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Perinatal brain injury: mechanisms and therapeutic approaches

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TABLE OF CONTENTS

1. ABSTRACT

Brain damage resulting from perinatal hypoxia-ischemia evolves slowly over time. While a small number of brain cells may die during a sufficiently profound period of hypoxia-ischemia, many will show initial recovery during a “latent” phase characterized by actively suppressed neural metabolism and activity. Critically, this transient recovery may be followed after ~6 hours by a phase of secondary deterioration, with delayed seizures, failure of mitochondrial function, cytotoxic edema, and bulk cell death over ~72 hours. This is followed by a tertiary phase of remodeling and recovery. Understanding the mechanisms of injury that occur during each phase may allow for the development of more targeted treatments. This review discusses the mechanisms of injury that occur during the primary, latent, secondary and tertiary phases of injury and potential treatments that target one or more of these phases. Treatment during the latent phase has the greatest potential to prevent injury. In the secondary phase of injury, anticonvulsants can attenuate seizures but show limited neuroprotection. By contrast, there is increasing preclinical evidence that neurorestorative therapies may improve long-term outcomes.

2. INTRODUCTION

Moderate to severe hypoxic-ischemic encephalopathy (HIE) resulting from perinatal hypoxia-ischemia (HI) occurs in approximately 1-3/1000 live

term and 1-8/1000 live preterm births (1-3). The only treatment available for term infants suffering from HIE is therapeutic hypothermia, while for preterm infants there are none. Although hypothermia significantly reduces death or disability, nearly half of infants presenting with HIE will still have an adverse outcome despite treatment (4). Therefore, new ways to further reduce the burden of injury are needed.

The partial protection with current hypothermia protocols found in clinical studies is likely related to the formidable difficulties involved in starting hypothermia within the optimal window of opportunity (5). It is clear from preclinical studies that hypothermia must be started during the latent phase, ideally within the first three hours after HI, to achieve the best possible neuroprotective effect (6-8). This is consistent with clinical data from a recent cohort study that suggested that asphyxiated neonates who were able to be cooled within three hours of birth had better motor outcomes than when hypothermia was started between three and six hours (9). However, in a randomized, controlled trial, hypothermia was only able to be started in 12% of neonates within four hours of birth (10).

This review explores the concept that the timing of treatment is imperative to its success and that the window of opportunity for a particular treatment is likely to be dependent on its mechanism of action relative to the evolution of brain injury. It is likely that some babies who are diagnosed late in the evolution of injury will not benefit significantly from neuroprotection alone and may require the combination of neuroprotection and neurorestoration strategies. These strategies may have benefit beyond the window of opportunity for successful treatment with hypothermia.

3. THE EVOLUTION OF INJURY

It has been widely confirmed across a wide range of *in vitro, in vivo* and clinical studies that HIE is not a single 'event' but rather is an evolving process that can continue for days or even weeks after the insult (Figure 1) (11-13). During the immediate period of HI (the "primary" phase), high-energy metabolites are depleted, with progressive depolarization of cells, severe cytotoxic edema (14), and extracellular accumulation of excitatory amino acids due to failure of reuptake by astroglia and excessive depolarization-mediated release (15). Although neurons may die during a sufficiently prolonged period of ischemia or asphyxia, many neurons initially recover, at least partially, from the insult in a so called "latent" phase, only to die many hours or even days later ("secondary" phase or delayed cell death). The latent phase is characterized by initial transient recovery of cerebral oxidative metabolism as shown by magnetic resonance spectroscopy (MRS), with actively mediated suppression of cerebral metabolism, cerebral blood flow and EEG activity. This is followed by a phase of secondary deterioration defined by cerebral energy failure from 6 to 15 hours after birth (16), accompanied by delayed onset of seizures and cytotoxic edema. These resolve over approximately 72 hours after the insult. The severity of the secondary failure of oxidative metabolism is closely correlated with neurodevelopmental outcome at 1 and 4 years of age (17), and infants with encephalopathy who do not show initial recovery of cerebral oxidative metabolism have extremely poor outcomes (16). An identical pattern of initial recovery followed by delayed energy failure is also seen after HI in the piglet, rat and fetal sheep where it is closely correlated to the severity of neuronal injury (13, 18, 19). The timing of energy failure after HI is tightly coupled with the appearance of histologic brain damage (20), implying that it is primarily a function of evolving cell death (Figure 1). There is now compelling evidence from the development of therapeutic hypothermia that the latent phase represents the key window of opportunity to interrupt the progression of delayed bulk cell death and so improve neural outcomes (14, 21, 22).

The timing of oligodendrocyte death after HI is also biphasic. Although, similarly to neurons, the majority of cells die after HI, there is evidence from treatment studies that the time course of delayed death in oligodendrocytes is shorter than for neurons. When therapeutic hypothermia was started 90 minutes or three hours after the end of global cerebral ischemia in term-equivalent fetal sheep, partial protection of oligodendrocytes was achieved, at a time when near-total protection of neurons was possible (7, 8). When hypothermia was started 5.5 hours after the end of global cerebral ischemia, no improvement in oligodendrocyte survival was seen, compared with partial but significant protection of neurons in the same study (8). These data suggest that there is a shorter window of opportunity for protecting oligodendrocytes compared to neurons after global cerebral ischemia in near-term fetal sheep, further emphasizing the importance of early intervention after HI.

