

Beyond Hypothermia: Alternative Therapies for Hypoxic Ischemic Encephalopathy

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Abstract: Neonatal encephalopathy remains a significant cause of death and disability worldwide. Therapeutic hypothermia has become a mainstay of therapy and has demonstrated the potential for neuroprotection and repair after neonatal hypoxic-ischemic brain injury. However, it has become apparent from published trials that hypothermia alone will not serve as complete protection nor benefit all neonates. The complicated cascade of events in a hypoxic-ischemic insult lends itself to multiple types of therapy, making a multi-faceted approach to treatment attractive. This review critically discusses the broad range of medical therapies currently being studied and summarizes the animal and human studies that have been done to date. Therapies that may act synergistically with cooling therapy are also discussed.

Keywords: Hypoxic ischemic encephalopathy.

INTRODUCTION

Hypoxic ischemic encephalopathy (HIE) continues to be a leading cause of neonatal morbidity and mortality worldwide. An estimated four million babies die every year during the neonatal period, and approximately one quarter of these deaths are attributed to perinatal asphyxia [1]. Of the infants who suffer from birth asphyxia, 15-20% will die in the newborn period, and among the survivors, one quarter will have permanent long term deficits [2]. Total body cooling has emerged as a therapy for these affected infants. Five multicenter trials demonstrated that treatment with mild hypothermia results in improved neurodevelopmental outcomes in infants 36 weeks gestation or greater who have suffered a hypoxic-ischemic event, leading to widespread acceptance of hypothermia as a mainstay of treatment for affected infants [3-7].

Despite the promising outcomes of hypothermia trials, the reduction in morbidity and mortality after cooling is modest, with one out of every nine neonates benefiting from the therapy [8]. Hypothermia does not fully protect an injured brain, and infants who suffer from the most severe form of HIE have exhibited the least benefit [6]. Because a clinician cannot distinguish a responder from a non-responder, much of the current research focuses on therapies that will be synergistic with hypothermia therapy. In addition, hypothermia may broaden the therapeutic window for other therapies to have a beneficial effect [9, 10]. If therapies can be found to act synergistically with hypothermia, overall intact survival may be improved.

PATHOPHYSIOLOGY OF CEREBRAL HYPOXIC-ISCHEMIC INJURY

The pathophysiology of injury during hypoxia-ischemia (HI) is essential to understand why and how synergistic

therapies may be promising. Initially, a disruption of cerebral blood flow decreases the delivery of oxygen and glucose to the brain. This event leads to anaerobic metabolism, an increase in lactic acid, decreased ATP, and decreased transcellular transport [11]. Intracellular sodium, water, and calcium levels rise. Membrane depolarization occurs, and excitatory amino acids such as glutamate are released, which leads to a flux of calcium into the cells via NMDA gated channels, perpetuating secondary injury [12]. Free fatty acids are released into the cytoplasm which leads to peroxidation by oxygen free radicals [13]. The culmination of energy failure, acidosis, glutamate release, lipid peroxidation, and nitric oxide (NO) toxicity all lead to cell death via necrosis and apoptosis [13]. Although this pathophysiology is complex, the multiple steps leading to cellular damage provide multiple opportunities for therapy (Fig. 1).

MAGNESIUM AND XENON – NMDA RECEPTOR ANTAGONISTS

Given the role that excitatory amino acids play in the cascade of events leading to cell death, it is logical to identify pharmacologic agents that would either inhibit their release or block their postsynaptic actions. Magnesium is a potential therapy because it blocks the NMDA receptor (and therefore blocks Ca^{2+} influx) in a voltage gated manner. Magnesium is safe and is used extensively in perinatal medicine to treat women with pre-eclampsia. Also, expectant mothers delivering prematurely are given magnesium because strong evidence shows better neurodevelopmental outcomes in these neonates [14, 15]. Animal data show that magnesium is effective in limiting injury in mature rat pups after induced focal stroke lesions [16, 17]. In addition, magnesium has been extensively studied in both hypoxic-ischemic animal models and human neonates with HIE.

In a hypoxic-ischemic model, rat pups given placebo, magnesium, or magnesium plus melatonin had reduced infarct volume and fewer cells that showed signs of apoptosis when they were in the treatment groups. However,

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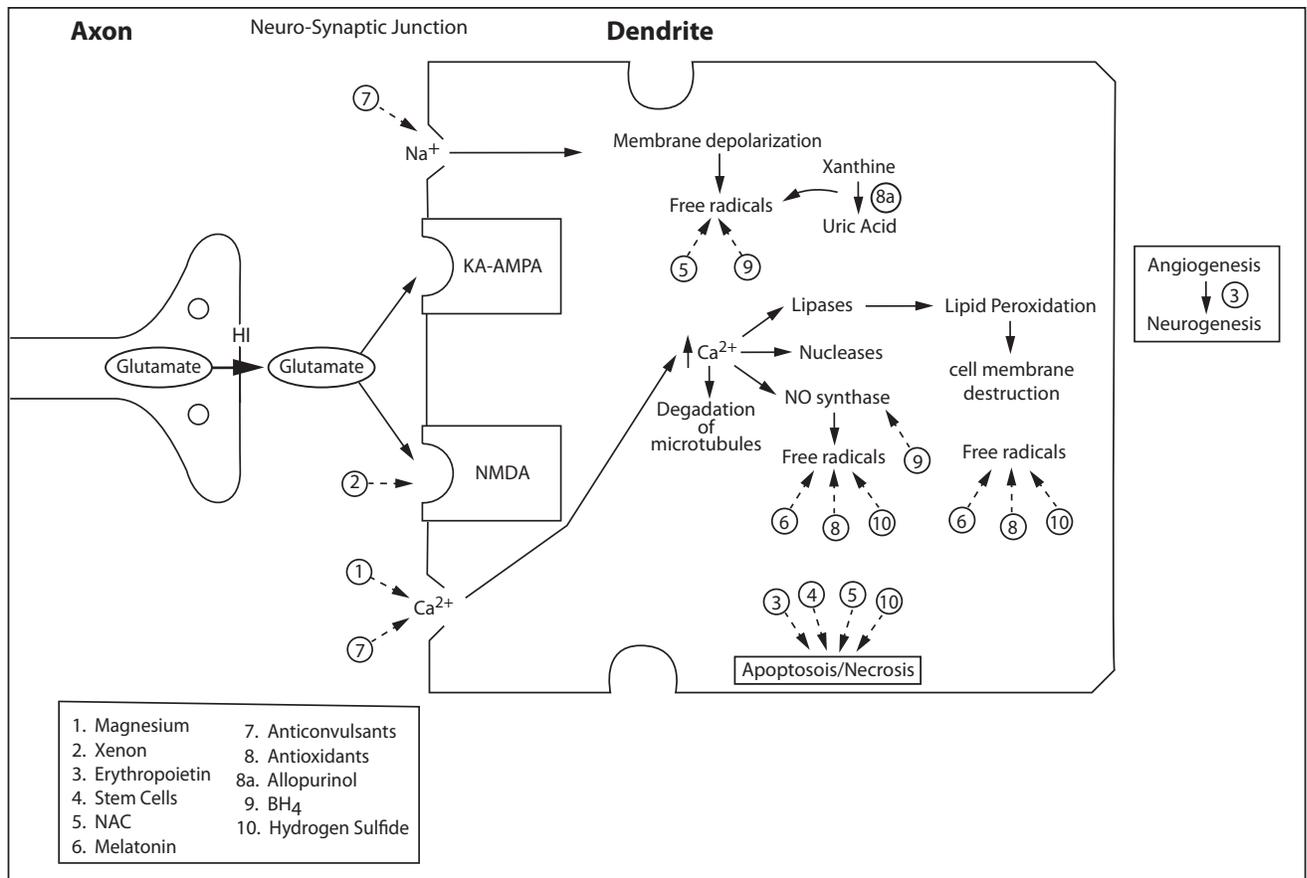


