoxin exposure.[38,39] When gene expression was analyzed in 7-day-old neonatal rats exposed to endotoxin, inflammatory and apoptosis genes were upregulated as early as 2 hours after LPS injection.[40]

Lipopolysaccharide also causes profound cardiovascular changes that affect the fetal brain. In the ovine model, umbilical blood flows are compromised within 1 hour of maternal lipopolysaccharide injection, resulting in hypotension, acidemia, and loss of cerebral autoregulation in the fetus.[41] When subsequently subjected to 1 minute to 2 minutes of hypoxia ischemia, the lipopolysaccharide -exposed ovine fetus had a 30-minute decrease in cerebral, placental, and carcass perfusion compared with non- lipopolysaccharide-exposed fetuses that exhibited a very brief decrease in cerebral blood flow. Lipopolysaccharide – hypoxic-ischemic lambs had a 50% decrease in cerebral oxygen saturation with loss of cerebral autoregulation,[37] mediated by inducible nitric oxide and endothelin-1.[42,43] With cerebral autoregulation impaired, 5 out of 7 lipopolysaccharide -exposed fetal sheep died within 1 hour of hypoxia-ischemia.

These studies give some insight into the pathophysiology of the intolerance of labor and delivery, which is classically seen in infants of mothers with chorioamnionitis. They are unable to accommodate even short periods of interrupted umbilical blood flow that typify most deliveries, and frequently have significant fetal heart rate decelerations, signs of fetal distress, and increased sensitivity to hypoxic-ischemic brain injury. Indeed, infants born to mothers with chorioamnionitis may have a clinical diagnosis of hypoxic-ischemic encephalopathy as well.

Oxidative Stress Mediates Inflammatory Injury

Oxidative stress directly depletes intracellular glutathione, allowing accumulation of reactive oxygen species and lipid peroxidation of cellular membranes. The immature brain handles oxidative stress poorly, with antioxidant activities that are very low in premature infants and only half of adult levels at term.[44] Reactive oxygen species damage lipid membranes of peroxisomes, which produce plasmalogens and very-long-chain fatty acids integral to myelin.[45,46] Reactive oxygen species and NF-κβ have also been shown to inhibit peroxisomal proliferation after injury, through inhibition of peroxisomal proliferator-activator receptor–alpha.[47]

Lipopolysaccharide injection of rat dams at E16-18 results in apoptosis of oligodendroglial precursor cells and in a reduction in peroxisomes in the oligodendroglial precursor cells that survive resulting in a dysfunctional population of OPCs.[49] By postnatal day 30 in the offspring, both OPC number and myelin are decreased, indicating that oligodendroglial precursor cells never recover their proliferative or functional abilities. This lack of catch-up growth by the remaining oligodendroglial precursor cells results in permanent loss of myelin, and therefore, loss of developmental potential.

Is Postnatal Neuroprotection Too Late?

Given the short timeframe for onset of inflammation in the fetus after endotoxin exposure and the synergistic effect of even mild hypoxia ischemia on brain injury, it is not surprising that in animal models of chorioamnionitis, neuroprotective therapies show little benefit if given after birth. Antenatal interventions early in the course of inflammation may have the best chance of preventing fetal brain injury from maternal chorioamnionitis, but the clinical issue of timing the onset of fetal inflammation is vexing and unresolved.

N-acetylcysteine is an endogenously produced, thiol-containing amino acid that serves as a glutathione precursor, preventing redox failure. N-acetylcysteine can directly scavenge oxygen free radicals by interactions with its thiol reducing group, therefore acting directly and indirectly as a potent antioxidant. N-acetylcysteine also decreases cytokine and iNOS expression through NF-κβ. N-acetylcysteine is transported across the placenta by selective amino acid transport[48] and *crosses the blood-brain barrier*, where N-acetylcysteine preserves peroxisomes, rescues oligodendroglial precursor cells, and restores myelination.[49,50] These properties make NAC a strong candidate to ameliorate some of the cascades of inflammation, as well as stimulate endogenous repair mechanisms in surviving OPCs.

N-acetylcysteine given to mother preserves OPC and myelination in the developing infant brain

In animal models of chorioamnionitis, N-acetylcysteine leads to improved survival of offspring after lipopolysaccharide injection in rat dams at E16-18.[47] N-acetylcysteine significantly decreased interleukin-6 in amniotic fluid and fetal serum, even when delayed 2 hours after lipopolysaccharide injection in E18 rats.[51,52] In addition, N-acetylcysteine restored oligodendroglial precursor cell numbers, markers of myelination, and peroxisomal function to control levels in rat pups sacrificed at postnatal day 30 (equivalent to older child) when given before or even shortly after endotoxin.[49] In animal models of chorioamnionitis, therefore, N-acetylcysteine can stimulate both the proliferative and self-repair mechanisms of oligodendroglial precursor cells, allowing development of normal postnatal white matter structure and function.

There is also experimental evidence that N-acetylcysteine can be delayed after lipopolysaccharide but needs to be given before hypoxia-ischemia. N-acetylcysteine treatment at 0 and 24 hours after hypoxia-ischemia in lipopolysaccharide -exposed 8-day-old rats dramatically improved pathology scores and decreased caspase 1 and 3 compared with saline controls, while N-acetylcysteine delayed until 2 hours and 24 hours after hypoxia-ischemia had no beneficial effect.[53]

These strong animal data suggest that N-acetylcysteine offers effective neuroprotection against white matter injury associated with oligodendroglial precursor cell apoptosis and dysfunction. We are conducting a pilot trial of N-acetylcysteine in maternal chorioamnionitis to determine the best dose of N-acetylcysteine, and the target serum levels that will decrease cytokine mediators of inflammation. This pharmacokinetic study will analyze different compartments for N-acetylcysteine concentrations, including maternal and neonatal serum at delivery, neonatal cerebrospinal fluid, and serum, and estimate transfer into these compartments across the placenta and blood-brain barrier.