It is important to appreciate that unlike in the laboratory, clinical insults are often not clearly defined and may begin many hours before birth or involve repeated or prolonged exposure to asphyxia (23). Thus, at the time of birth, the evolution of injury may already be well into the latent phase, leaving a very short window of opportunity for treatment with therapeutic hypothermia. Further, the duration of the latent phase is dependent on the severity of the insult. More severe insults are associated with reduced duration of the latent phase and a more rapid transition to irreversible cell death (24). Not surprisingly, after severe HI, some cells may never

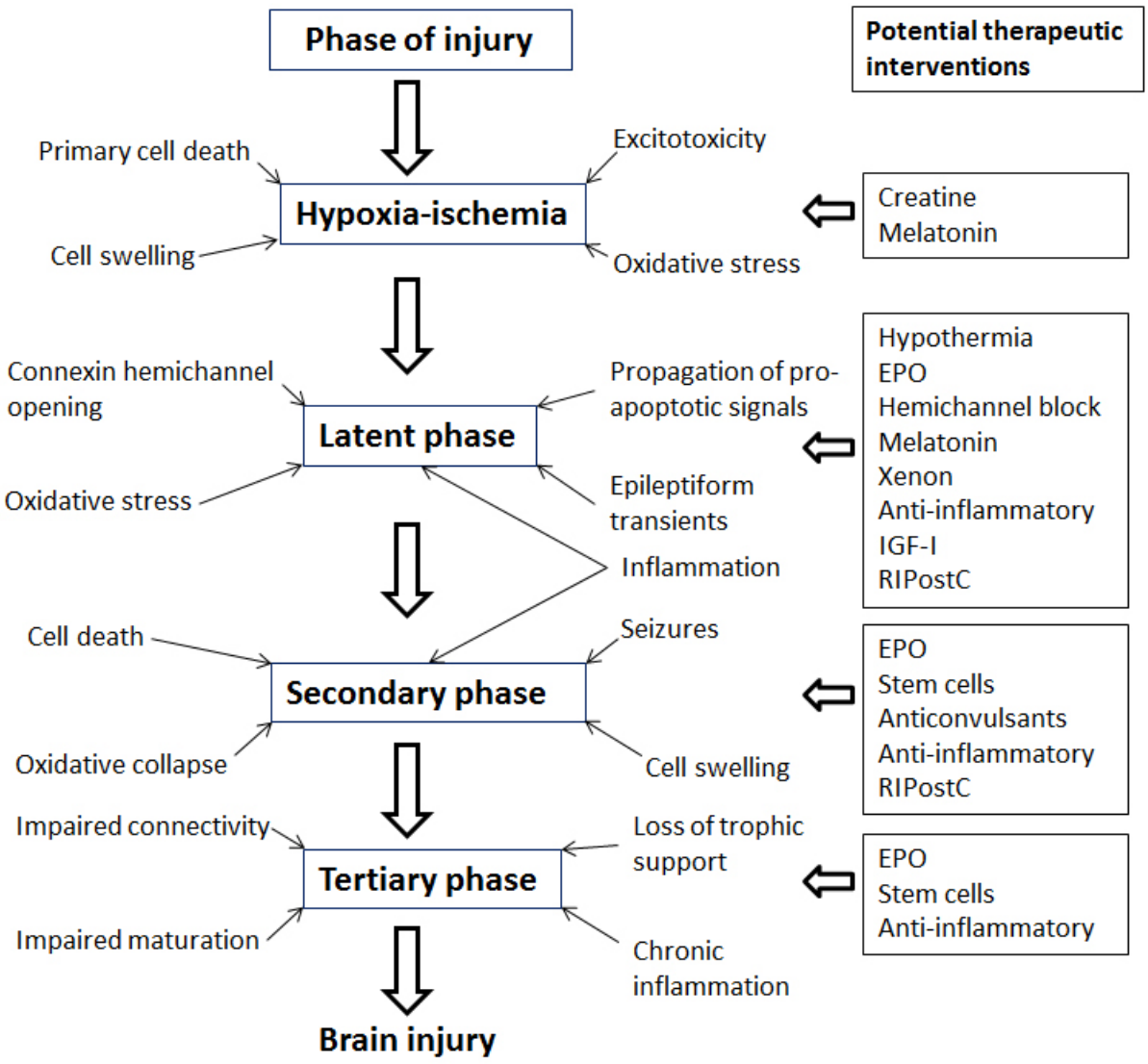

Figure 1. Diagram illustrating the phases of injury after hypoxia-ischemia and potential therapeutic interventions targeting specific mechanisms of injury in each phase.

fully restore their mitochondrial function (25). Further, brain damage in very preterm babies may result from a sequence of injurious events, including intrauterine growth restriction and infection, in addition to HI (26).

3.1. Delayed cellular maturation after hypoxic-ischemic injury

In addition to the biphasic pattern of death of oligodendrocytes and neurons in the brain following HI, there is increasing evidence for delayed impairment of cellular growth and maturation in a "tertiary" phase, starting after resolution of the delayed seizures and brain edema in the secondary phase, and continuing for many months afterwards. Diffuse white-matter injury is characterized by acute death of pre-myelinating oligodendrocytes (preOLs) (27-30). PreOLs are highly present in the human white matter between 23–32 week's gestation, which is the period of greatest risk for white matter injury (31). By term age, preOLs mature into immature/mature oligodendrocytes that produce myelin, allowing normal axonal signaling. preOL cell death, leading to reduced numbers of mature oligodendrocytes, was considered to cause the diffuse deficits in white matter myelination observed in preterm neonates. More recently, human and experimental studies found that preOLs in the white matter exhibit a remarkable plasticity, whereby they rapidly regenerate following injury. However, these regenerated preOLs fail to mature into myelinating cells, and thus fail to produce myelin. (32). Thus, chronic deficits in white matter myelination observed in modern cohorts of preterm infants are now considered to mainly reflect failure of oligodendrocyte maturation, rather than chronic oligodendrocyte cell death (33-35).

