Title: The fetus at the tipping point: modifying the outcome of fetal asphyxia

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Running title: Modulating fetal asphyxia

Research Category: Preclinical Research

Word Count: 3882

Figure Count: 2

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Abstract

Brain injury around birth is associated with nearly half of all cases of cerebral palsy. Although brain injury is multifactorial, particularly after preterm birth, acute hypoxia-ischemia is a major contributor to injury. It is now well established that the severity of injury after HI is determined by a dynamic balance between injurious and protective processes. In addition, mothers who are at risk of premature delivery have high rates of diabetes and antepartum infection/inflammation and are almost universally given treatments such as antenatal glucocorticoids and magnesium sulphate to reduce the risk of death and complications after preterm birth.. We review evidence that these common factors affect responses to fetal asphyxia, often in unexpected ways. For example, glucocorticoid exposure dramatically increases delayed cell loss after acute hypoxia-ischemia, largely through secondary hyperglycemia. This critical new information is important to understand clinical treatment of women at risk of asphyxia.

Fetal asphyxia and HIE

Acute perinatal hypoxia-ischemia (HI) remains a major cause of life long disability. Half of all cases of CP are now associated with perinatal brain injury (Reid *et al.*, 2016). A third of cases are related to preterm birth (Committee on Understanding Premature Birth and Assuring Healthy Outcomes). There is considerable evidence that in term infants, perinatal HI is the primary antecedent of acute neural injury (Cowan *et al.*, 2003). Preterm brain injury is notoriously complex and multifactorial (Galinsky *et al.*, 2017b). However, acute, profound asphyxia at birth with early onset hypoxic-ischemic encephalopathy (HIE) is more common that at term; for example from 2008 to 2011, in a cohort of 115,502 deliveries in the USA, 37.3/1000 infants born before 37 weeks of gestation had moderate to severe HIE (Manuck *et al.*, 2016).

Fetal HI has distinct characteristics that limit extrapolation from studies of neonatal or adult HI. Firstly, the insult is most often global, affecting the whole fetus. Thus the fetal systemic and cardiovascular responses are critical to understanding the determinants of outcome. Secondly, the insult is generally reversible, whether spontaneously or therapeutically (e.g. delivery and resuscitation) and so can be associated with an evolving pattern of cerebral dysfunction and delayed injury after the insult. Thirdly, the injury may be a single acute episode, or repeated insults. Fourthly, many of the insults occur in the stable and warmer thermal environment of the uterus. Finally, the maturity of the brain has a considerable effect on how neurons and glia respond to asphyxia.

It is now understood that the fetal response to asphyxia is not stereotypical, but rather depends upon both the nature of the insult and the condition of the fetus (Figure 5.1). The fetus is spectacularly good at defending itself against such insults, and injury occurs only in a very narrow window between intact survival and death. Further, even after severe HI, there can be significant transient recovery of cell function, followed by delayed evolution of cell death,

modified by a balance between endogenous protective and damaging pathways from hours to weeks after the insult (van den Heuij *et al.*). Not surprisingly this fine balance is affected by external factors, such as is the significant neuroprotection provided by mild "therapeutic" hypothermia (Wassink *et al.*, 2014). There is increasing evidence that other common clinical therapies and metabolic changes also modulate neural outcome.

Evolution of neural injury after asphyxia

It is now well established in term infants and animals that there can be considerable cell survival after severe HI, followed by progressive evolution of bulk cell death over hours to days (Wyatt *et al.*, 1989; Lorek *et al.*, 1994). Distinct phases of injury can be broadly defined. During the "primary" phase of HI ischemia per se high energy metabolites are depleted, with progressive depolarization of cells, leading to severe cytotoxic edema (cell swelling) (Gunn *et al.*, 1997) and extracellular accumulation of excitatory amino acids due to failure of reuptake by astroglia and excessive depolarization mediated release (Tan *et al.*, 1996).

After reperfusion, in term piglets there is marked recovery of high energy phosphates on magnetic resonance spectroscopy for many hours, in a "latent" phase. The extent of recovery in the latent phase is related to the severity of injury (Iwata *et al.*, 2008); milder injury may even be associated with recovery to above baseline values for many hours. This is followed by secondary deterioration with secondary cytotoxic edema and seizures (Gunn *et al.*, 1997), and ultimately cerebral energy failure from 6 to 15 hours after birth (Azzopardi *et al.*, 1989). The timing of energy failure after HI is tightly coupled with the appearance of histologic brain damage (Vannucci *et al.*, 2004), implying that it is primarily a function of evolving cell death (Figure 1). From 3 days after HI, there is evidence of a tertiary phase of injury, involving repair and reorganisation, but also chronic inflammation and epigenetic changes lasting for weeks to months after injury (Fleiss & Gressens; Galinsky *et al.*, 2017b). It is this delay before secondary

deterioration in a ‘latent’ phase that enables delayed therapeutic hypothermia to significantly improve clinical outcomes (Wassink *et al.*, 2014).