In neuroprotection for chorioamnionitis, the following concepts inform the clinical trial design:

- Antenatal treatment with N-acetylcysteine has the best chance of effective neuroprotection and may be essential to successful rescue of immature oligodendroglial cells.
- The therapy will need to be administered to mother, but the target is the fetal brain.
- Not many drugs will be able to cross the placenta to the fetus and penetrate the blood-brain barrier, be safe and effective.

N-acetylcysteine is a strong candidate for a clinical translational trial because it is already FDA-approved, has good safety data in pregnant women who have suffered acetominophen overdose, and is in current use in clinical practice in neonates as part of an antioxidant cocktail given to neonates in liver failure. There are also good pharmacokinetic and safety data in preterm neonates from a European study of N-acetylcysteine to prevent chronic lung disease.[54,55] In this randomized clinical trial, 194 infants received N-acetylcysteine by continuous infusion during the first 6 days after birth, and although the incidence of chronic lung disease was unchanged, the authors noted a 39% decrease in PVL in the N-acetylcysteine group.[55]

Pilot Study Design

We wish to assess N-acetylcysteine distribution across different compartments in pregnancy: mother's blood, placenta, fetal blood, and brain. The placental clearance and how much will get to fetal plasma and brain are unknown. We also do not know if any side effects will be evident in infants when given before birth. Major expected side effects include rash and itching, or more advanced anaphylactoid reaction in mother, with rare hypotension, all responding to diphenhydramine. Slight prolongation of prothrombin time has also been reported, but there is no clinical indication of increased risk of bleeding in either mothers or in preterm infants.[56,57]

After obtaining consent we are enrolling 30 mothers within 4 hours of fever onset or clinical diagnosis of chorioamnionitis. We will obtain baseline blood umbilical blood flow studies and infuse N-acetylcysteine over 60 minutes, per FDA recommendation. The first 20 patients will be randomly assigned to one of 2 groups: 100 mg/kg or saline control. If no toxicities are seen, then we will enroll 10 mothers into the high N-acetylcysteine dose of 150

mg/kg. This staged approach with randomization allows us to be cautious and analyze safety variables in an unbiased manner.

We are also stratifying based on gestational age into preterm (24 weeks to 33 weeks gestation) and near-term- term neonates (≥34 weeks). N-acetylcysteine will also be infused intravenously every 12 hours for 2 days in the infant at a dose level based on mother's expected plasma concentration and projected infant clearance. We have safety stops at each dose level if any major complications develop. We will measure fetal cerebral artery blood flow, cerebral oxygenation, and superior vena cava flow after N-acetylcysteine, as well as serum cytokines, to correlate with N-acetylcysteine levels. We will obtain an MRI with spectroscopy and diffusion tensor imaging within 2 weeks after discharge to detect diffuse white matter injury, and collect 12-month neurological outcomes by standard tests.

We have designed this pilot trial to determine what doses or target plasma concentrations of N-acetylcysteine have little toxicity and evidence of short-term efficacy in preterm and term infants. These safety, pharmacokinetic, and efficacy outcomes will be used to evaluate the relationship between plasma concentration of N-acetylcysteine and outcomes, to determine the range of plasma concentrations of N-acetylcysteine that have evidence of effect but no major toxicities, to pick an appropriate dose of N-acetylcysteine and help estimate effect and sample sizes for a subsequent full-scale trial.

The target N-acetylcysteine plasma concentrations identified within this pilot trial may be used to design N-acetylcysteine dosing in future trials with greater accuracy. First, we are measuring actual N-acetylcysteine concentrations in several compartments, and then we will couple these pharmacokinetic data with markers of safety and efficacy. As opposed to the more common approach of measuring pharmacokinetics without measuring evidence of in vivo effect, or using a drug dose without measuring effective concentrations, we are gathering data on variability in N-acetylcysteine pharmacokinetics which we believe will be critical in evaluating efficacy in this patient population. N-acetylcysteine doses, safety outcomes, and sample size estimates in future trials may then be based on pharmacokinetic data specific for this disease process and gestational age participants, with evidence of safety and biologic effect. The preliminary data in this pilot trial should lead to future randomized controlled clinical trials in which N-acetylcysteine effect is either robustly confirmed or disproved because target plasma concentration levels will have been identified and reached.

We are continuing N-acetylcysteine dosing for 2 days postnatally in the infants because experimental evidence suggests it is the best design, covering the period of greatest lipid peroxidation in inflammatory and hypoxic-ischemic brain injury. Future studies can be designed to test the best length of therapy with a particular dose or concentration identified in this pilot trial, or to identify particular groups, possibly by gestational age or cytokine levels, that may benefit from longer therapy.

Because clinical chorioamnionitis is dramatically underdiagnosed in the preterm population and the risk of white matter injury so high in premature births, some neuroprotective therapy, which is proved quite safe, may be potentially started in mothers upon presentation in preterm labor, allowing protection against oxidative stress before the fetal inflammatory response begins. This approach would move therapy from the realm of rescue to true neuroprotection. If safety is shown in this safety trial and future efficacy trials, N-acetylcysteine would seem to be an ideal candidate for this type of prophylaxis.

Lastly, experimental treatment of mothers antenatally for fetal brain injury will continue to be a theme of future research with this compound and others. The pharmacokinetic data we obtain in this pilot trial, with different gestational age groups and multicompartment

measurements, will help model future clinical trial design of this and other pharmacologic therapies.

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