Recent magnetic resonance imaging (MRI) studies suggest that preterm birth is also associated with persisting changes to grey matter structures of the brain, including reductions in cortical and subcortical volumes (e.g., striatum, thalamus, hippocampus) (36-41), decreased cortical surface area, complexity and folding, and delayed gyral maturation (40), which persist into childhood (36), adolescence (42, 43) and early adulthood (44), without evidence of gross pathology. These grey matter deficits likely underlie the adverse neurodevelopmental outcomes associated with preterm birth. For example, reduced cortical growth in preterm babies is strongly associated with poor neurodevelopmental outcomes, impaired cognition, and lower IQ in later life, but not with motor function (40). Reduced growth of cortical and hippocampal structures is also associated with impaired memory, learning ability, and processing speed at 7 years of age after very preterm birth (45, 46), while abnormal cortical folding in preterm infants is associated with poor reading recognition scores at 8 years of age and lower IQ scores at 19–21 years (44). Further, reduced cortical and thalamic volumes are associated with impaired executive function and memory scores in adolescence and early adulthood after very preterm birth (47, 48).

Until recently, failure of grey matter growth was thought to reflect irreversible death of neurons (49). Indeed, in historical cohorts of preterm infants with evidence of severe cystic white matter injury, post-mortem studies have found widespread death of neurons (50, 51), including subplate neurons, layer V pyramidal neurons (49), and late-migrating interneurons (52). However, more recent post-mortem studies of prematurely born infants with diffuse non-cystic white-matter changes found no evidence of acute neuronal degeneration or overt grey-matter injury (53). In part, these findings are consistent with findings from both imaging in humans and histology in preterm fetal sheep that the preterm brain is highly tolerant of HI, and that this tolerance is lost from caudal to rostral, consistent with the pattern of myelination, such that acute HI is associated with subcortical injury but sparing of the cortex (54-57). The long-term impairment of development of the cortex may reflect, in part, the key role of neuronal activity in maintaining normal brain growth. Loss or attenuation of neural activity from a severely damaged region can lead to secondary Wallerian degeneration in other regions due to loss of trans-synaptic activity, as reviewed (58). Consistent with this, a strong relationship between impaired cerebellar development and cerebral white-matter lesions has been shown using volumetric MRI (59-61). Further, subcortical white-matter injury can lead to isolation of the overlying cortex with long-term abnormalities of structural and functional development (50). In particular, axotomized pyramidal neurons can transform into local circuit interneurons with hypertrophy of interneurons and the intracortical neuropil, and so the effects of acute injury can ultimately resemble areas of cortical dysplasia.

Further, the dramatic growth and connectivity of the cerebral cortex and other grey-matter structures in humans over the last trimester (62) primarily reflects a prolific increase in growth and complexity of neuronal processes (dendrites) (63). Using a preterm animal model of diffuse white-matter injury, impaired cortical and striatal growth was found to be driven by reduced growth and complexity of neuronal dendrites and reduced neuronal synapses (64, 65). This failure of neuronal maturation was associated with loss of the normal maturational decline in MRI-derived fractional anisotropy (FA); cortical FA values in humans and other species decline during normal fetal development, reciprocally with increased neuronal complexity (66, 67). Preterm neonates were found to have higher cortical FA values at term than term neonates. Higher cortical FA values were strongly associated with impaired cortical growth and worse neurodevelopmental function at 2 years of age (67, 68). Further, in a small case series, some preterm neonates showed reduced dendritic complexity and numbers of neuronal spines in the visual cortex, consistent with arrested neuronal development (69). Supporting these findings, there is now evidence for abnormal brain connectivity in preterm neonates, including reduced structural connectivity between the thalamus and cortex in childhood (70) and impaired functional connectivity in language-associated areas of the cerebral cortex at school age (71) and in the thalamocortical network in adolescence (72). These findings support a paradigm shift from supposedly irreversible brain injury to a dysfunction of brain maturation (33-35). This strongly suggests that therapies that stimulate oligodendrocyte and neuronal development may help to repair preterm brain dysmaturation, and promote healthy brain development.

This concept, that an acute global insult can trigger evolving injury and that characteristic events are seen at different times, including large-scale death of neurons and/or oligodendrocytes within the first 72 hours, an acute inflammatory response that may become chronic, followed by delayed impairment of brain maturation. Understanding these events is central to the rational development of therapeutic interventions targeted to the specific phases of evolving injury. These interventions may include prophylactic treatments to reduce injury occurring during the primary phase, or acute intervention after HI to protect neurons and oligodendrocytes during the latent and secondary phases, or neuro-restorative strategies to suppress inflammation and promote maturation and repair during the tertiary phase (Figure 1). Some potential therapeutic interventions, which target a number of injury mechanisms, may be effective in multiple phases of injury.