Targets during hypoxia-ischemia

Ultimately neuronal loss requires a period of sustained anoxic cell depolarisation due to lack of oxygen and substrate. The triggers of delayed neuronal loss include excessive entry to calcium, combination of biophysical damage, excessive entry of calcium into the cell though multiple channels, including the excitatory amino acid receptors, and damage-associated molecular patterns (DAMPs). After reperfusion, there is increasing evidence that astrocytic and microglia responses contribute to spreading injury from the most severely affected regions to previously undamaged areas of the brain, in part by opening of cell membrane channels such as connexin43 hemichannels, leading to release of excitatory small molecules such as ATP and glutamate (Davidson et al., 2013; Hartings et al., 2017). These all contribute to activate multiple intracellular programmed cell death pathways leading to delayed apoptosis and necrosis (Thornton *et al.*, 2017).

In preclinical studies, these damaging events are associated with endogenous inhibitory neuroprotective responses that help to limit the degree of injury. For example, the dramatic rise in extracellular glutamate during HI in grey matter is closely accompanied by accumulation of endogenous inhibitory neuromodulators such as gamma-aminobutyric acid (GABA) and adenosine in term-equivalent (0.8 to 0.9 of gestation) fetal sheep (Tan *et al.*, 1996; Hunter *et al.*, 2003; Lotgering *et al.*, 2003). In striking contrast with the adult brain, the relative rise in GABA in near-term fetal sheep is many times greater than that of glutamate (Tan *et al.*, 1996). There is strong evidence that metabolism of ATP to adenosine during HI mediates the initial rapid depression of EEG activity, and that this reduces hippocampal, striatal, and parasagittal neuronal loss after 72 hours recovery (Hunter *et al.*, 2003).

During the latent phase after HI there is delayed-onset regulated suppression of cerebral metabolism. In preterm fetal sheep for example, this regulated suppression reached a maxima 1 h after occlusion and gradually resolved at the end of the latent phase, with increased cortical tissue PO_2, and cerebral hypoperfusion. This hypometablism and hypoperfusion are actively mediated by inhibitory neuromodulators including allopregnanolone (Yawno *et al.*, 2007), and alpha-2 adrenergic receptor activity (Dean *et al.*, 2006). Blocking these factors markedly increased cerebral injury, strongly denoting that these responses are beneficial (Dean *et al.*, 2006; Yawno *et al.*, 2007).

In addition to neuronal inhibition, multiple neuroendocrine responses also help protect the brain (Robertson *et al.*, 2012). For example, in newborn piglets and postnatal day 7 (P7) rats there is release of melatonin early in the latent phase, and delayed upregulation of multiple anti-apoptotic growth factors such as erythropoietin (Epo) and insulin like growth factor 1 (IGF-1) in the secondary and tertiary phases after HI (Guan *et al.*, 2003; Robertson *et al.*, 2013; Ohls *et al.*, 2015). Evidence that it is possible to further improve outcomes by augmenting these responses is discussed later.

Multiple hit hypothesis

A recent MRI study in preterm infants demonstrated that a synergy between prenatal and postnatal insults, such as intrauterine growth restriction and prolonged mechanical ventilation had a cumulative effect on white matter injury, as shown by lower white matter fractional anisotropy at term equivalent age, and impaired neurodevelopmental outcomes at 20 months corrected age (Barnett *et al.*, 2018). It is also plausible that the developing fetus can potentially be exposed to multiple injurious factors *in-utero*, such as chronic hypoxia, intra-uterine infection/inflammation, antenatal treatments (e.g. glucocorticoids and magnesium sulphate), and maternal health and lifestyle associated issues that may modulate fetal response to asphyxia and resultant neural injury.