Fig. (1). Hypoxia-ischemia (HI) leads to a complex cascade of events producing cellular damage and destruction. Globally, cerebral perfusion is reduced secondary to decreasing cardiac output depleting the cells in the brain of both oxygen and energy substrates. At the cellular level ATP is rapidly depleted due to the inefficient shift to anaerobic metabolism which is further compounded by a decrease in glucose delivery. The rapid depletion of ATP results in severe compromise in the basic metabolic processes of the cells leading to a series of secondary intracellular events resulting in cellular damage. These events can be grouped into three main categories: excitatory neurotransmitter toxicity, intracellular calcium overload, and free radical formation. During HIE, an excessive amount of the excitatory amino acid glutamate is released from the pre-synaptic terminal. This excess glutamate leads to overstimulation of the glutamate receptors (AMPA, KA, and NMDA) located on the post-synaptic neuron and leads to excitotoxicity. Overstimulation of the KA and AMPA receptors causes sodium and chloride to enter the cell which increases cell osmolality. Increased cell osmolality leads to the influx of water, resulting in subcellular edema. If the edema is severe enough, the cell lyses. Overstimulation of the NMDA receptor triggers the influx of calcium. Calcium is the predominant second messenger in the cell and when present in pathological amounts activates a series of enzymes which results in the destruction of the cell. Calcium also contributes to the production of free radicals such as NO. Free radicals are also generated from fatty acid and prostaglandin metabolism, leading to the formation of harmful amounts of superoxide and hydrogen peroxide. The three aberrant cellular processes lead to both apoptosis and necrosis. The various neuroprotective agents are illustrated at the points in which they interfere with the pathophysiologic cascade. Key: Solid arrows represent the pathophysiologic cascade which is unleashed as a result of hypoxic-ischemic injury. The dashed arrows represent interruption of the cascade by the various neuroprotective agents (circles with numbers) reviewed in the manuscript. The numbers in the circles correspond to the agents in the lower left panel.

magnesium plus melatonin did not show an additive effect [18]. When given within one hour of insult, magnesium decreased the amount of HI-induced brain injury, including hippocampal damage. However, when given within two hours of insult, the protective effect decreased, demonstrating that timely administration is crucial [19]. Animal studies have also demonstrated long-term benefits of magnesium therapy. When tested one month after injury, rats treated with magnesium after HI injury did significantly better on sensorimotor tests than the control group. Furthermore, the animals treated with magnesium showed

improved skills throughout the training period compared with the animals that did not receive magnesium [20].

Several randomized trials in asphyxiated term infants have been reported. The dosing (250 mg/kg or 125 mg/kg) was similar among the studies, and none of the studies combined magnesium with hypothermia [21-24]. Magnesium improved short-term outcomes: decreased seizures, shortened time to full oral feeding, improved EEG tracing and head CT results, and an improved neurological exam at discharge, however magnesium did not change overall mortality [21-24]. Negative side effects such as hypotension or hypoventilation were not observed [23]. Only

one study to date has reported long term outcomes, and those infants treated with magnesium were compared to historical controls of asphyxiated infants. Magnesium-treated infants had improved neurological outcomes at 4, 10, and 18 months of age [24]. Limited side effects have been described. Doses of 250 mg/kg demonstrated no difference between the treatment and control groups with regard to apnea, need for assisted ventilation, oxygen requirement, and use of blood pressure medication [25]. However, when higher doses were used (400 mg/kg) incidence of hypotension increased [26].

Magnesium therapy appears to be safe and well-tolerated. Magnesium therapy overall shows a predominance towards improvement for these infants. It is a relatively easy intervention and may have an additive effect when combined with hypothermia therapy. More research needs to be done on this combination therapy.

Xenon, a noble gas currently used as an inhaled anesthetic, has also shown promise in neuroprotection. Like magnesium, xenon is an NMDA receptor antagonist and may have other neuroprotective qualities such as effects on other ion channels and a reduction in neurotransmitter release in general. It is an attractive agent for use in affected infants because it easily crosses the blood-brain barrier, has limited cardiovascular side effects, takes rapid effect, is rapidly reversible, and has myocardial protective properties [27].

In animal studies with and without hypothermia, xenon demonstrated significant benefit [28-34]. By itself, xenon improved neuropathology scores by 80% [29]. Xenon and hypothermia administered together improved regional pathology scores and improved motor function in a dose-dependent manner when compared to control and hypothermia alone [34]. Xenon plus hypothermia also reduced the infarct volume. This reduction was also significant when xenon was given within one hour of hypothermia as compared to the group that received xenon within five hours [33]. When given both therapies, rats had significantly improved hemispheric weight, less apoptotic cell death and increased viable cell count, and improved performance on functional studies 30 days after injury [35]. Xenon also provided long-term benefit. Rats that received both xenon and hypothermia improved the most on long-term behavioral and functional testing, obtaining almost complete restoration of function and significant improvement on repeated testing [31].