4. POTENTIAL PROPHYLACTIC TREATMENTS

4.1. Creatine

There is increasing evidence that dietary supplementation during the second or third trimester of pregnancy, with the amino acid derivative creatine, could reduce the risk of antenatal or perinatal brain injury (73, 74). Creatine is involved in cellular energy production and can help to maintain adenosine triphosphate (ATP) turnover, acid base balance and mitochondrial function after HI. Creatine has also been shown to have antioxidant actions, stabilize lipid membranes, and reduce excitotoxicity through interaction with glutamate and $GABA_A$ receptors (74, 75). An advantage of creatine therapy is that it has been shown to effectively protect a number of major organs in addition to the brain. Antenatal creatine treatment increases overall survival from 50 to 88% after birth asphyxia in the spiny mouse pup, as well as being neuroprotective, reducing structural and functional damage to the diaphragm, and preventing acute kidney injury (76-78). The clear disadvantage of creatine therapy is that it requires long-term treatment of all pregnant women.

4.2. Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring indolamine secreted by the pineal gland to regulate circadian rhythm that has anti-oxidant properties (79). Melatonin has clinical potential as a prophylactic treatment for fetuses at high risk of perinatal HI as it readily crosses the placenta (79). When given before and immediately after HI, melatonin is neuroprotective in postnatal rodents (79). In term-equivalent fetal sheep, maternal prophylactic melatonin (1 mg total) given before 10 min umbilical-cord occlusion was associated with reduced brain-lipid peroxidation, neuronal death, microglial activation and astrogliosis (80). In preterm fetal sheep at 0.7. gestation, maternal low-dose melatonin infusion was associated with faster fetal EEG recovery, delayed onset of seizures, improved survival of mature oligodendrocytes, and reduced microglial activation in the periventricular white matter (81).

There is emerging evidence for a neuroprotective effect of melatonin treatment during the latent phase. In preterm fetal sheep at 0.6. gestation, fetal infusion of high-dose (20 mg/kg) melatonin for 6 h from shortly after umbilical cord occlusion was associated with reduced apoptosis and microglia in the white-matter, although cell survival was not quantified (82). High-dose (5 mg/kg/h over 6 h) melatonin given immediately after HI in postnatal term piglets strikingly augmented protection from therapeutic hypothermia, both for MRS markers of anaerobic stress, and histopathology (83).

An important potential limitation is that melatonin is a hydrophobic molecule and therefore ethanol is frequently used as a diluent, which has been shown to induce widespread caspase-mediated apoptotic neurodegeneration in the brain of developing rats and mice (84-90). In postnatal term piglets, very high dose (10 mg/kg) melatonin dissolved in ethanol was associated with hypotension and increased inotrope requirements after HI. It is unknown whether the melatonin or ethanol—or the combination—mediated this adverse effect (83). Further, in a recent study of prophylactic maternal melatonin before severe asphyxia in preterm fetal sheep, there was evidence that although melatonin was associated with faster recovery of the fetal EEG and improved white matter recovery compared to the 2% ethanol vehicle, melatonin and the ethanol vehicle were independently associated with similar improvement in neuronal survival in the striatum and reduced post-asphyxial seizures (81). Ethanol was also associated with greater neuronal loss in the CA3 and CA4 regions of the hippocampus and reduced white matter proliferation, with greater induction of amoeboid microglia. These findings strongly suggest that even small amounts of ethanol may partly confound any neuroprotective effects of melatonin, and thus, that it is essential to test alternate diluents. Small human studies suggest that melatonin has not been associated with adverse outcomes and may improve survival of neonates with septic shock and reduce ventilator-associated lung injury in preterm infants (91).

5. POTENTIAL NEUROPROTECTIVE THERAPIES DURING THE LATENT PHASE

5.1. Connexin hemichannels

One of the most striking features of hypoxic-ischemic brain injury is that injury consistently spreads from severely affected regions to areas that were originally intact (92). This pattern is consistent with the long-standing hypothesis that cell to cell communication might contribute to spreading injury. The gap junctions that link adjacent cells to allow transport of small molecules, ions and second messengers (93), are formed by docking of hexamer hemichannels (connexons) from adjacent cells. There is increasing evidence that these connexin hemichannels are not just passively waiting to dock, but are themselves active under normal physiological conditions, for example through purinergic signaling by regulated release of ATP (93). Critically, pathological conditions such as ischemia may cause unregulated opening of these channels, compromising the resting membrane potential and allowing transmitters such as ATP or glutamate into the extracellular space (93).

An elegant study from Orellana *et al* showed that Connexin43 hemichannels open *after*

hypoxia in cultured astrocytes (94). They showed increased dye uptake in Connexin43-containing astrocytes, but not Connexin43-deficient astrocytes, and that blockers of Connexin43 hemichannels prevented dye uptake and death of astrocytes. In fetal sheep, intracerebroventricular (ICV) infusion of a mimetic peptide at a dose concentration that blocks Connexin43 hemichannels (95), started 90 minutes after either cerebral ischemia or profound asphyxia and continued for 25 hours, improved EEG recovery and reduced white and grey matter damage (96, 97). In the term-equivalent fetal sheep this mimetic peptide infusion was associated with a striking reduction in status epilepticus after ischemia, consistent with the hypothesis that connexin hemichannels play a key role in propagating these intense seizures (96). Conversely, blockade of connexin 43 hemichannels *during* ischemia in term-equivalent fetal sheep had no effect on subsequent EEG recovery or cell death (98). When blockade of connexin 43 hemichannels was delayed until three hours after global cerebral ischemia, no improvement in EEG recovery or cell death was seen, despite a significant reduction in seizure activity and cytotoxic edema (99). Taken together these data suggest that connexin hemichannels play a key role in the downstream propagation of injury within the first three hours after ischemia. However, combined treatment with delayed connexin hemichannel blockade and therapeutic hypothermia, both started at three hours, was not associated with additive neuroprotection after global cerebral ischemia in the near-term fetal sheep (99).