Modification of neural outcome by multiple insults

Many preterm deliveries are associated with the presence of intrauterine inflammation. Histological chorioamionitis is reported in nearly 95% of preterm births at 21 – 24 weeks of gestation, and in about 10% of deliveries at 33 – 36 weeks (Kim et al., 2015). The evidence from preclinical studies in neonatal rats suggests that exposure to inflammation can either exacerbate or induce tolerance against HI neural damage depending on the order, intensity and time of the insults (Eklind *et al.*, 2005; Hickey *et al.*, 2011).

Sensitisation

Clinical data suggest that prior inflammatory stimulus can enhance metabolic decompensation during subsequent HI. For example, near-infrared spectroscopy in preterm infants showed that intrauterine inflammation was associated with an increase in cerebral oxygen consumption after birth (Stark *et al.*, 2016). Numerous preclinical studies have provided evidence that an acute inflammatory insult can worsen neuroinflammation after subsequent HI. In P8 mice systemic inflammation induced with injection of TLR 2 agonist (Pam3CSK4) suppressed ADP induced oxidative phosphorylation in mitochondria isolated from the brain 14 hours after the injection (Mottahedin *et al.*, 2017). The combination of TLR 2 activation and HI was associated with greater loss of neural tissue 5 days after HI, suggesting that inflammation induced dysregulation of mitochondrial function might contribute to neural damage during HI.

In P2 rat pups, 0.5 mg/kg of lipopolysaccharide ((LPS), a component of cell wall of gram negative bacteria) given 2 hours before HI augmented microglia activation, cerebral cytokines, blood brain barrier damage and white matter damage compared to HI alone (Wang *et al.*, 2010). Similarly, the interaction between inflammation induced by the viral protein mimetic polyinosinic-polycytidylic acid (poly(I:C)) and subsequent HI in P8 mice, increased pro-inflammatory cytokines and apoptotic proteins in the brain, and increased infarct size compared

with HI alone (Stridh et al., 2013). This preclinical literature is supported by clinical evidence that exposure to sepsis and HI during the perinatal period have a cumulative effect on the risk of cerebral palsy in very premature infants (Wang *et al.*, 2014). Similarly, the combination of fetal growth restriction, denoting prenatal hypoxia and postnatal inflammation markedly increases risk of impaired neurodevelopmental scores at 2 years of age compared to either alone (Leviton *et al.*, 2013).

Tolerance

It is important to appreciate that exposure to multiple insults is not consistently deleterious. Depending on the insult severity, the time interval between insults and maturational stage of the brain, the interaction between inflammation and HI insults can be protective. Mild or sub-threshold insults can activate endogenous neuroprotective pathways and modify evolution of injury after the subsequent insult. For example, in P7 rat pups, exposure to low-grade inflammation (0.3mg/kg LPS) 24 hours before HI, reduced neural injury two weeks after HI (Eklind *et al.*, 2005). The neuroprotective effect of low dose LPS (0.3mg/kg) for subsequent HI was only observed if the time interval between the insults was 24 hours, whereas exposure 2, 6 or 72 hours before HI increased neural damage (Eklind et al., 2005). This timing likely reflects at least in part the time needed for upregulation of type I interferon and interferon regulatory factors (Marsh *et al.*, 2009).

Correspondingly, data from preterm fetal sheep shows that pre-treatment with low dose LPS (50–100 ng/kg) differentially regulated toll-like receptors mRNA expression and increased protein expression of interferon-beta only in the animals that were exposed to HI at 24 hours after LPS treatment, whereas no effect was seen with the time interval of 4 hours. Furthermore, LPS preconditioned fetuses had reduced microgliosis and astrogliosis, and reduced loss of oligodendrocytes at 5 days after HI (Dhillon et al., 2015). Developmentally regulated

expression of inflammatory pathways can also determine the outcome of the interaction between the insults. For example, an age dependent difference in the neuroprotective effect of preconditioning with inflammatory stimuli (LPS or poly(I:C)) before HI was reported in neonatal rats (Hickey et al., 2011). The neuroprotective interaction may be dose dependent; however, there is no consensus on the dose of LPS required to induce preconditioning in P7 rats (Lin *et al.*, 2009; Hickey *et al.*, 2011). Similarly, the neuroinflammatory response after HI was attenuated in P7 rats preconditioned with transient asphyxia in utero or mild hypoxia postnatally, and the interaction between the insults was found to be neuroprotective (Park *et al.*, 2011; Vlassaks *et al.*, 2013).

Thus, there is considerable pre-clinical evidence for a protective effect of interactions between various insults. In practice, it is unlikely that it will be possible to directly regulate such highly time dependent interactions. However, understanding the protective mechanisms underlying these interactions warrants careful study to help identify potential new therapeutic strategies.