Two studies used a piglet model, which better replicates the global insult of HI injury and its effects on different organ systems. These studies compared hypothermia and xenon alone and together and demonstrated a clear advantage with combination therapy [28, 30]. Xenon plus hypothermia decreased the evidence of damage as indicated by a decreased lactate peak on MRI [30]. Also, global and regional pathology significantly improved with combination therapy, providing 75% neuroprotection. Each brain region analyzed (gray matter, white matter, thalamus, basal ganglia, hippocampus, cerebellum, and brain stem) had significantly less histological injury than either treatment alone [28]. Xenon at doses of 50% appeared to be without significant side effects while 70% xenon often resulted in sedation, respiratory depression, and CO₂ retention [36].

Xenon appears to be relatively safe and well-tolerated. Duration and timing of treatment require more investigation. Also, because the delivery devices used to deliver this medication are expensive, more cost effective devices will need to be developed. Currently, affected infants who may need xenon therapy would have to be intubated and mechanically ventilated to receive treatment. Also, human studies need to be done, and several groups will be doing this in the near future.

ERYTHROPOIETIN

Erythropoietin (EPO) is a naturally occurring glycoprotein frequently used to stimulate erythropoiesis and is a safe and efficacious treatment for anemia of prematurity [37, 38]. EPO is naturally produced in the CNS and is upregulated in the cord blood of infants who have suffered perinatal asphyxia [39]. While EPO is a large protein, it will cross the blood-brain barrier. HI injury disrupts the blood-brain barrier, which may increase the penetration of EPO [40]. EPO has already demonstrated neuroprotective qualities in studies of anemia of prematurity. In premature infants treated with EPO, EPO both decreased IVH and white matter injury and improved neurodevelopmental scores [41, 42]. EPO has a protective effect in a variety of neurologic injury models: spinal cord injury, traumatic brain injury and ischemic stroke [43-45].

EPO has many possible mechanisms for neuroprotection. It provides neuroprotection against apoptosis and has an anti-inflammatory effect when bound to EPO receptors on astrocytes and microglial cells [13, 46-49]. It prevents NO-induced death of neurons and protects neurons from glutamate toxicity [50, 51]. EPO is neurotrophic and has demonstrated effects on neurogenesis, differentiation and repair after injury [52, 53]. It seems to be particularly neuroprotective for damage related to ischemia-reperfusion stress [54].

Several studies in animal models of HI injury have shown EPO to be neuroprotective. EPO treatment, before or after cerebral insults in neonatal rats or mice, resulted in improved histological analysis, decreased neuronal injury, and/or less functional impairment [55]. After neonatal focal ischemic stroke, both single- and multi-dose treatments reduced infarct size and improved sensorimotor and cognitive function, even when administered 24 hours after injury [48, 56-59]. In the mouse model of HI, multiple doses of EPO given over a 48 hour period improved behavioral outcomes in all mice but only improved histological outcomes in the female mice [57]. Also, seizures associated with HI injury had increased latency and decreased duration [60, 61]. Two other studies using the rat model of HI demonstrated less damage to the brain on histological analysis (improved cortical volumes) and a decreased level of apoptosis and increased neuroprotection [62, 63].

The human data on the use of EPO for HI brain injury are limited but promising. In the study by Elmahdy *et al*, term infants with mild/moderate HIE were randomized to treatment with EPO (2500 IU/kg q day for five days) or no treatment, and a group of healthy term infants was used as a control. The infants randomized to the EPO group had an improved EEG tracing, fewer seizures, decreased NO concentrations, and improved neurodevelopmental outcome

at six months when compared to the no treatment group. MRI findings did not differ between the groups [64]. In the study by Zhu *et al.*, a much lower dose of EPO was used (300 u/kg or 500 u/kg every other day for two weeks), with the first dose given within 48 hours of birth. In the study, 167 term infants with moderate/severe HIE were randomized to either receive EPO or conventional treatment. None of the infants had hypothermia therapy. Infants who received EPO therapy had improved neurological scores at 7, 14, and 28 days of life. Death and moderate/severe disability were decreased in the EPO group ($p=0.017$) at 18 months, and the outcome was the same between the 300 u/kg and 500 u/kg doses. These improvements were seen only in moderate, not severe HIE. EPO levels were elevated in the cerebrospinal fluid suggesting adequate penetration [65].

While EPO therapy seems quite promising, many questions remain to be answered. The dosings in the two human studies were remarkably different (300 or 500 IU vs 2500 IU), but both studies showed clinical improvement. Given the natural course of HIE, EPO therapy over an extended timeframe appears to be beneficial, as in the Zhu study (over two weeks). Neither of these two studies demonstrated any negative side effects, but a large multicenter trial in adult patients using extremely high doses (40,000 IU) raised safety concerns [43]. Also, as hypothermia treatment is now becoming widely available, a study to examine the synergy between EPO and hypothermia should be performed. Another concern is that gender effects have been demonstrated in several animal studies, suggesting females respond better to EPO therapy than males [57, 66]. However, as apoptotic injury can progress for several weeks after the initial injury, and neurogenesis is a lengthy process, a treatment such as EPO is particularly attractive because it targets these processes and can be administered over a longer period of time.

Recently, EPO has been used in combination with hypothermia in rat models of HIE. Two studies suggested a difference in gender response without a clear advantage of additive treatment [67, 68]. Both studies raised methodology concerns since hypothermia alone was not globally protective in the pups [67, 68]. The safety of EPO for use in human neonates undergoing hypothermia has also recently been evaluated. In a dose escalation study, a starting dose of 250 U/kg EPO was escalated to 2500 U/kg. The higher doses did not produce any adverse effects and attained serum values corresponding to levels seen in animal models for neuroprotection [69]. Based on the strength of this study, EPO may be tested in combination with hypothermia in the next several years in a large multicenter clinical trial.

STEM CELL THERAPY

Stem cell therapy is another adjunctive therapy attractive to researchers because of its potential for benefit through several different mechanisms. In HI injury, neurons, glia and endothelial cells are damaged, leading to widespread necrosis and apoptosis. Stem cell transplantation may aid in the replacement of damaged cells, and the release of trophic and anti-apoptotic factors, which have anti-inflammatory effects that lead to the preservation of endogenous tissue [70-73]. Researchers have studied stem cell therapy in a variety of adult neurological diseases such as: cerebral

ischemia, Alzheimer's, and Parkinson's with good results [74-76]. Stem cell therapy is attractive for neonatal use secondary to the plasticity of the neonatal brain. The subventricular zone has continuous cell division, which provides intact cell signaling. Such cell signaling improves migration and differentiation of transplanted cells [77]. Stem cells offer the potential for a long-term effect even with just a single intervention.