5.2. Xenon

Xenon is an inert noble gas used for its anesthetic properties, mediated via competitive binding at the glycine binding site of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor (100). In addition to potentially attenuating excitotoxicity, xenon may also activate pro-survival kinases, such as p-Akt and the anti-apoptotic factor Bcl-2, and potentially inhibit opening of the mitochondrial permeability pore (101). There is evidence of an additive neuroprotective effect when adding xenon to hypothermia treatment. Xenon and hypothermia administered together, immediately or as late as 4 hours after HI in neonatal rats significantly reduced apoptotic cell death and loss of brain matter while improving long-term neurological motor function and coordination (79). In the newborn piglet, the combination of xenon with whole body cooling was associated with a 75% reduction in global neuropathology after perinatal asphyxia (102). In a similar paradigm others found that xenon-augmented hypothermia reduced cerebral MRS abnormalities and cell death markers in some brain regions compared with no treatment, although the effect was not significant compared to hypothermia alone (103).

The feasibility of treating with xenon during therapeutic hypothermia has been shown in a recent study in which infants with hypoxic-ischemic encephalopathy received up to 50% xenon for up to 18 hours during cooling, with no apparent adverse effects seen at 18 months follow-up (104). The limited natural availability of xenon and thus high price means that it needs to be used with a recirculating ventilator (105), and thus it is unlikely to ever be available outside of tertiary units.

5.3. Magnesium sulfate ($MgSO_4$)

Magnesium has natural anti-excitotoxic effects, mediated by binding to the magnesium site on the NMDA receptor, raising the possibility that it may be neuroprotective (106). Meta-analysis of randomized controlled trials of maternal $MgSO_4$ treatment for preterm birth suggests that it may be associated with a small but significant reduction in the risk of cerebral palsy and gross motor dysfunction in early childhood (107). However, there was no significant effect on the combined outcome of death or disability, and so it is unclear whether there is long-term benefit (108).

However, its effects on hypoxic-ischemic injury at term are unclear. A recent systematic analysis of preclinical studies of $MgSO_4$ for neuroprotection at term-equivalent found that while 7 of 15 studies reported improved neuronal outcomes with $MgSO_4$ treatment, these studies did not adequately control environmental or body temperature (106). In contrast, studies that controlled environmental or body temperature did not find significant neuroprotective effects of $MgSO_4$. This analysis strongly suggests that the apparent protective effects of magnesium may be confounded by iatrogenic hypothermia, and that further investigation is essential before considering large clinical trials of $MgSO_4$ for HIE in term neonates (109).

It has been shown in apparently uninjured post-mortem human infant brains that all NMDA receptor subunits are expressed at higher levels in the white matter and cortex compared with the adult brain (110). Increased expression of the magnesium sensitive receptor subunit NR2A was seen on glial cells at mid-gestation and progressively increased with gestational age in the cortex, while increased NR2B function has been shown in white matter during the time of rapid myelination and subcortical neuronal growth (110). Furthermore, infusion of $MgSO_4$ was associated with a modest but significant suppression of EEG activity before asphyxia and a reduction in seizure activity after asphyxia in preterm fetal sheep, suggesting that $MgSO_4$ effectively binds to its endogenous inhibitory site on NMDA receptors in the developing brain (111). These effects cannot have

been mediated by neuroprotection, since there was no improvement in neuronal survival or recovery of EEG activity after $MgSO_4$ infusion.

5.4. MicroRNAs

MicroRNAs (miRNAs) are a novel class of endogenous small single-stranded non-protein coding (20-24) nucleotides. miRNAs play a critical role in the control of gene expression at the post-transcriptional level. An extensive body of literature from adult experimental and clinical models suggests that dysregulation of miRNA biogenesis, and their regulatory role, is a common theme associated with the development of neurological injury and disorders (112-121). The role of miRNAs in perinatal HI has been evaluated in NG2-specific Dicer1 knockout mice (122). These authors demonstrated that knockdown of Dicer1, the dsRNA nuclease essential to the production of functional miRNAs, after perinatal HI increased the number of mature oligodendrocytes and MBP expression and was associated with improved motor co-ordination performance.

Hypoxic regulation of miR-210, a member of a specific group of miRNAs known as hypoxamirs, has been shown to be consistently upregulated under various hypoxic conditions (123, 124) as well as being transiently upregulated after focal ischemia (125). ICV administration of miR-210 mimic suppressed neuronal apoptosis in P7 neonatal rats after middle cerebral artery occlusion, by inhibiting caspase activity and through controlled regulation of bcl-2 and bax levels, suggesting a neuroprotective effect of miR-210 (126). In contrast, Ma et al (127) demonstrated that miR-210 directly targets the 3'UTR region of the glucocorticoid receptor (GR) in the neonatal rat brain and down-regulates GR protein following HI resulting in increased susceptibility to injury. Silencing of miR-210 through ICV administration of complementary locked nucleic oligonucleotides (miR-210-LNA), 4 hours after HI, significantly ameliorated neuronal injury and infarct size and was associated with a reduction in brain miR-210 levels. Interestingly, intranasal administration of miR-210-LNA under the same conditions resulted in similar effects. The reason for the discrepancies between these studies is unknown, but may reflect differences in insults.