Modification of neural outcome by antenatal treatment

Antenatal glucocorticoids and hyperglycemia

Exposure to antenatal treatments can also modify the neural outcome after fetal asphyxia. For example, antenatal glucocorticoids are administered to mothers at the risk of preterm delivery, to reduce mortality and morbidity associated with complications of being born prematurely. There is no clinical information on the interaction between antenatal glucocorticoids and hypoxic-ischemic encephalopathy in preterm infants, as such cases were excluded from the many randomised controlled trials (Roberts *et al.*, 2017). Preclinical studies in preterm fetal sheep have illustrated that a clinical dose (maternal intramuscular injection of 12 mg) of dexamethasone modulated the fetal response to asphyxia. Dexamethasone treatment was

associated with increased glucose concentrations before asphyxia, and both dexamethasone administration and hyperglycemia alone, induced by glucose infusion, in preterm fetal sheep were separately associated with improved neurophysiological adaptation during asphyxia (Lear et al., 2017). However, despite this improved adaptation, both dexamethasone treatment and hyperglycemia were associated with hyperactive EEG and increased seizure burden after asphyxia, and caused cystic neural injury by one week after asphyxia (Lear et al., 2017). Similarly, some studies in neonatal rats have also reported exacerbation of HI induced neural injury with prior dexamethasone exposure (Chang *et al.*, 2013; Yeh *et al.*, 2016). For example, administering a tapering course of dexamethasone 0.5, 0.3 and 0.1 mg/Kg on postnatal day 1-3 in neonatal rats, and subsequently subjecting them to HI on P7 showed greater loss of oligodendrocytes, reduced myelin thickness, and worse functional outcome in the long-term as compared to animals subjected to HI alone (Yeh et al., 2016).Treatment with a clinical dose of dexamethasone *after* asphyxia in preterm fetal sheep was also associated with hyperactive EEG, evidence of increased mitochondrial metabolism on near-infrared spectroscopy (Lear *et al.*, 2014), and increased white and grey matter injury (Koome *et al.*, 2013).

The underlying cause of exacerbation of neural damage with a combined exposure to dexamethasone and HI is not completely understood. Studies in neonatal (P7) rats have suggested a role of dexamethasone induced excitotoxicity in exacerbation of neural damage after HI (Chang et al.; Yeh et al.). For example, a reduction in the basal expression of glutamate transporter GLT1 after dexamethasone treatment from P1 to P3 in rats was associated with worse neural outcome after HI induced at P7, and pharmacological intervention that increases GLT1 transporter expression significantly reduced dexamethasone mediated exacerbation of HI neural injury (Chang et al., 2013). Furthermore, the effect of dexamethasone on neurophysiological recovery and neural outcome after asphyxia were replicated with glucose induced hyperglycemia in preterm fetal sheep, suggesting that hyperglycemia plays a role in

mediating the dexamethasone induced neural damage (Lear et al., 2017). Treatment with dexamethasone after asphyxia was likewise associated with hyperglycemia (Lear *et al.*, 2014). However, in contrast with the cystic lesions observed with dexamethasone pretreatment (Lear et al., 2017), only mild exacerbation of neural injury was found with treatment after asphyxia (Koome *et al.*, 2013). This strongly suggests that the main detrimental effect of hyperglycemia occurs *during* HI. *In vitro* evidence supports this concept and further suggests that increased opening of connexin hemichannels may be a key factor in the detrimental effects of hyperglycemia during HI (Orellana *et al.*, 2010).

Hyperglycemia

There is increasing evidence that hyperglycemia independently increases hypoxic-ischemic injury. This is especially pertinent considering given that infants with HIE show highly variable blood glucose levels during the early period after birth (Nadeem *et al.*, 2011). Preclinical studies in preterm and near-term fetal sheep and newborn piglets have shown exacerbation of neural injury induced with HI in combination with hyperglycemia (LeBlanc *et al.*, 1993; Petersson *et al.*, 2004; Lear *et al.*, 2017). Clinical data from term infants with HIE shows adverse neurodevelopmental outcomes associated with both hyperglycaemia and hypoglycaemia during the first day after birth (Chouthai *et al.*, 2015; Basu *et al.*, 2016). Similarly, hyperglycaemia during postnatal period in very preterm infants has been associated with impaired neurodevelopment at two years of age (van der Lugt *et al.*, 2010).