Two key issues arise with stem cell therapy, those being delivery to the target area and then differentiation into the appropriate cell type. Several studies have demonstrated that stem cells migrate to the site of injury specifically and preferentially [35, 78-81]. When animals that did not have HI injury were transplanted, stem cells were found to be evenly distributed throughout the brain. In animals that had injury, transplanted stem cells were localized around the area of damage [81]. When transplanted into the non-injured half of the brain, stem cells were shown to migrate to the side that the injury was present [82]. Stem cells have also differentiated into neuronal cells. This differentiation also appears to be a specific process. When stem cells are injected into animals without injury, little to no differentiation occurs [35, 78, 81, 83-85]. In one study, bone marrow mesenchymal cells differentiated into oligodendrocytes, benefiting remyelination of damaged areas, and also into neurons, helping to replace lost neurons [85]. Stem cell transplantation also improved brain histology as evidenced by less grey and white matter loss and decreased lesion size [80, 85]. When stem cells were transplanted by intravenous injection or by intracranial injection, both routes of transplantation showed a marked decrease in the loss of neurons in the hippocampus, one of the areas most sensitive to damage in HI injury [86]. The IV route of administration is more attractive given the invasiveness of intracranial transplantation.

However, the most appealing aspect of stem cell therapy may not be the direct replacement of damaged neuronal cells, but rather the trophic, anti-inflammatory and paracrine effects that stem cells may provide. In several studies, animal brains have shown significant histological and functional improvement even though the number of transplanted cells to survive was low and differentiation was limited [87, 88]. In the rat model, even though multipotent adult progenitor cells had low graft survival (<1%) motor and behavioral outcomes significantly improved [86, 89]. Similar results were seen using human bone marrow derived mesenchymal cells transplanted *via* intracardiac injection. Brain volume restoration was limited, but functional testing showed much improvement [83]. Stem cells upregulate important growth factors after transplantation. Also, stem cells are anti-inflammatory in HI injury by decreasing the amount of microglia, which are a component of the inflammatory process and prevent neurogenesis [80, 85, 90]. Gene therapy may be added to stem cell transplantation to increase its multi-factorial effect. When stem cells were altered to express neurotrophin-3, the number of neural stem cell-derived neurons increased from >5% to 20% [79].

Stem cell therapy is promising, but many questions remain. The best type and source of cells to be transplanted are unknown although most studies have used neural stem cells or mesenchymal stem cells with positive effects. In

most of the animal studies to date, the route of administration for transplantation is intracranial, but for ease of use in the human population intracardiac (similar to using the umbilical artery), intravenous, and intranasal methods have all been used with good results and minimal complications [80, 83, 86]. Other questions remain: the concurrent use of immunosuppressives, the number of cells to be transplanted, the timing of transplantation, and the effects of stem cell transplantation combined with hypothermia therapy. To date, human studies have not been published, but a clinical trial is underway at Duke using autologous cord blood as the source of stem cells. The purpose of the trial is to determine the safety and outcomes of stem cell transplant in children up to one year of age [91]. However, stem cell therapy appears to have much promise given its multi-factorial potential for improvement. Stem cell therapy appears to help repopulate the damaged area and also may improve the actual healing environment.

N-ACETYL-CYSTEINE (NAC)

NAC is a treatment known previously because of its use in treating liver injury after acetaminophen overdose. NAC has several different mechanisms for treatment of HI injury: glutathione precursor, antioxidant and free radical scavenger, anti-inflammatory agent and has anti-apoptotic properties. In adult models of stroke NAC was shown to be a free radical scavenger and decreased reperfusion injury and NO production [92]. In spinal cord injury studies the combination of NAC and hypothermia significantly improved the degree of injury when compared to each treatment alone [93]. In neonatal rats with induced excitotoxic brain injury, NAC reduced the area of damage and inhibited apoptosis [94].

Studies using NAC in the HI model are limited but promising. In a piglet model of HI injury, NAC reduced H₂O₂ production (and therefore decreased oxidative stress), decreased lactate accumulation, decreased lipid peroxidation, and improved cerebral perfusion. However, NAC had no effect on NO production as predicted [95]. No histological or functional outcomes were measured. In another piglet model, NAC enhanced cerebral blood flow and also decreased levels of caspase-3, which plays a role in cell death and is considered to be a specific marker for apoptosis [96]. In the only study to combine NAC with hypothermia, combination therapy reduced infarct size 48 hours after insult, and preserved brain volume two and four weeks after insult, suggesting a therapy with continued benefit. Combination therapy also improved functional outcomes and reflexes in the rat models [97].

NAC appears to be an attractive therapy given its multifactorial approach and previous use in other disease models with minimal side effects. More studies will need to be done to investigate combination therapy and use in human subjects. A consensus on a therapeutic dose does not exist yet (the studies reviewed here used between 20 -150 mg/kg), and so an optimal dose and length of therapy need to be determined.

MELATONIN

Melatonin is a naturally-occurring substance produced mainly from the pineal gland. Melatonin is widely known for

its use in regulating the circadian rhythm, but it has many other effects that may benefit infants with HI injury. Melatonin serves as a free radical scavenger of OH, O₂⁻, and H₂O₂ [98]. It decreases inflammatory cytokines and stimulates anti-oxidant enzymes such as glutathione peroxidase and reductase, glucose-6-phosphate dehydrogenase, and superoxide dismutase [99, 100]. It will cross the blood brain barrier and has beneficial effects on decreasing microglial activation [101-103]. Melatonin has improved outcomes in adult humans after stroke and has no documented toxicity for humans [102, 104]. When given to infants with sepsis, it reduced inflammatory markers without adverse effects [105].

Several promising studies examined melatonin for use in the prenatal period and also in preterm infants. When given to pregnant mice at the end of pregnancy, melatonin decreased apoptosis and neuronal inflammatory cells in newborn mice exposed to asphyxia at delivery [106]. Also, maternal administration of melatonin prevented the subsequent increase in free radical production in fetal sheep exposed to intrauterine asphyxia [107]. When melatonin was given to the fetuses of mid-gestation sheep that were then subjected to umbilical cord occlusion, the number of TUNEL -positive cells (a marker of DNA fragmentation secondary to the apoptotic signaling cascade) decreased in the white matter and also attenuated the increase in activated microglia and 8-isoprostane production, a marker of oxidative stress [108]. Animals treated with melatonin had a slower recovery of blood pressure after occlusion, but a difference in overall mortality was not observed. In newborn rat pups subjected to hypoxia on pregnancy day 17, melatonin administered from birth to day of life three decreased microglia and increased mature oligodendrocytes. Oligodendrocyte death was unchanged as demonstrated by TUNEL staining [109]. This study looked at a wide range of doses (0.002 mg/kg to 20 mg/kg) with significant benefit shown with 0.2 mg/kg and higher. In one day old rats subjected to hypoxia, three doses of melatonin (10 mg/kg, with the first dose before the insult) decreased NO levels, decreased VEGF production (which contributes to cerebral edema), decreased malondialdehyde levels (formed during lipid peroxidation and serving to further augment tissue damage by free radicals), and increased glutathione levels, a powerful antioxidant [110]. In mouse pups exposed to excitotoxic injury, melatonin decreased the size of white matter lesions (with no change demonstrated on gray matter lesions), decreased neuronal cell death, and decreased microglial activation. Melatonin also demonstrated a long term beneficial effect by preventing the loss of conditioning ability in pups with the induced brain lesions [111].