5.5. Remote ischemic postconditioning

In 1986, Murry *et al.*, showed decreased infarction size in dogs after acute myocardial injury when preceded by short ischemic episodes, termed preconditioning (128). The same protective mechanism was later reproduced by means of short periods of non-lethal ischemia to a hind limb *after* the ischemic insult in a stroke model in adult rats, termed remote ischemic postconditioning (RIPostC) (129). The tissue-protective effects of RIPostC have been investigated in different organ systems and currently the cardioprotective effect of RIPostC is being investigated in adult human trials (130). RIPostC has been found to be neuroprotective after stroke in several experimental studies in adult mice and rats (131). In two studies using neonatal rats, delayed and immediate RIPostC showed neuroprotective properties after HI (132, 133). Only two studies have investigated the potential neuroprotective effect of RIPostC in a larger animal model (134, 135). Ezzati *et al.*, found a reduced white matter lac/NAA-ratio, and increased levels of whole brain ATP, 48-hours after the HI insult in piglets (134). They also found reduced apoptosis in the periventricular white matter, internal capsule, and corpus callosum (134). Rocha-Ferreria *et al.*, investigated the possible mechanisms of RIPostC at 48 hours after HI in piglets (135). They found reduced nitrosative stress with a reduced amount of nitrotyrosine deposits, reduced iNOS and increased eNOS expression in all assessed regions of the brain (135).

Although the underlying mechanism is not yet fully understood, RIPostC has an advantage as a potential neuroprotective strategy due to the opportunity of immediate intervention through simple means that does not require tertiary center equipment or facilities. There is currently no evidence that RIPostC has an ameliorating effect when combined with therapeutic hypothermia and since current pre-clinical evidence is still sparse further studies are needed.

5.6. Insulin-like growth factor-1 (IGF-1)

There is good histological evidence that activation of apoptotic pathways is a significant contributor to post-HI cell death in the developing human brain. But, post-HI cell death is not purely apoptotic, but rather includes elements of both apoptotic and necrotic processes, with one or the other being most prominent depending on factors such as maturity and the severity of insult (136). Consistent with the hypothesis that apoptotic processes are a key therapeutic target, hypothermia started after severe HI was reported to reduce apoptotic cell death, but not necrotic cell death in the piglet (137). Similarly, protection with post-HI hypothermia in fetal sheep has been closely linked with suppression of activated caspase-3 (138).

IGF-I is potently anti apoptotic, as well as promoting neural stem cell proliferation, differentiation, maturation, myelination, neurite outgrowth and synaptogenesis. There is marked up-regulation of endogenous IGF-I in injured areas of the brain, which is suggested to contribute to cerebral repair and functional recovery (139). However, in the neonatal rat, during the preceding latent phase there is a global *reduction* in all components of the IGF

system, suggesting the hypothesis that relative lack of neurotrophic support after injury may contribute to delayed cell death (140).

There is consistent evidence that administration of exogenous IGF-I shortly after HI can attenuate subsequent severe, delayed, neuronal and oligodendrocyte cell death and associated demyelination in rats (141), and near-term fetal sheep (139). For example, in term-equivalent fetal sheep, IGF-I given as a 1 h ICV infusion was associated with reduced loss of oligodendrocytes in the intragyral white matter, reduced demyelination, reduced tissue swelling, but upregulation of astrocytes and microglia (142). Delayed co-treatment with IGF-I plus hypothermia after cerebral ischemia did not improve white matter damage compared to hypothermia alone, suggesting that their mechanisms of neuroprotection are overlapping (143).

IGF-I treatment was associated with reduced caspase-3 activation and increased glial proliferation in a dose-dependent manner (144). Caspase-3 was only expressed in oligodendrocytes that showed apoptotic morphology. Proliferating cell nuclear antigen co-localized with oligodendrocytes, astrocytes, and microglia. Thus, increased oligodendrocyte numbers after IGF-I treatment is partly due to suppression of apoptosis, and partly due to increased proliferation. In contrast, the increase in reactive glia was related only to proliferation. These intriguing data raise the possibility that protective effects by reactive glia may partly mediate white matter protection by IGF-I (145), and thus speculatively, that chronic treatment with this or other growth factors could help restore production of oligodendrocytes in premature infants.

6. POTENTIAL NEUROPROTECTIVE THERAPIES DURING THE SECONDARY PHASE

6.1. Anticonvulsants

Although seizures in infants suffering hypoxic-ischemic encephalopathy are associated with adverse outcomes (146, 147), it remains unclear whether these seizures are the cause of injury or simply reflect the evolution of ongoing injury. Thus, it is unknown whether blocking seizure activity reduces the development of brain injury (148). Mild hypothermia does seem to reduce the overall burden of seizures after moderate HI encephalopathy (149), although seizures remain common during cooling and are highly associated with adverse outcomes (150). There is considerable interest as to whether anticonvulsant therapy can augment hypothermic neuroprotection. In neonatal rats, phenobarbital treatment from 15 minutes after HI in combination with hypothermia started either one or three hours after HI was associated with a significant improvement in sensorimotor performance and reduced brain damage compared to hypothermia alone (151). However, hypothermia was only induced for three hours and was markedly delayed compared to injection of phenobarbital.

In near-term fetal sheep, infusion of the NMDA receptor antagonist, dizocilpine, six hours after the end of HI, completely suppressed seizure activity, but only reduced neuronal cell death in the less susceptible lateral cortex (temporal lobe) and hippocampus, but not in the highly susceptible parasagittal cortex (152). Potentially, this may indicate that to achieve neuroprotection, treatment with anticonvulsants would need to be initiated *before* seizures start. Consistent with this concept, in preterm fetal sheep, dizocilpine infusion started shortly after severe asphyxia was associated with selective neuroprotection of the striatum (153), but combined treatment with delayed mild therapeutic hypothermia showed no additive neuroprotective effect. (109). These findings suggest that hypothermia may in part act by suppressing neural injury related to excessive glutamatergic activity.