Neuroprotective effect of dexamethasone and hyperglycemia in neonatal rats?

Given the evidence suggesting that hyperglycemia aggravates HI ischemic injury (LeBlanc *et al.*, 1993; Lear *et al.*, 2017) and mediates the adverse neural effects of dexamethasone in preterm fetal sheep after HI (Lear et al., 2017), it is interesting to note that there is contrary evidence that hyperglycemia is both independently protective and at least in part mediates the

protective effects of dexamethasone in P7 rats after HI (Vannucci & Mujsce, 1992; Tuor *et al.*, 1993; Tuor *et al.*, 1997) Overall, these data suggest species and age-dependent differences in the effect of dexamethasone on on HI neural injury, with neuroprotection only occuring in neonatal rats. The most likely explanation is the low utilisation of and ability to transport glucose in the neonatal rat brain (Vannucci, 1994). Therefore, the reassuring neuroprotective effects of dexamethasone for HI induced neural injury observed in neonatal rat studies might not translate into human infants. Consistent with this, a recent meta-analysis reported lack of evidence for antenatal glucocorticoid treatment to have a preventive effect on cerebral palsy (Shepherd *et al.*, 2017).

Magnesium sulphate

Evidence from meta-analyses and systemic reviews show that magnesium sulphate administered to women at risk of preterm labour is associated with small, but significant reduction in the risk of cerebral palsy at 18 months to two years of age (Doyle *et al.*, 2009). However, the long-term follow-up studies show that magnesium sulphate treatment is not associated with significant improvement in neurodevelopmental outcomes at school age, although these were small studies (Chollat *et al.*, 2014; Doyle *et al.*, 2014). Preclinical studies in term equivalent animals of effects of magnesium sulphate for HIE have reported highly inconsistent outcomes, ranging from neuroprotection, to no effect or increased neuronal loss; it is highly likely that apparent neuroprotection was mediated by drug induced hypothermia, as reviewed in (Galinsky *et al.*, 2014).

Magnesium's primary neural effect is to inhibit glutamatergic signalling through binding its specific site on the N-methyl-D-aspartate receptor (Zeevalk & Nicklas, 1992). Consistent with this, reduced basal brain activity was reported in preterm infants treated with magnesium sulphate (Stark *et al.*, 2015), and in preterm fetal sheep (Galinsky et al., 2016). There is some

evidence for anti-oxidative and anti-inflammatory effects (Maulik et al., 1999; Sugimoto et al., 2012). In preterm fetal sheep, magnesium sulphate for 24 hours before and after asphyxia was associated with a significant reduction in basal EEG activity and seizure burden after asphyxia, but no effect on microglial activation, infiltration, astrogliosis or neuronal loss. Indeed, it was associated with increased loss of oligodendrocytes 72 hours after injury (Galinsky *et al.*, 2017a). A recent study in P7 rats suggests that the interaction between magnesium sulphate and HI is time dependent, with neuroprotection when it was administered between 6 days and 12 hours before HI, but not at 3 hours or 30 minutes before HI (Koning *et al.*, 2017). This effect was likely mediated by improved mitochondrial resistance to HI. Overall, these studies suggest the impact of magnesium sulphate on HIE is complex and possibly time dependant. Thus, further careful investigation into the effect of magnesium sulphate on the pathogenesis of HIE in preterm and term-equivalent translation animal models are essential before undertaking further clinical trials for HIE.

Maternal lifestyle associated risks

Preclinical studies have examined the effect of maternal health on the outcome of hypoxic-ischemic neural injury in the offspring. For example, a study in neonatal rats showed that there was an increased induction of microgliosis, astrocyte hypertrophy and neuronal loss after HI, in the pups of mothers that were given high fat diet (Teo *et al.*, 2017). Another study in mice showed that maternal exposure to cigarette smoke exacerbated cellular damaged after neonatal HI in male offspring (Chan *et al.*, 2017). However, few clinical studies have examined such interactions.

Improving outcomes by augmenting endogenous protective responses

As discussed, HI triggers multiple protective responses. Here we will review two promising examples of acute and delayed but long-lasting responses with evidence that augmenting these responses can protect the brain.