In the studies examining HI injury, the results are also promising. In a cohort study of asphyxiated newborns, melatonin (10 mg PO given every two hours for eight doses) reduced malondialdehyde and nitrate/nitrite levels compared to the infants given placebo. These reduced levels demonstrate a reduction in lipid peroxidation and oxidative damage [112]. In rats subjected to HI injury, melatonin reduced oxidative stress and glial cell activation [113]. In newborn rats exposed to HI injury on day of life seven, melatonin was dosed at 15 mg/kg for three doses. This dose of melatonin increased the number of surviving neurons and decreased reactive gliosis [114]. Newborn rats received

melatonin in varying doses (5 or 15 mg/kg), for one or three doses, and for varying time frames. The rats showed benefit when melatonin was administered either before or after HI injury, and in a dose-response fashion. On histological analysis, hippocampal injury was significantly decreased. In addition, the rats exposed to melatonin had behavioral outcomes that improved even into adulthood and had less behavioral asymmetry [115].

While the body of evidence regarding melatonin as a safe neuroprotective therapy is impressive, further studies need to be done that examine the optimal timing and dose of the medication. Also, further inquiry is needed into melatonin's use in infants with HI and concurrent use with hypothermia. To date studies which combine melatonin with cooling therapy in an animal or human model have not been done. Melatonin therapy is very attractive given its multifactorial potential for benefit, extensive use in humans with limited side effects, and ease of use.

ANTICONVULSANTS

Anticonvulsant medications have been studied as possible neuroprotectants for multiple reasons. Infants affected by HI injury have an increased likelihood of seizures, and seizures can amplify damage that has already occurred [116, 117]. Early prevention or reduction of seizure activity could help attenuate brain injury because the severity of seizures in HIE is independently associated with brain injury [118]. Also, seizure activity and HI injury cause damage similarly by an excessive release of excitatory amino acids and increased neuronal activity [119]. Most anti-epileptic medications have various pathways of action which could provide varied pathways toward neuroprotection.

Topiramate is used commonly for the treatment of seizures in children and has been proven safe and with minimal side effects [120]. It has several mechanisms of action. Topiramate inhibits carbonic anhydrase isozymes, modulates AMPA/kainate and GABA-activated ion channels, and modulates voltage activated sodium and chloride channels. All of these actions reduce excitotoxicity and glutamate release. Topiramate is neuroprotective in adults with cerebral ischemia [121, 122]. When given before ischemia in rats, topiramate decreased the likelihood of resultant seizures. When given to rats after induced status epilepticus, topiramate improved cognitive function [123, 124]. It also decreased neuronal cell death in rats exposed to prolonged periods of hyperoxia [120]. Topiramate stabilizes cell membranes and blocks fast conductances in neuronal cells exposed to oxygen/glucose deprivation [125].

In a rodent model of HI injury designed to induce periventricular leukomalacia, topiramate was given at various doses (10, 30, or 50 mg/kg), each for four doses. The authors concluded that topiramate decreased white matter injury and improved functional outcome at 21 days of life. These effects were achieved primarily by blocking the AMPA/kainate receptor. However, the only dose that showed benefit was 30 mg/kg [126]. In an experiment examining whether topiramate could restore the time period of cooling efficacy, a dose of 30 mg/kg was given in a rat model of HI shortly after the injury occurred. After a three hour delay, hypothermia was started. While neither therapy alone showed benefit, combination treatment decreased the

severity of brain pathology and improved functional performance one and four weeks after injury [10]. Since many centers cannot offer active cooling for infants, this therapy has the potential to extend the cooling window. When given either before and after HI injury, or just after the injury, topiramate provided neuroprotection and improved long term outcomes in rat pups. This study used several different dosing regimens and compared oral and IV dosing. The pups given topiramate as pretreatment had the best outcomes. When topiramate was given only after injury, it was less effective and the treatment was delayed. Both oral and IV dosing demonstrated efficacy. Interestingly, the higher dose of 100 mg/kg showed limited benefit. The 20 and 50 mg/kg doses were the only ones that showed statistical improvement [127]. In a piglet model of HI injury, topiramate decreased neuronal cell loss when given at a higher dosing regimen (50 mg/kg, followed by 20 mg/kg for two doses) but less benefit at the lower dose (20 mg/kg followed by 10 mg/kg for two doses). No side effects (decreased vigilance, change in neurological or feeding behavior) were noted. Behavioral deficits showed a trend toward improvement after topiramate therapy but were not statistically significant. Seizure activity was similar in both groups. Also, topiramate at the higher doses increased incidence of TUNEL positive cells [128].

There are few studies done in infants with HI. In infants given topiramate while undergoing hypothermia, oral absorption was maintained. Because hypothermia can affect pharmacokinetics, researchers were not surprised that infants undergoing cooling had increased plasma concentrations when compared to normothermic infants. Also, plasma concentrations were increased in deep hypothermia vs mild hypothermia. Hypothermia slowed the clearance of the drug, which resulted in higher plasma drug concentrations over all time points and a longer half-life [129]. The same group also examined topiramate's potential for benefit in conjunction with hypothermia. Two different doses were used. The first dose was given at the onset of hypothermia and continued daily for three days. Limited side effects were noted, however, no statistical difference was seen in the short-term outcomes, survival rate, or pathology on MRI [130].