In a retrospective study of neonates given phenobarbital before treatment with therapeutic hypothermia for hypoxic-ischemic encephalopathy, the combination was not associated with improvement of the composite outcome of neonatal death or an abnormal post-treatment brain MRI (154). Thus, at present this strategy requires further robust preclinical testing before formal controlled trials can be considered.

7. TREATMENTS TARGETING NEURORESTORATION IN THE TERTIARY PHASE

It has been shown that neurorestoration is possible, even a long time after HI. For example, intravenous administration of epidermal growth factor or brain derived neurotrophic factor increased striatal neurogenesis in adult mice who were exposed to neonatal hypoxic ischemic brain injury induced by unilateral carotid artery ligation and inhalational hypoxia (155). There are a number of potential treatment strategies that target both neuroprotection and neurorestoration that have shown promise in preclinical and clinical trials.

7.1. Acute and long-term inflammation

Brain injury leads to induction of the inflammatory cascade with increased release of cytokines (156) and induction of microglia, the resident immune cells of the brain (6, 7). Inflammation may exacerbate both acute and delayed injury and could therefore be considered a target for both neuroprotection and neurorestoration. Experimentally, cooling can potently suppress the acute inflammatory

reaction (5). For example, *in vitro*, hypothermia inhibits microglial proliferation and superoxide and nitric oxide production. In adult rats, hypothermia suppresses the post-traumatic release of interleukin-1β, and accumulation of polymorphonuclear leukocytes. Similarly, post-insult hypothermia was associated with partial suppression of microglial activation in fetal sheep (6-8, 138).

However, it is worth noting that the role of microglia is far more complex than previously believed. There is a broad spectrum of phenotypes ranging from pro-inflammatory M1 microglia (classical activation) at one end of the continuum, to M2 anti-inflammatory/ reparative microglia (alternative activation) at the other end (157). Evidence from the adult mouse brain after middle cerebral artery occlusion suggests that there is a distinct time course of microglial activation state after injury, with a rapid but transient induction of genes associated with the M1 state followed by a gradual transition to M2 (158). However, this is in contrast with evidence from the neonatal mouse brain, where there was rapid and transient induction of genes associated with both classical and alternative activation states within 24 hours after HI followed by a gradual return towards baseline expression, with increased numbers of microglia still evident at seven days in the ipsilateral compared to the contralateral hemisphere (159).

Despite the fact that microglial activation is associated with brain injury, the net effect of microglial depletion is detrimental. For example, microglial depletion after neonatal stroke increased the production of pro-inflammatory mediators, the volume of the infarct, frequency of hemorrhage and disruption of the blood brain barrier in P7 rats (160, 161). Therefore it appears that microglia play a dual role in the response to injury in the immature brain with both neurotoxic and neuroprotective functions, likely depending on microglial phenotype and the phase of injury evolution (157).

Given these complex responses of microglia after injury, it follows that rather than preventing microglial activation, it may be more useful to modulate the inflammatory phenotype to reduce brain injury. For example, genetic disruption of IL-18, a pro-inflammatory cytokine expressed by microglia, reduced brain injury after HI in P9 mice (162). Neonatal mice lacking MyD88, a protein adapter of TLR4, were no longer sensitized by lipopolysaccharide (LPS) to greater brain injury as a result of HI induced by unilateral carotid artery ligation and hypoxia at P9 (163). Galectin-3 is an endogenous paracrine TLR4 ligand released from activated microglia to stimulate pro-inflammatory M1 microglial activation. Depletion of galectin-3 was neuroprotective and anti-inflammatory after global brain ischemia in mice, as well as in a model of LPS-induced neuronal inflammation in cell culture and in mice after intranigral LPS injection (164).

Treatment with a single dose of the synthetic second-generation tetracycline derivative minocycline at the time of reperfusion shifted microglial phenotype towards the alternative M2 activation state and significantly reduced infarct size and tissue loss, as seen by MRI, and blood-brain barrier permeability up to four weeks after middle cerebral artery occlusion in the adult hypertensive rat (165). Furthermore, Cikla *et al* showed a biphasic microglial response with an early increase in microglial number and activation in the hippocampus followed by a delayed increase in the cortex and striatum in the P9 (equivalent to the term human brain) and P30 (juvenile-equivalent brain) mouse brain after HI (166). Administration of minocycline at two hours and 24 hours after HI suppressed numbers of microglia and their activation at day one in both P9 and P30 mice and prevented the delayed increase in the group that received HI at P9 only. Interestingly, although the group that received HI at P9 showed a reduction in neuronal injury after two days and nine days, they showed significant atrophy on MRI at day 60 as well as impaired learning and memory on the Morris water maze test, while the group that received HI at P30 showed significant improvements in brain volume and cognitive performance. The authors suggest that the suppression of the delayed increase in microglial activation in the animals that received HI at P9 may have been detrimental to long-term outcome (166). Thus, modulation of microglial number and activation state may be a useful therapeutic target after hypoxia ischemia but more research is required to better understand the temporal and spatial evolution of the inflammatory response in the neonatal brain.