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is primarily released from the pineal gland and helps entrain circadian rhythms (McMillen *et al.*, 1995). It is rapidly induced after HI in many species (Robertson et al., 2013), and in adult animals pinealectomy increases focal ischemic injury (Kilic *et al.*, 1999), strongly inferring that it is neuroprotective. It appears to act primarily on effects during HI and in the reperfusion phase and early latent phase. Consistent with this, in term piglets, exogenous infusion of melatonin 15 min after HI dramatically augmented hypothermic neuroprotection (Robertson *et al.*, 2013). Similarly, in preterm fetal sheep, infusion of melatonin to the mother starting before severe asphyxia reduced oxidative stress, inflammation and apoptosis in the white matter, with improved survival of oligodendrocytes and increased myelin thickness (Yawno *et al.*, 2017) (Drury *et al.*, 2014). Intriguingly its reported effects may be related in part to the diluent, 2% ethanol, which had regional specific effects to improve neuronal survival in the caudate nucleus, but increase neuronal loss in regions of the hippocampus (Drury *et al.*, 2014), illustrating the complexity of treatment studies.

Erythropoietin (Epo)

By contrast with the very rapid release of melatonin, the endogenous growth factor Epo shows much slower upregulation, corresponding with the secondary and tertiary phases after HI. Epo and Epo receptor protein expression were increased in the injured hemisphere of neonatal (P7) rats at 24 hours and one week after HI (Sun et al., 2004). Similarly, Epo receptors were shown to be upregulated in the P2 rat brain after exposure to transient HI *in-utero* at embryonic day

18 (Mazur *et al.*, 2010). Interestingly, Epo and Epo receptor mRNA expression were differentially regulated after HI alone, or a combination of HI and LPS. Further the discrepancy in mRNA levels of Epo receptor and ligand (Epo) at P15 was associated with adverse neural outcome at P28 (Jantzie *et al.*, 2014). Recently, elevated serum Epo concentration was reported in full term infants exposed to perinatal asphyxia on day one and two after birth, and the serum Epo concentration was associated with severity of HIE on MRI (Sweetman *et al.*, 2017). Endogenous upregulation of Epo after HI is delayed but prolonged, suggesting that it likely has a role both in limiting injury in the secondary phase and promoting neurorepair in the long-term.

Studies of exogenous treatment support this hypothesis. Delayed treatment with 5000 U/Kg human recombinant Epo (rEpo) at 24, 48 and 72 hours after HI in P7 rats was associated with decreased neuroinflammation and improved neural outcome (Sun *et al.*, 2005). Furthermore, delayed treatment with rEpo starting 48 hours after HI in P7 rats did not reduce tissue volume loss, and yet was able to increase oligodendrogenesis at 5 days after HI, with improved oligodendrocyte maturation, reduced white matter injury and increased neurogenesis at 14 days after injury (Iwai et al., 2010). Clinically, postnatal treatment with Epo improved neurodevelopmental outcomes in several trials of term neonates with hypoxic-ischemic brain injury (Zhu *et al.*, 2009; Elmahdy *et al.*, 2010; Wu *et al.*, 2012; Rogers *et al.*, 2014; Wu *et al.*, 2016; Malla *et al.*, 2017). Moreover, retrospective studies of cohorts of preterm infants that received Epo for erythropoiesis compared to controls suggest improved outcomes (Bierer *et al.*, 2006; Brown *et al.*, 2009; Neubauer *et al.*, 2010). A recent meta-analysis of 1133 infants randomized to early Epo for neuroprotection, demonstrated a reduced incidence of children with MDI < 70 at 18-24 months PMA, with an odds ratio (95% confidence interval) of 0.51 (0.31–0.81), P < .005 and a number needed to treat was 14 (Fischer *et al.*, 2017).

Conclusions

Preclinical studies have provided significant evidence for interaction between multiple insults modifying neural outcome after asphyxia and have demonstrated that time and dose dependent interactions could act in synergy to exacerbate or attenuate the damage induced by asphyxia. However, there is only limited clinical data examining the effect of multiple interactions on neurodevelopmental outcome. In addition, there is a significant gap in knowledge of mechanisms underlying the interactions between various factors. Nevertheless, the evidence presented above highlights the importance to assessing the effect of multiple hits on neural outcomes in infants with HIE. Potentially, identification of high-risk groups can inform the development of future treatments. Furthermore, there is a need for more preclinical studies examining the efficacy of neuroprotective treatments for injury induced with multiple insults to examine the realistic clinical scenario. Identification of endogenous neuroprotective mechanisms has provided a rationale for exogenous treatment with these factor agents to further augment neuroprotective effects. It remains to be determined if multiple treatments given in a similar temporal profile to their endogenous upregulation will have an optimal neuroprotective effect.

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