Levetiracetam was first approved for use in 1999 and since then has become one of the first-line anticonvulsant medications used in children. Like topiramate, levetiracetam affects AMPA and NMDA receptor-mediated excitatory synaptic transmission and reduces glutamate release, inhibiting the amplitude of excitatory currents [131]. This drug has good solubility, little acute toxicity, and neuroprotective activity [132]. It is protective in kainate-induced toxicity, reducing the amount of lipid peroxidation [133]. When compared to other anti-epileptic medications, it does not induce cell death, even at increased doses [134]. Levetiracetam improved outcomes in focal ischemia but not in global insults [135]. Levetiracetam did not provide neuroprotection after oxygen/glucose deprivation in *in vitro* rat hippocampal cells even though several other anti-seizure medications showed neuroprotection [136]. Similar results were seen in an *in vitro* study using neuronal cells exposed to ischemia [125].

Levetiracetam shows promise, but studies have not been done to specifically examine its use in neonatal HI injury. As

a relatively new drug used in newborns, studies have examined levetiracetam's efficacy and safety profile in that population. Several different studies have been promising, showing it to be a potent, safe, and more effective than conventional medications (phenobarbital and phenytoin) [137, 138]. The study used levetiracetam doses ranging from 10-60 mg/kg in the newborn and preterm populations [137, 138]. A study has also found levetiracetam safe for long-term use in children [139]. In studies examining brain ischemia, levetiracetam stabilized hippocampal cells by decreasing excitatory potassium and calcium currents [140, 141]. Also, rats subjected to middle cerebral artery occlusion had significantly reduced infarct volume with the use of levetiracetam [142].

Phenobarbital is the current drug of choice for seizures in the newborn setting and has several different routes of possible neuroprotection. It decreases cerebral metabolic rate, reduces lipid peroxidation, limits free radical damage, and stabilizes the cell membrane [2, 143]. It significantly and dose dependently decreased cell death *in vitro* after oxygen/glucose deprivation [136]. However, phenobarbital has potential adverse effects as well. It increased neuronal apoptosis *in vitro* and has demonstrated adverse effects on the developing brain such as interference with cell proliferation and migration, axonal arborization, synaptogenesis, and synaptic plasticity [144, 145]. Normal rats given multiple doses of phenobarbital had an increased incidence of spatial learning deficits in adulthood [146]. Evidence exists that phenobarbital may induce cognitive impairment in infants and toddlers [147].

Study results are varied in the use of phenobarbital for neuroprotection in HI injury and in combination with hypothermia therapy. In a rat model, the evidence was promising. Phenobarbital improved short- and long-term neurodevelopmental outcomes, but a difference in overall mortality was not found [148]. With regards to human studies, a meta-analysis of heterogeneous interventions showed a trend toward improved neurodevelopmental outcome, but a difference in death or severe neurodevelopmental disability was not noted [149]. Phenobarbital treatment within six hours of birth decreased death and/or disability in infants at three years of age [150]. In addition, studies have shown phenobarbital to have antioxidant effects, decreasing lipid peroxides, superoxide dismutase, and glutathione peroxidase on CSF analysis when given to asphyxiated infants [151, 152]. A dose of 20 mg/kg did not change mortality or frequency of abnormal neurological outcome at discharge, but it did reduce the incidence of seizures without notable adverse effects, such as a need for increased ventilator support in treated infants [152]. In a retrospective study, cooled infants were compared between two groups: those who received a prophylactic dose of phenobarbital at 40 mg/kg and those who did not. The treated infants had a significantly reduced incidence of seizures. On univariate analysis, there was no statistical significance in moderate to severe impairment or death in the phenobarbital group, and multivariate analysis suggested a trend toward improved outcome after treatment [153]. Another retrospective study compared similar groups and noticed an increased risk of death and abnormal MRI at discharge in the treatment group. Infants who received the prophylactic dose were clinically worse and already at risk of

adverse clinical outcome. However, the authors cautioned the use of phenobarbital in cooling for infants with HI [154].

In conclusion, anticonvulsants have many potential benefits and are attractive for continued study in the area of neuroprotection after HI. Physicians extensively prescribe anticonvulsants to infants and children. Therefore, safety profiles and dosages for seizure treatment are well documented. Also, anticonvulsants' intended role as anti-epileptics is a benefit of HI treatment given the role of seizures in HI. However, all three anticonvulsants reviewed here had mixed reviews with regards to benefit and potential for harm. Further research should be performed to examine their effectiveness, potential for apoptosis, and deleterious effect on the developing brain.

ANTIOXIDANTS

Hours after an initial hypoxic-ischemic injury, a secondary phase of neuronal injury occurs as a result of the production of oxygen free radicals, inflammatory mediators and apoptosis [155]. Investigators have considered the role of various antioxidant substances that may prevent or ameliorate this secondary phase of brain injury. Antioxidants are any substances that reduce oxidative damage such as that caused by free radical molecules. Many naturally-occurring, readily-available antioxidants exist, including vitamins A, C and E, and trace minerals like selenium, copper, zinc and manganese. Additionally, flavonoids, phenols, and polyphenols possess antioxidant properties. These compounds are present in many fruits, vegetables and beverages such as green tea and wine.

POLYPHENOLS

Polyphenols are products of plant metabolism that possess antioxidant properties and affect gene expression. One particular polyphenol, resveratrol has received particular attention after studies demonstrated that this compound could activate the "anti-aging" gene family known as sirtuins. In human cells, these genes seem to blunt the activity of the tumor-suppressor gene *p53*, blocking programmed cell death [156]. Dietary supplementation with foods rich in polyphenols provides neuroprotection in animal models of focal brain ischemia and periventricular white matter injury [157, 158]. In a study of an adult model of stroke in rats, resveratrol protected the brain when the rodents were pre-treated for three weeks or longer with resveratrol-containing drinking water [159]. One of the richest food sources of these polyphenol compounds is pomegranate juice [160].

To date, two trials that used polyphenols for neuroprotection in the neonate are published. Loren, *et al.* investigated whether maternal dietary supplementation with pomegranate juice could protect their pups in a murine model of HI injury. In a controlled study, dams were provided ad libitum access to drinking water with pomegranate juice (at three concentrations) during the last third of their pregnancy and throughout the duration of litter suckling. Their pups underwent hypoxic-ischemic injury at postnatal day seven. On histologic examination, pups whose dams received dietary supplementation with pomegranate juice showed markedly decreased brain tissue loss compared to controls that received water, sugar water, or vitamin C water. This effect was dose-

dependent with the greatest significance ($p \leq 0.0001$) in those pups that received the highest dose of pomegranate juice [161].