7.2. Erythropoietin

Erythropoietin (EPO) has a central role in erythropoiesis and is now routinely used as a treatment for anemia in the premature infant. Endogenous EPO production is upregulated after chronic hypoxia via increased expression of hypoxia inducible factor one (167). In addition, there is increasing clinical and experimental evidence suggesting that recombinant EPO (rEPO) may be neuroprotective in both preterm and full-term neonates after HIE as well as adults after acute ischemic stroke (168), acting via the EPO receptor (EPOR) on neurons and glia as well as erythroid precursors. For example, prolonged infusion of rEPO from 30 minutes until 72 hours after complete umbilical cord occlusion in the preterm fetal sheep, was associated with partial neuronal and oligodendrocyte protection, reduced inflammation, more rapid recovery of brain activity and reduced seizure activity (169).

Although EPO has a high molecular weight, the increased permeability of the blood-brain barrier after hypoxia-ischemia allows rEPO to enter the brain in a dose-dependent manner (170). rEPO appears to have a broad range of mechanisms of action, which makes it a suitable candidate both for acute neuroprotection and long-term neurorestoration. EPO binding to EPOR can suppress apoptosis by promoting expression of the anti-apoptotic genes Bcl-2 and Bcl-xL (171). In addition to being anti-apoptotic, rEPO has also demonstrated anti-inflammatory, neurotrophic and antioxidant properties, which likely contribute to acute neuroprotection (171). rEPO has also shown a number of long-term effects that may promote neurorestoration, including anti-inflammatory effects, angiogenesis, neurogenesis and oligodendrogenesis (172-175).

Furthermore, there is reasonable clinical evidence that rEPO treatment is safe. A recent meta-analysis of five studies involving 233 patients, including very low birth weight neonates and premature neonates, showed that rEPO administration was not associated with adverse effects and was associated with improved neurodevelopmental outcome (176). In full-term neonates with hypoxic-ischemic encephalopathy, studies have reported that rEPO treatment is safe. A phase 1 trial showed that 500, 1000 and 2500 U/Kg rEPO given in conjunction with hypothermia was well-tolerated beyond the neonatal period (177, 178). A phase II study showed that 1000 U/Kg rEPO given in conjunction with hypothermia resulted in less brain injury on MRI and improved 1-year motor function compared to hypothermia alone (177, 179). Low-dose rEPO (300 or 500 U/kg) was associated with a reduced risk of death or disability in term infants with moderate, but not severe, hypoxic-ischemic encephalopathy (177-180). Further, high-dose rEPO (2500 U/kg) started within the first 48 hours of life was shown to improve neurodevelopmental outcome in term neonates with mild/moderate HIE and was also associated with a significant reduction in seizure activity, improved abnormal EEG background at two weeks, and decreased neurologic abnormalities at six months (181).

7.3. Stem cell therapies

Over the past decade, there has been increasing interest in the use of stem cells as a treatment for infants suffering hypoxic-ischemic encephalopathy and even for children with cerebral palsy. There is increasing evidence that significant functional improvements can occur without significant functional engraftment (182).This suggests that the effects of stem cells are likely mediated by neurotrophic and immunomodulatory/immune suppressive factors. Consistent with this, in rabbits exposed to intrauterine HI at 70% gestation, subsequent infusion of human umbilical cord blood cells at birth was associated with a dose-dependent improvement in motor function despite little penetration of the cells into the brain (183). Promisingly, combined treatment with mesenchymal stem cell therapy and 24 hours of hypothermia, started 6 hours after the end of HI in P7 rats, showed greater improvement with combined treatment than either treatment alone (184) as measured at P42 by MRI and functional behavioral tests.

In addition to being neuroprotective, stem cells may also have great potential as a neurorestorative therapy. A study showed that mesenchymal stem cells given intranasally at three or 10, but not 17 days after HI, improved cognitive and sensorimotor function as well as histological outcome at nine weeks after unilateral carotid artery ligation and hypoxia in P9 mice (185). Furthermore, improved cognitive and sensorimotor function has been shown to persist until 14 months after intranasal treatment with mesenchymal stem cells at 10 days after hypoxia-ischemia in the P9 mouse (186). A recent study showed that human amnion epithelial cells administered on days one, three and 10, after 25 minutes of complete umbilical cord occlusion in preterm fetal sheep, improved brain weight, oligodendrocyte maturation, myelination and subcortical neuronal survival as well as reducing inflammation (187).

A feasibility study in full-term human neonates has shown that it is both possible and safe to administer autologous umbilical cord blood cells to neonates with hypoxic-ischemic encephalopathy, including those being treated with therapeutic hypothermia (188). A small double-blind randomized, placebo-controlled clinical trial in 96 children with cerebral palsy receiving rehabilitation therapy found that treatment with a combination of umbilical cord blood and recombinant erythropoietin, ameliorated motor and cognitive dysfunction after 6 months of treatment more than rehabilitation therapy with or without erythropoietin (189). Interestingly, a pilot study suggested that treatment with allogenic umbilical cord blood from healthy infants (n=3) may reduce pro-inflammatory plasma cytokines compared with autologous umbilical cord blood (n=4) (190). Thus, potentially the specific origin of cord blood cells may be important.

Taken together, these studies suggest that stem cell therapy may be a promising treatment for HIE, whether given alone or in combination with other treatments like therapeutic hypothermia or erythropoietin (183). In order to optimize the potential of this treatment, further systematic studies of the mechanisms of action, optimal dosing and timing and type of stem cells are needed, as well as studies investigating the effect of delayed stem cell therapy after therapeutic hypothermia.

8. CONCLUSION

Brain damage after HI in the preterm or full-term fetus/neonate evolves over time. Understanding how potential new treatment strategies affect each phase of injury and when they will be most effective may allow for more successful treatment of infants with HIE.

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