Building on the previous data, West *et al.* tried to demonstrate that the polyphenols in pomegranate juice specifically conferred the neuroprotection. They performed a similar study with pomegranate polyphenol extract (PPE) instead of pomegranate juice. In their study, the pregnant dams drank sugar water with PPE. Pups from these dams were subjected to neonatal hypoxia-ischemia at postnatal day seven and caspase-3 activity was measured in the left and right hippocampus at 24 hours following the injury. Caspase-3 activation is a reliable and readily quantifiable measure of the extent of apoptotic neuronal cell death in response to neonatal hypoxic ischemic injury [162, 163]. Pups of dams drinking PPE had significantly less caspase-3 activation in the left hippocampus than pups of dams drinking sugar water [164]. This work suggested that the polyphenols in pomegranate juice were responsible for the neuroprotection observed in both trials.

ASCORBIC ACID (VITAMIN C)

Ascorbic acid (vitamin C) is a well-known antioxidant. At low concentrations, it scavenges and neutralizes reactive oxygen species and regenerates other antioxidants such as α -tocopheroxyl, urate and β -carotene [155]. Ascorbic acid completely protects lipoproteins from peroxidative damage by aqueous peroxy radicals [165]. Animal and human studies have evaluated ascorbic acid as a neuroprotective agent in hypoxia ischemia.

Several studies have demonstrated that ascorbic acid is neuroprotective in animal models of hypoxia-ischemia. Ranjan *et al.* demonstrated that a short course of high dose ascorbic acid given to adult monkeys prior to induction of focal cerebral ischemia significantly reduced the macroscopic infarct size [166]. Also, intraventricular administration of ascorbic acid in newborn rats significantly reduced the macroscopic brain injury and the number of necrotic cells at 7 days post injury [167].

Ascorbic acid has also been evaluated in combination with other compounds known to exhibit antioxidant properties. Ascorbic acid exhibits its antioxidant properties in the aqueous phase of plasma. As mentioned previously, ascorbic acid also interacts with alpha-tocopheroxyl radicals, regenerating the antioxidant form of alpha-tocopherol, and thereby maintaining alpha-tocopherol concentrations in the plasma [168]. Thus, a combination of alpha-tocopherol and ascorbic acid offers antioxidant protection in both lipid and aqueous phases. Nakai *et al.* studied the administration of ascorbic acid, alpha-tocopherol or a combination to pregnant rat dams before and after induction of transient intrauterine ischemia. Cerebral neocortical mitochondrial respiration was measured as a marker of secondary brain injury. Ascorbic acid treatment alone demonstrated a modest improvement (64% of non-ischemic controls) of the secondary mitochondrial dysfunction. Alpha-tocopherol alone demonstrated a similarly-modest improvement (62% of non-ischemic controls) of the secondary mitochondrial dysfunction. Treatment with ascorbic acid and alpha-tocopherol combined caused a normalization of mitochondrial activity to 91% of non-ischemic controls [169].

Despite positive results in animal studies, a recent randomized controlled trial of ascorbic acid in combination with ibuprofen for neonates with HIE failed to show an effect on outcomes including mortality, the incidence of neurologic abnormalities at hospital discharge, or the incidence of developmental delay at 6 months of age [155]. To date, no other studies evaluate ascorbic acid alone or in combination with other agents for neuroprotection in the human neonatal brain.

ALLOPURINOL

Allopurinol is a purine analog that inhibits the enzyme xanthine oxidase. In the postasphyxial reoxygenation phase, xanthine-oxidase converts hypoxanthine, produced in large amounts during the actual hypoxic ischemic insult, into uric acid. This reaction produces large amounts of the free radical superoxide. By inhibiting this reaction, allopurinol functions as an antioxidant. Additionally, allopurinol directly scavenges the toxic hydroxyl free radical and chelates nonprotein-bound iron, a potent pro-oxidant. Allopurinol reduces delayed cell death in animal models of perinatal asphyxia and in human patients with other forms of organ perfusion injury [170].

For many years, researchers have investigated allopurinol for the prevention and treatment of cerebral hypoxia ischemia. Immature rats treated with allopurinol at fifteen minutes after cerebral hypoxia-ischemia had markedly reduced acute brain edema and long-term cerebral injury [171]. Using a rat model of stroke, Lin *et al.* demonstrated that oxypurinol, the active metabolite of allopurinol, administered 30 minutes prior and 24 hours after the onset of focal ischemia significantly reduced the development of the ischemic infarct, attenuated tissue swelling, and ameliorated the neurological deficits [172]. Additionally, research in asphyxiated pigs that received allopurinol postnatally demonstrated beneficial effects on cerebral energy status and cytotoxic edema [173].

Furthermore, pregnant ewes undergoing intermittent partial umbilical cord occlusion received allopurinol antenatally. This study demonstrated a good transfer of allopurinol from the mother to the fetus and a significant suppression in superoxide production in fetal brain microdialysis perfusate [174]. Also, in newborn piglets, allopurinol crossed the placenta and achieved therapeutic blood levels [175].

Results of human neuroprotection trials with allopurinol are conflicting. Studies have investigated antenatal and neonatal allopurinol administration. An earlier study by Van Bel *et al.* investigated the effect of high dose allopurinol on free radical status, postasphyxial cerebral perfusion and electrical brain activity in severely asphyxiated newborns. This study was small, with 22 patients divided between the treatment and control groups. The study demonstrated that after allopurinol treatment pro-oxidant activity was lower and lipid peroxidation was reduced in the plasma. Additionally, the researchers found a relative preservation of postasphyxial cerebral blood volume and electrical brain activity in the allopurinol-treated infants [176]. However, in a randomized, double blind, placebo-controlled study of allopurinol for severely asphyxiated infants, the study was ended early after interim analysis showed that mortality was

high and did not differ between allopurinol and vehicle-treated infants. The researchers speculated that the selection of only extremely severe asphyxiated babies made the expectation of beneficial effects from postnatal interventions unrealistic because these infants already had substantial neuronal damage [177].

In 2008, a Cochrane review was performed to evaluate the effectiveness of allopurinol on mortality and morbidity in newborn infants with suspected HIE. The review identified three randomized or quasi-randomized controlled trials that compared allopurinol administration with placebo or no drug in affected infants. In total, 114 infants were in the studies. Meta-analysis did not reveal a statistically significant difference in the risk of death during infancy, nor in the incidence of neonatal seizures. Only one trial assessed neurodevelopment in surviving children and did not find a statistically significant effect. The authors concluded that available data are not sufficient to determine whether allopurinol has clinically significant benefits for newborn infants with hypoxic ischemic encephalopathy [170]. Notably, the analysis only included infants treated postnatally with allopurinol.

Previously, Boda *et al.* showed that pharmacologic plasma levels were achieved in the human fetus after allopurinol administration to the mother [178]. In a more recent randomized, double blind feasibility study, 53 pregnant women in labor with a gestational age of >36 weeks and fetal hypoxia received 500 mg of allopurinol or placebo intravenously. Researchers evaluated maternal and cord blood concentrations of allopurinol and oxypurinol in relation to cord blood levels of the brain injury marker S-100B. They found that S-100B was significantly lower in the subgroup of infants whose allopurinol levels were therapeutic compared to placebo and subtherapeutic allopurinol groups [179].

Presently, the ALLO-Trial is underway in the Netherlands, which is a randomized double blind placebo controlled multicenter study in pregnant women at term in whom the fetus is suspected of intra-uterine hypoxia. The study expects to enroll 220 patients over the course of two years. Enrolled women will receive allopurinol or placebo when fetal hypoxia is suspected. Primary outcome measures are the amount of S-100B and the severity of oxidative stress, both measured in umbilical cord blood. Secondary outcome measures are neonatal mortality, serious composite neonatal morbidity and long-term neurological outcome.

While antioxidant results are definitely mixed, they remain a promising avenue of investigation. Most antioxidants are naturally occurring substances, and they seem to have minimal side effects. Notably, antioxidants could be given maternally or to affected infants after delivery. No studies have been performed which combine hypothermia with antioxidant treatment, and this interaction should be examined.

TETRAHYDROBIOPTERIN (BH₄)

BH₄ is an essential cofactor for a number of enzymes, such as tyrosine hydrolase and all three forms of nitric oxide synthase (NOS) [180,181]. It may serve as an antioxidant, in particular as a scavenger of superoxide [182]. BH₄ is known to cross the blood brain barrier and is safe in humans with no

adverse events on long term follow up [183-186]. In patients with dystonia, BH₄ treatment can improve functionality indicating possible benefit in the prevention of motor problems [187]. BH₄ has a wide range of uses including treatment for phenylketonuria and a potential therapy for autism [184,186].

Neurons with intrinsically elevated levels of BH₄ are resistant to oxidative stress [188]. L-sepiapterin is a precursor of BH₄ and significantly and dose dependently protects against oxidative stress as well [189]. Researchers have discovered that neonatal HI injury can cause a relative BH₄ deficiency, which results in increased superoxide production. This process can be reversed with BH₄ administration [190]. In pregnant rats subjected to uterine ischemia, HI decreased BH₄ levels in the thalamus, basal ganglia, and frontal cortex. This BH₄ deficiency increased vulnerability of the brain to injury. Maternal treatment with BH₄ increased levels in the aforementioned brain regions, decreased motor deficits, and reduced the number of still births. The BH₄ levels increased even more when given L-sepiapterin, which is more stable than BH₄ [191]. However, when researchers gave BH₄ to mice with intracerebral hemorrhage, brain edema did not decrease and neurological outcomes did not improve at 24 hours [192].

BH₄ is a fascinating avenue of study and has potential in both maternal and neonatal administration. It appears to be an influential factor in determining the vulnerability of the developing brain to HI, and its safety and efficacy make it attractive for further study. The interaction of BH₄ with hypothermia, the best dosing route and schedule, and the appropriate length of treatment remain unknown.

HYDROGEN SULFIDE

Hydrogen sulfide (H₂S) is a clear water-soluble gas that is a vital signaling molecule for the inflammatory, cardiovascular, and central nervous systems. H₂S is now known as the third endogenous gaseotransmitter along with CO₂ and NO [193]. H₂S is a lipophilic compound that easily permeates cell membranes without needing a specific transporter, which makes it an attractive neuroprotective agent [193]. It is a free radical scavenger, has anti-apoptotic properties, and is either pro or anti-inflammatory, depending on the administered dose [194]. H₂S acts as an antioxidant by increasing glutathione levels and the activity of superoxide dismutase. It demonstrated an *in vitro* reduction of reactive oxygen species in cardiomyocytes subjected to ischemia/reperfusion [195-197].

Inhaled H₂S can induce a state of suspended animation which decreases energy expenditure [198,199]. Multi-organ failure secondary to stress (HI) may be an adaptive, hypometabolic response aimed at organ preservation. This induced hibernation state may be beneficial and prevents further damage from occurring [200]. Through this proposed mechanism, H₂S markedly improved myocardial and neurological function after induced cardiac arrest and resultant resuscitation in mice [89]. Mice exposed to otherwise lethal hypoxia and rats undergoing lethal hemorrhage that were pretreated with inhaled H₂S for twenty minutes had markedly prolonged survival [201,202]. H₂S protects a variety of tissues after ischemia-reperfusion injury including lung, liver, kidney, and heart [203-206].

The research regarding H₂S seems promising, but it has all been done in animal models and has produced controversial results. At high concentrations H₂S is toxic but is beneficial at low concentrations [194,198,202]. When inhaled, H₂S can be a pulmonary irritant but also has an intravenous route of administration. Further studies must examine the safety profile of H₂S and determine the best route of administration.

ALTERNATIVE THERAPIES

Patients with HIE have received several non-traditional alternative therapies such as hyperbaric oxygen, acupuncture, cerebrolysin, citicoline, and B vitamins [207-209]. The studies have involved limited numbers of patients and a recent Cochrane Review examined these therapies [209]. Most of the studies included in the review either did not specify the age of the infant at treatment or provided treatment to neonates between one and two months of age which is not in the acute period of HIE. While the Cochrane review was ultimately inconclusive, it did show a trend that intervention with either acupuncture and/or various IV medications (cerebrolysin, citicoline, ganglioside, and vitamins B1, B6, and B12) decreased the incidence of cerebral palsy at six and twelve months of age [209].

CONCLUSION

HIE continues to be a significant source of morbidity and mortality among newborn children, and the effects of this injury are far-reaching. Hypothermia has emerged as a mainstay of therapy and has been extensively studied, with many of these studies now demonstrating results further into childhood. While hypothermia has proven effective, often the more severely-affected infants benefit the least. An adjunctive therapy or therapies to use along with hypothermia are an immensely attractive avenue of study and could potentially benefit many infants. Given the mechanism of HIE and its chain of events that occur over several days, a treatment that acts along one or more of those steps could provide significant benefit. All of the therapies outlined in this paper have promise, and many have a multi-factorial approach to treatment given their mechanism of action. Currently, the most attractive therapies are erythropoietin and xenon. Much of the future research is directed at examining these two medications. However, the possibilities are endless, and other therapies or undiscovered therapies could prove to be the most beneficial. Neonatal HIE is an exciting area of the current study with much potential for saving and improving the lives of affected infants.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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